

Development of Multiphasic Scaffolds for Tissue Engineering of Bone- Ligament Interface



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Abstract

Due to their low cell density and low nutrient and oxygen requirements, ligaments have a poor regeneration capacity and injuries frequently lead to persistent impairment of joint function. The integration of ligament into bone occurs at a specialised interface known as the enthesis, which is designed to allow smooth transfer between tissues of vastly different mechanical properties. Healthy enthesis exhibits a continuous gradient of interfacial tissue classified into four distinct zones, with varying cellular compositions, mechanical properties, and functions. Current reconstructive treatment methods are unable to recreate the natural smooth transition that typifies the original attachment site. Therefore, the aim of this research was to capture the biological and mechanical behaviour of natural entheses by developing multiphase collagen-based scaffolds which could be used to regenerate the compositionally complex interface and facilitate functional graft-to-bone integration.

Although enthesis regeneration has increasingly become a subject of intense interest within the field of orthopaedic and tissue engineering research, the development of a successful technique has been hindered by a lack of understanding of the structure and composition of the enthesis. Therefore, the first part of this thesis reports the composition and mechanical behaviour of the entheses of pig knees. The gradient of stiffness through the enthesis was presented by utilising a nanoindentation technique. Dry and wet nanoindentation tests were performed on the anterior cruciate ligament (ACL) to tibia insertions to measure the elastic modulus of each region and observe the time-dependent behaviour of biological material.

The second part of this thesis reports the development of various collagen-based scaffolds utilising monomeric forms of the protein. Extruded collagen fibres were developed and used to reinforce porous scaffolds which typically have less than optimal mechanical strength. Composite scaffolds exhibited superior mechanical properties and strong fibre-matrix bonds

were formed. Furthermore, highly porous (>97%) collagen-based constructs were created to mimic the various regions of the enthesis: a soft tissue layer composed of Type I collagen; an uncalcified fibrocartilage layer composed of Type II collagen and chondroitin sulphate (CS); a calcified layer composed of Type I collagen and hydroxyapatite (HAp). These scaffolds were then used to fabricate two-phase scaffolds with great integration of layers. Preliminary *in vitro* studies on the single-layer porous scaffolds showed the biocompatible nature of the scaffolds and their viability as an implantable device for tissue growth.

Preface

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Chapter 1 - Background and Literature Review

1.1. Introduction

The musculoskeletal system involves the complex interactions of bones, muscles, cartilage, tendons, ligaments and other connective tissue, to provide support and stability to living beings. Connective tissues, such as ligaments, are joined to bone via highly specialised interfaces which integrate their structures into bone and facilitate joint motion. Injuries to ligaments are common and can destroy these specialised interfaces and operative interventions to attempt to recreate the natural organisation of these insertion sites are required. However, at present there is no optimal method for the management of these injuries and even the gold standard treatment procedures fail to heal sites to their pre-injury state. Therefore, there is a need for a suitable treatment that can fully restore the functionality of these crucial regions.

This chapter will explore the distinct tissue organisation and composition of the ligament-bone interface. Describe the current strategies and associated outcomes related to the reconstruction of these regions, specifically regarding anterior cruciate ligament (ACL) repairs, and previous strategies in tissue engineering of each region of tissue present. Furthermore, this chapter will explore the use of nanoindentation on biological tissue and discuss the influence of scaffold design on cell behaviour.

1.1.1. Anatomy of Knee

The knee is one of the largest and most complex joints in the body. The knee joins the thighbone (femur) to the shinbone (tibia). A smaller bone runs alongside the tibia (fibula) and the kneecap (patella) sits in front of the joint to provide some protection. Two C-shaped pieces of cartilage called the medial and lateral menisci act as shock absorbers between the femur and tibia. Tendons connect the knee bones to the leg muscles that move the knee joint, whilst ligaments

are bands of tough elastic tissue that act like strong ropes to hold bones together and give support to joints (Figure 1.1).

The major ligaments in the knee joint are:

- **Patella ligaments-** It attaches the tibial tuberosity, a continuation of the quadriceps femoris tendon distal to the patella.
- **Collateral ligaments-** two strap-like ligaments found on the sides of the knees that control the sideways motion of the knee, bracing against excessive medial and lateral movement
- **Cruciate ligaments** - found on the inside of the joint connecting the femur and the tibia. In doing so, they cross each other to form an “X” with each other, hence the term ‘cruciate’. They control the back-and-forth motion of the knee.

The anterior cruciate ligament (ACL) runs diagonally in the middle of the knee and prevents the tibia from sliding out in front of the femur, as well as providing rotational stability to the knee. The ACL courses anteriorly, medially and distally across the joint and it passes from the femur to the tibia. As it does, it turns on itself in a slight outward (lateral) spiral.

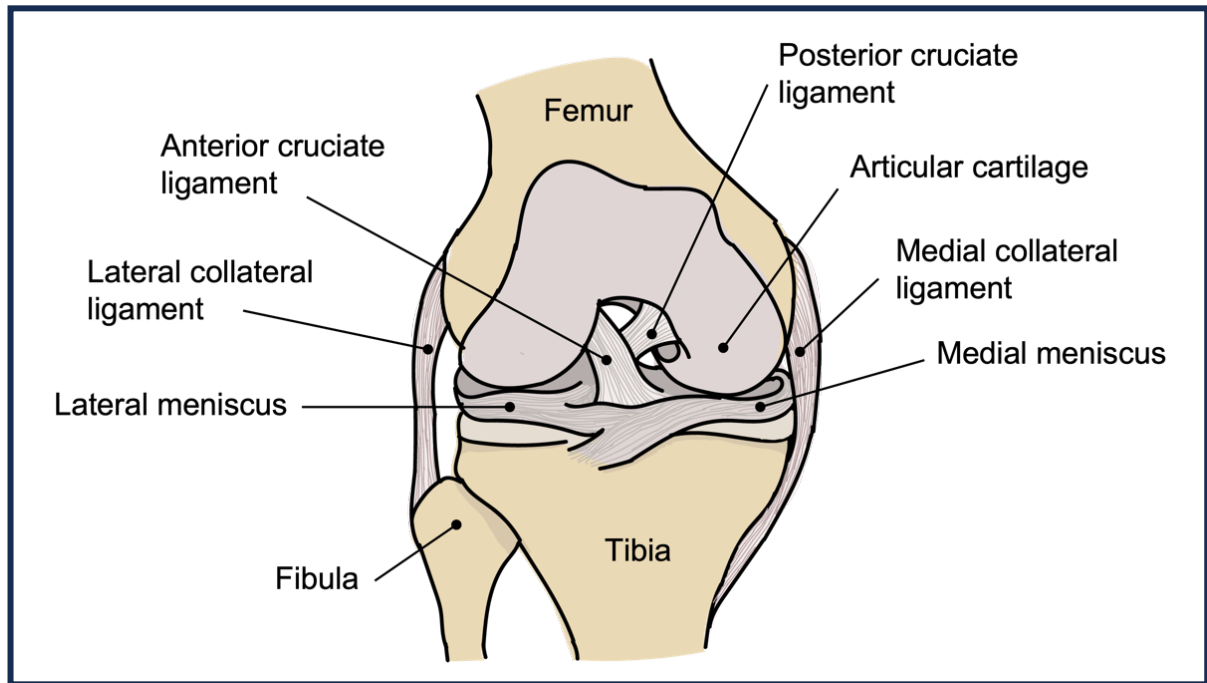


Figure 1.1: Anatomy of Knee

1.1.2. Anterior Cruciate Ligament Injuries

ACL injuries are common in young individuals who participate in sports activities associated with pivoting, decelerating and jumping [1], with more than 200,000 ACL rupture cases recorded annually in the US alone; accurate prevalence studies are difficult due to not all injuries being diagnosed [2], [3], [4]. The ACL is responsible for a large part of the knee's stability, therefore injury to the ACL is characterised by joint instability and an ACL tear often leads to the knee “giving out” and may often require surgical repair. Knee instability leads to poor quality of life, decreased activity, and increased risk of secondary osteoarthritis of the knee [5], [6], [7]. Approximately 70% of ruptures are sustained with minimal or no contact at the time of injury, such as sudden deceleration combined with changing direction or pivoting or landing with the knee in nearly full extension after a jump [8], [9].

In England, an estimated 30,000 primary ACL reconstruction surgeries are done each year [10], [11]. Based on the conservative estimate (n=15,000), in 2015 the annual cost of ACL

reconstruction to the UK National Health Service (NHS) was estimated to be £63 million [12]. Furthermore, the age-standardised rate of ACL reconstruction in the UK increased 12-fold between 1997 and 2017 from 2.0 surgeries per 100,000 population to 24.2 surgeries per 100,000 population [10]. Therefore, the financial strain that ACL reconstruction surgeries pose on the NHS is expected to rise each year with increasing needs.

1.2. Anatomy of Enthesis

The enthesis is a specialised tissue at the ligament-to-bone interface that connects two mechanically dissimilar materials: ligament (with a modulus $\sim 200\text{MPa}$), a compliant proteinaceous material with high toughness, and bone (modulus $\sim 20\text{GPa}$), a hard-mineralised tissue with a significantly higher modulus [13].

The attachment of dissimilar materials is prone to stress concentrations due to the mismatch in the deformation between the two phases. However, to alleviate these stress concentrations and allow for effective stress transfer, the healthy enthesis exhibits a complex hierarchical structure across multiple length scales. Interfacial tissues are integrated into a continuous gradient populated by a variety of cell types, and these cell types are accompanied by chemical factors and signalling modules that influence the maturation of these tissues and maintain homeostasis [14], [15], [16]. Following enthesis injury, the enthesis is unable to regain its uninjured morphological appearance, which increases the risk of recurrent injury [13], [17], [18].

1.2.1. Classification and Structure of Enthesis

Soft tissue insertion sites can be characterised as either direct or indirect. Direct entheses have a fibrocartilaginous region between the bone and the highly organised collagen fibres of the ligament. Conversely, indirect entheses, also known as fibrous, are usually observed on the shafts of long bones and have fibres that connect directly into bone (Sharpey's fibres). This report will focus on fibrocartilaginous insertions as they are more common and include the Anterior Cruciate Ligament (ACL).

The architecture of the enthesis is complex consisting of hierarchical arrangements of collagen fibrils, proteoglycans, and apatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ crystals. The molecular composition,

spatial distribution, and nano- to micro- scale assembly of these components dictate the properties of each tissue region and are critical for defining the mechanical stability of the entire enthesis.

As the name suggests, fibrocartilaginous entheses are sites where chondrogenesis has occurred. A typical fibrocartilaginous enthesis has four distinct zones that create a structurally continuous gradient from calcified bone to uncalcified ligament. These zones, in order, are bone, calcified fibrocartilage, uncalcified fibrocartilage, and oriented soft tissue, illustrated in Figure 1.2 [13], [19], [20]. Each of these different zones has a unique extracellular matrix (ECM). The ECM is the non-cellular component present within all tissues which provides essential physical scaffolding for the cellular constituents and initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis.

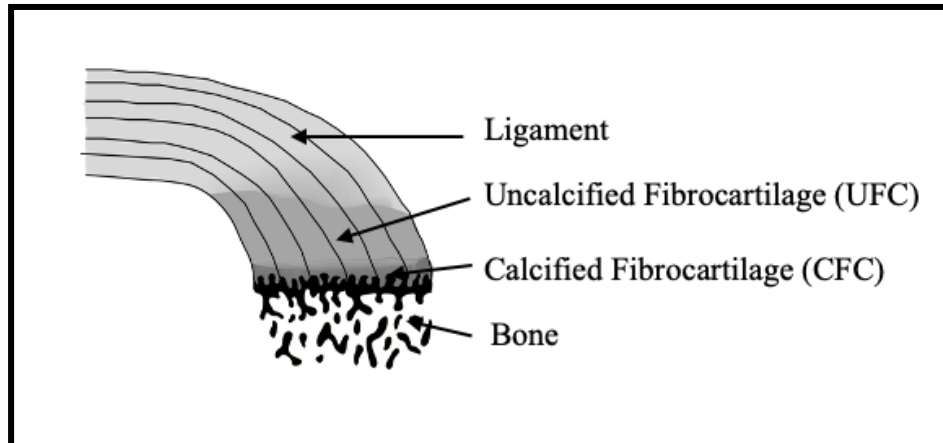


Figure 1.2: Illustration of fibrocartilaginous insertion

1.2.1.1. Bone

Bone is a natural solid bio-composite with a unique hierarchical structural organisation at multi-scale. The ECM of bone comprises an organic and an inorganic (calcified) portion. The inorganic portion which represents 50% of the dry weight of bone is composed of calcium and

phosphorus ions. This portion primarily consists of carbonate-substituted hydroxyapatite (CHA), with a composition of $\text{Ca}_{10}(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{OH})_2$ [21]. The organic portion consists of 95% type I collagen (which is very sparse in both fibrocartilage zones [14], [22]) with small amounts of ground substance containing glycoprotein, proteoglycans and glycosaminoglycan accounting for the remaining 5%.

1.2.1.2. Calcified fibrocartilage

Calcified fibrocartilage is a zone of calcified fibrocartilage consisting predominantly of collagen type II and a significant amount of type X, stiffened by mineral deposits and aggrecan [16].

1.2.1.3. Uncalcified fibrocartilage

Compositionally, the uncalcified fibrocartilage matrix consists of components typical of cartilage and is mainly composed of collagen type II, and also has high levels of pericellular collagen III [14], together with small amounts of types I, IX, and X as well as proteoglycans (mainly aggrecans) with associated chondroitin 4- and 6- sulphate (CS) glycosaminoglycans (GAGs). The collagen fibril bundles are not strictly parallel.

1.2.1.4. Ligament

This region consisting of fibrous connective tissue is characterised by the presence of parallel types I and III Collagen fibres with interspaced spindle-shaped fibroblasts organised in arrays of 70-150 μm in diameter. Type II collagen is only present in trace amounts, and is concentrated near the insertion site [23]. The ECM of ligaments is composed of a complex macro-molecular network with water making up about two-thirds of the weight, fibrillary protein collagen (predominantly types I and III) making up the majority of the remaining dry weight with small

amounts of elastin and ground substances accounting for the remaining weight [22], [23]. A normal ligament consists of about 90% fibrillary type I collagen with less than 10% small rectangular fibres of collagen type III situated in between the collagen type I bundles. Other collagen types are present in smaller quantities.

1.2.1.5. Tidemark

Traditionally, the outer limit of calcification was demarcated by a tidemark, signifying a sharp transition between the calcified and uncalcified fibrocartilage regions [26], [27]. However, recent evidence indicates the presence of a mineral gradient distributed across the insertion site, suggesting that this shift in composition may be more gradual and less distinct than previously thought [15], [20], [26]. The exact structure of the tide-mark for the ligament-to-bone interface is difficult to define as there is very little work done on this specific region. However, Lyons *et al.* [29] believe that the tidemark in articular cartilage is formed by two juxtaposed laminae with differing biochemical characteristics. They suggest that it inhibits hydroxyapatite crystal formation and growth after musculoskeletal maturity, protecting the cartilage from progressive mineralisation.

1.2.2. Enthesis Native Cell type

The interfacial region consists of a spatial distribution of cell types. Each cellular type found in the four regions of the enthesis has a distinctive behaviour, cell shape, matrix production profile, and generic expression that defines its phenotype. Moving along the interface, osteoblasts, osteoclasts, and osteocytes are the three main cell types found in bone. The three cells work together to maintain bone formation and homeostasis. Osteoblasts initiate new bone formation, while osteoclasts resorb and remodel bone, and osteocytes (a terminally differentiated osteoblast that resides in the mineralised matrix) maintain bone homeostasis. The

first transitional zone, calcified fibrocartilage, is regulated by hypertrophic fibrochondrocytes. Hypertrophy is the process in which chondrocytes increase in size and secrete type X collagen in a mineralised cartilaginous matrix [30]. Fibrochondrocytes that reside in the uncalcified fibrocartilage region of the enthesis are named as such because they exhibit phenotypic markers of both fibroblasts and chondrocytes [31]. Ligament and tendon cells are often described as exhibiting a fibroblastic phenotype due to their spindle shape and production of type I collagen [32]. These cell types are accompanied by biochemical and biomechanical cues that also vary by region, often with temporal and spatial gradients in concentration [32], [33], [34], [35].

1.3. Hierarchical Structure of Bone

Bone is a heterogeneous material containing three main phases; mineral, collagen and water, arranged in a series of hierarchical structures, as shown in Figure 1.3. Collagen contributes to bone's elastic and viscoelastic behaviour while mineral stiffens the overall material. The degree of mineralisation and collagen orientation highly influences mechanical properties. Mineralisation is limited by the underlying construction, thus restricting the range of mechanical properties found in a single type of tissue. Bound and unbound water contribute to elasticity and ductility.

1.3.1. Macrostructure

At the macrostructure, bone can be distinguished into the cortical (compact) and cancellous types (trabecular) types. In cross-section, the end of a long bone such as the femur (which attaches to the ACL) has a dense cortical shell with a porous, cancellous interior. Cortical bone forms the outer wall of all bones and, in the adult human skeleton, represents 80% of the skeleton mass. It is associated with mechanical properties and thereby, responsible for the supportive and protective function of the skeleton. The remaining 20%, cancellous bone, type is typically associated with metabolic functions, but also plays a role in biomechanical functions [34], [35].

1.3.2. Microstructure

Mineralised collagen fibres form into planar arrangements called lamellae (3-7 μm wide). In some cases, these sheets wrap in concentric layers (3-8 lamellae) around a central canal to form what is known as an osteon or a Haversian system. Cortical bone is composed of repeating osteon units, most of which are calcified (80 to 90%). The osteon looks like a cylinder about 200-250 μm in diameter running roughly parallel to the long axis of bone. The space between

osteons is occupied by interstitial lamellae, which are the remnants of osteons that were partially resorbed during the process of bone remodelling.

Cancellous bone is composed of bony trabecular struts and marrow-filled cavities where only 15 to 25% of the volume is calcified and the empty spaces that are usually filled with bone marrow are the source of undifferentiated cells. Trabecular struts are about 50-300 μ m in diameter. The degree of porosity of cancellous bone contrasts with the denser structure of the cortical bone irrigated by a series of fine channels filled with blood vessels. The outer and inner bone surfaces are covered by the periosteum and the endosteum membranes, respectively, which play important roles in the nutrition of the bone tissue and supply of osteoprogenitor cells, which divide by mitosis and differentiate into bone-forming cells for bone formation and repair.

1.3.3. Nanostructure

The trabeculae and osteon units are composed of collagen and apatite crystals (CaP). The most common perception of the arrangement of the collagen fibres in a lamella of an osteon is that they lie in parallel in each lamella, with a change in the orientation of fibrils from one lamella to the next described as “a twisted plywood or helicoidal structure” [38]. The mature CaP crystals are plate-shaped despite dahllite’s normal hexagonal crystal structure [39] (~50x25x2.5 nm [38], [39]). These plate-like apatite crystals of bone occur within the discrete spaces within the collagen fibrils and are roughly parallel to the long axes of the collagen fibrils. The primary organic component of the matrix is Type I collagen which is secreted by osteoblasts and self-assembled into fibrils. The collagen fibrils include a 67nm periodicity and 40nm gaps between collagen molecules. The CaP crystals are embedded in these gaps between collagen molecules and increase the rigidity of the bone. Water is present in the collagen

channels in bone unmineralised and mineralised bone (10 wt% constituents of bone [42]), and contributes to the structural and mechanical properties of bone [41], [42].

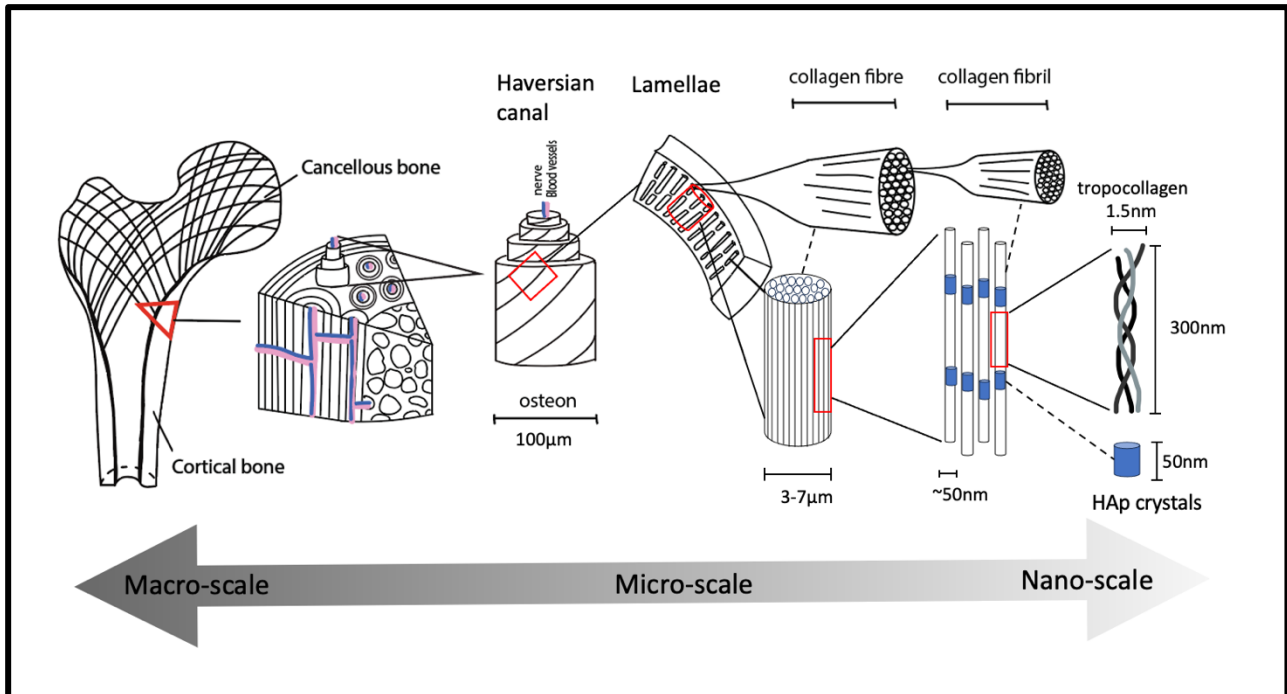


Figure 1.3: Scheme showing the hierarchical organisation of bone from the macro- to the nanoscale. Adapted from Gao et al. 2017 [45], and Feng et al. 2011 [46].

1.3.4. Mineral phase

To mineralise bone, bone-forming osteoblasts secrete vesicles containing alkaline phosphatase, which cleaves the phosphate groups and acts as the foci for calcium and phosphate precipitation [47]. Bone mineral is mainly composed of hydroxyapatite (HAp) with a Calcium/Phosphorus (Ca/P) ratio of less than 1.67 and contributes to bone stiffness. Natural HAp contains impurities such as carbonate (4-6%), sodium (0.9%) and magnesium (0.5%) ions as replacement groups in phosphate and hydroxyl sites, resulting in poorly crystalline, calcium-deficient and carbonated HAp [40], [44]. During the process of biomineralisation *in vivo*, the mineral content shows a pronounced local variation and increases from low to intermediate and complete

mineralisation [49]. The mineral phase of bone, as the stiff and brittle reinforcement of the collagen matrix, strongly influences the stiffness and breaking strain of bone: a highly mineralised bone matrix is stiffer but breaks earlier [46], [47].

1.4. Hierarchical structure of Ligament

1.4.1. Macrostructure

Ligaments are tough, silvery-white, dense connective tissue that connects bones directly and indirectly and stabilises kinematic articulations. The structure of the ligament is hierarchical in nature and is shown in Figure 1.4. On closer inspection of ligament surfaces, they often have a membrane on them (an ‘epiligament’ for extraarticular ligaments or synovium on intra-articular ligaments) which can obscure their fibrous detail. As this membrane is stripped away, the fibrous architecture of the ligament becomes more apparent. The epiligament can be seen as extremely cellular and vascular, also containing some sensory and proprioceptive nerves. The ligament itself is much less vascular and has limited access to blood supply.

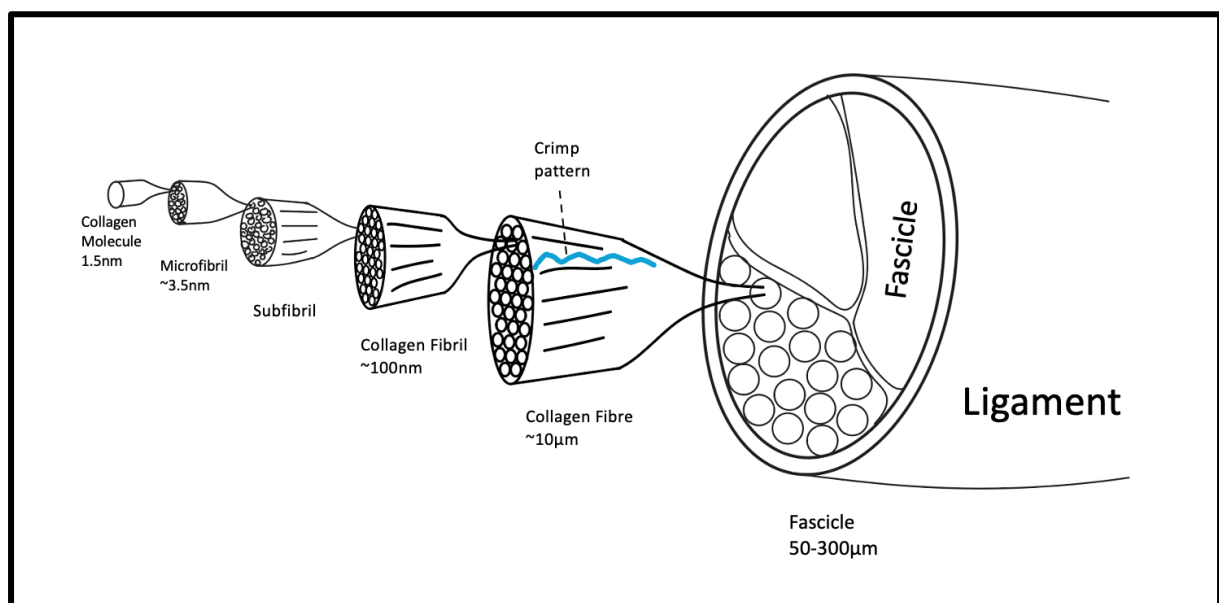


Figure 1.4: Scheme showing the hierarchical organisation of ligament from the macro- to the nanoscale. Adapted from Kastelic et al. 1978 [52].

1.4.2. Microstructure

At the microscopic levels, ligaments are much more complex, being composed of cells called fibroblasts which are surrounded by a matrix. Ligament microstructure can be visualised using polarised light and reveals ligaments are composed of bundled fascicles that are 50 to 300 μ m in diameter. The fibres have a multimodal distribution of diameter, and the diameter of the fibres varies along the length. It includes both small and large fibres (10 to 500nm) [53]. Most collagen fibres are aligned along the long axis of the ligament with a few small fibres appearing to ‘cross-connect’ in some way. It is difficult to separate these bundles, suggesting that they are interconnected in some ways. With joint movement, it is evident that there is interaction between fibres as they maintain the appearance of a single structure whilst individual fibres can be observed tightening or loosening depending on the bone position and the forces applied.

Ultrastructural studies have revealed that collagen fibres are composed of smaller fibrils, existing in a variety of sizes from about 60nm to about 4000nm in diameter [54] and that elastin, proteoglycans and minor collagens (e.g., type VI collagen) are found within the inter-fibrillar spaces [55]. Collagen fibres display in some areas a wavy appearance described as ‘crimping’ which can be observed in histology and scanning electron microscopy images along longitudinal sections. In ligaments, the periodical crimps of collagen fibres are composed of single collagen fibrils that sharply change directions called fibrillar crimps. Collagen fibrils and fibres exhibit crimp patterns at every 67 nm and 45 μ m, respectively [52]. The crimp pattern, at different levels of magnification, results in gradual stiffening under tensile loading. Therefore, fibrillar crimps forming fibre crimps represent functional shock absorbers of ligaments during motion. At the lower level, collagen fibrils are made up of microfibrils (3.5 nm).

1.4.3. Nanostructure

At the molecular level, the fundamental units from which collagen fibrils are assembled are tropocollagen molecules about 14 Å wide and 2800 Å long. Collagen appears to be secreted in this form by the connective-tissue cells called fibroblasts, and the tropocollagen molecules assemble extracellularly to form striated collagen fibrils [56].

1.5. ACL Reconstruction Treatment

Entheses are of particular concern to orthopaedic surgeons because of the need to re-attach ligament to bone – e.g. during the reconstruction of an anterior cruciate ligament. It is uniquely challenging to recreate the natural smooth transfer of load from ligament to bone that typifies the healthy, original attachment site [57]. A variety of techniques have been pioneered surgically that attempt to do so, but most simply involve stapling the ligament to the bone. More recently, various materials with viscous properties (e.g. hydroxyapatite) have been coated on the attachment site to encourage tissue regeneration [57].

1.5.1. Natural Grafts

Natural grafts can be classified as (i) autografts, (ii) allografts, and (iii) xenografts. Grafts harvested from the patient's tendon (partly) for the reconstruction of the torn ACL are termed autografts. Autografts reduce foreign body rejections, potential allergic reactions, and any disease transmission. The most common choices for autografts are bone-patellar-bone (BPTB) quadriceps tendon, and hamstring tendon (HT). Autograft-based ACL surgical methods demand more surgery time as well as recovery time due to additional incisions in the patient's body [58].

Since its advent in 1963, BPTB graft has been widely used in the primary surgical reconstruction of the ACL due to its strength, stiffness and potential for bone integration (due to the presence of bone plugs at its ends) [59]. Biomechanical studies on cadavers have shown that the middle third of the BPTB graft has an initial strength and stiffness comparable to, or maybe even greater than, those of native ACL [59]. However, Figure 1.5, illustrates the tensile strength of ACL and the patellar tendon (PT) used in BPTB grafts highlighting the difference in mechanical behaviour. The difference in the composition of ground substance and fibre

arrangement causes the tendon to stretch shorter and absorb higher loads. However, due to the shorter strain characteristics of PT autografts, there may be a mismatch in laxity in a PT-implanted knee as compared to the contralateral knee with a natural ACL.

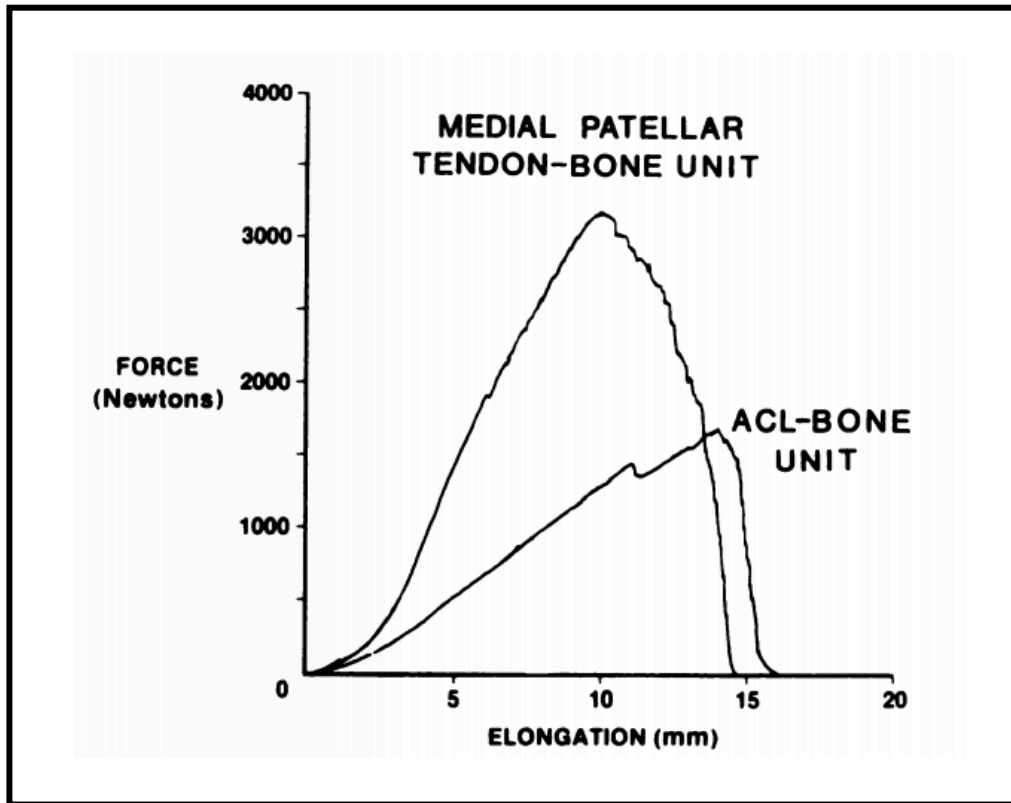


Figure 1.5: Tensile strength of ACL and the patellar tendon. Note the much larger stiffness, maximum load and failure energy for the tendon, and its abrupt failure [58].

After ACL reconstruction, the patellar or hamstring tendon undergoes several phases of healing in the intra-articular graft region and at the site of graft-to-bone incorporation. The changes in the biological and mechanical properties of the healing graft in its intraarticular region are referred to as ‘ligamentisation’ which occurs over 24 months after surgery. The biological changes from the time of reconstruction until around the 4th postoperative week is the early graft healing phase, characterised by graft necrosis, and hypocellularity. Graft site morbidity may also have had a detrimental effect on the process. The width of the graft and availability

of bony insertion points decide the success of the autograft for ACL replacement. The proliferation phase, between the 4th and 12th postoperative week, is characterised by a maximum of cellular activity and changes in the extra-cellular matrix, which is paralleled by the lowest mechanical properties of the reconstructed knee during healing. The ligamentisation phase follows directly after the proliferation phase and involves the ongoing process of continuous remodelling of the healing graft toward the morphology and mechanical strength of the intact cruciate. All autografts undergo weakening. Hence the initial strength of the autografts at the time of harvesting should be sufficiently larger than native ACL to make up for the loss in strength arising due to tissue necrosis after implantation. Noyes *et al.* [58] showed that the BPTB graft had a tensile strength four times greater than that of normal ACL. Historically, tendon grafts have undergone changes towards ligaments, but collagen distribution remains unchanged or minimally changed with grafts being unable to match the mechanical behaviour of native tissue even after ligamentisation [60], [61]. Furthermore, autograft surgeries have reported residual anterior knee laxity in reconstruction. [62], [63], [64], with one study reporting residual anterior knee laxity greater than 5 mm with BPTB grafts [63]. Autografts have the drawback of possible post-operative complications related to donor site morbidity. In the case of a BPTB graft, such complications include patellar fractures, weakening of the quadriceps muscles, patellar tendon rupture and patellar tendonitis [62], [63]. The frequency at which medium and long-term complications occur varies. Loss of extension of more than 5° in 2% of cases, anterior knee pain in 17% of cases, and infections at the surgical site in 0.5% of cases [63].

Grafts obtained from human cadavers are known as allografts. Patellar tendon, Achilles tendon, hamstring tendon and anterior/posterior tibialis are various options for allografts. Allograft-based replacement surgeries offer several advantages: reduced surgical time on the patient and

therefore a reduced recovery time, less donor site morbidity and a lower risk of post-operative pain [63], [64]. On the other hand, it has an increased surgical cost. Availability of donor, donor medical history, and sterilisation processes of allografts affect the quality of the graft for replacement. However, there are high chances of infection of certain diseases and rejection of the graft altogether. Also, the high temperature and pressure during the sterilisation process may alter the biomechanical properties. Disadvantages of allografts include the risk of transmission of infectious diseases (viral infections like HIV and HCV), immune rejection, delay in the remodelling and integration processes due to the sterilisation methods (gamma rays), as well as an inevitable increase in costs [66], [67].

A third source of natural grafts is from other animal species such as porcine and bovine, and these grafts are collectively termed xenografts. These grafts are similar to allografts but with a greater risk of transmission of diseases and foreign body rejection. Attempts at using xenografts have been unsuccessful mainly due to poor biocompatibility, insufficient biomechanical properties of the graft before implantation, and a lack of host integration [71].

1.5.2. Synthetic grafts

Silver, stainless steel, nylon and silk strings, and so on are a few materials experimented on for synthetic ligaments. Studies involving these materials did not clear animal studies due to early rupture and unsatisfied results. However, there currently exists clinical studies on patients who underwent ACL reconstruction with synthetic grafts and showed good results at medium and long-term follow-up. Lavoie *et al.* [72], at 8–45 months follow-up, reported 0 failures out of 47 patients, while Ventura *et al.* [73] reported 14 failures at 19-year follow-up of 51 patients. In both studies the Knee injury and Osteoarthritis Outcome Scores (KOOS) were good to excellent, ranging between 73.5 and 93 for the first study, and between 81 and 91 for the second

one. Although the synthetic graft decreases surgery time and eliminates complications due to donor site morbidity, it is still not widely used. This is down to negative previous experiences and the fact that it is judged based on poor results.

However, synthetic grafts include augmentation devices and permanent replacement. Augmentation devices provide initial protection to autografts until it matures, and revascularisation happens. Permanent replacement grafts include carbon fibre and polymer fibres. Various carbon fibre-based commercial grafts are no longer available, failing to make an impact due to high rupture rate, deposition of carbon particles in the liver tissues, and inflammatory responses in surrounding tissues. Whilst, commercial polymeric fibre-based grafts have been withdrawn due to leaching effects, one such graft was the polytetrafluoroethylene- (PTFE-) based “Gore-Tex” [74]. Other reasons for failure of polymeric grafts include low biocompatibility, poor abrasion and torsion resistance causing high rupture rates, and wear debris causing complications.

1.5.3. Engineered Biografts

Several attempts have been made to improve the repair of the enthesis *in vivo* with the use of novel materials being used to augment healing at the bone-tendon junction in animal models. Bone cement, most commonly used to fill large bone defects, are now being used to fill the gap between the tendon and bone during surgical repair, providing an osteoconductive material to induce bone ingrowth at the site of repair [75], [76], [77], [78], [79]. In general, increases in ultimate failure strength when compared to control groups [70], [71], positive histological effects at the junction with new bone formation [71], [72], improved regeneration of the fibrocartilaginous insertions, and reduced scar tissue formation [76] has been reported.

Healing of the bone-tendon interface has been facilitated through the use of polyglycolic acid (PGA) sheets which provide a rapidly degrading scaffold that stimulates cell growth and supports the newly forming tissue [80]. However, this method failed to promote the formation of a fibrocartilaginous interface, resulting in interface mechanical properties being lower than in a normal tendon model. Whilst PGA-tendon transition improved, PGA-bone interface remained weak.

Another material proposed to enhance healing at the bone-tendon interface is demineralised bone matrix (DBM), which provides a robust, osteoinductive scaffold at the site of repair [81]. DBM-treated animals showed significantly more fibrocartilaginous tissue and similar bone formation than the control group in an ovine patellar model after 6, 9 and 12 weeks. However, the mechanical strength of the interface has yet to be determined. Furthermore, Wong *et al* [82], reported on the use of an autologous cartilage plug that can be inserted between soft and hard tissue at the repair site, increasing fibrocartilaginous tissue formation.

In addition, bone marrow stem cells (BMSCs) resulted in no difference in cartilage production, collagen orientation or failure strength in the treatment group of a rat rotator cuff repair model [83]. However, coating the ends of a tendon allograft with BMSCs in a fibrin gel led to the formation of a zone of fibrocartilage and increased mechanical properties at the interface in a rabbit ACL replacement model [84]. Furthermore, the addition of BMSCs in fibrin glue alone was shown to increase the mechanical properties of the interface and result in an organised enthesis 45 days after surgery in a rat Achilles tendon model [85]. Similarly, MSCs transduced with a matrix metalloproteinase (MMP) gene exhibited increased fibrocartilage, load, stress, and stiffness at the insertion site after 4 weeks [86]. Finally, a chondrocyte pellet facilitated the formation of a fibrocartilaginous zone in a rabbit model [87], however, while the addition of

chondrocytes within a fibrin glue matrix resulted in increased mechanical properties when compared to controls, there was a lack of organised tissue structure at the interface [85].

1.6. Tissue Engineering Approaches

Current surgical methods for the repair of soft-to-hard tissue interface through the application of grafts still present several major problems, including tissue harvesting, slow or lack of tissue integration, donor tissue availability and risk of disease transmission. Additionally, following repair, graft sites are prone to reinjury, joint laxity and long rehabilitation periods [18], [85], due to the formation of neo-fibrovascular tissue, compromising the stability and consequently the mechanical performance of the newly formed tissue [88], [89]. Given the importance of these interfaces to joint mobility and quality of life, there is a critical need to be able to regenerate this complex region of tissue. In the past decades, a field of tissue engineering has arisen aiming at the recreation of complex and hierarchical interfacial tissues [90]. Tissue engineering is the development of biological substitutes capable of restoring damaged or diseased tissues to their original function [91].

Interface tissue engineering strategies can be focused on the generation of the interface alone or as a part of an integrated complex such as a bone-ligament graft. Commonly used biomaterials for the generation of bone include calcium phosphate ceramics (HAp in particular), natural materials (such as alginate) and collagen. Similarly, commonly used biomaterials for ligament/tendon tissue engineering include natural polymers (such as collagen and silk). The use of synthetic polymers has also been explored in tissue engineering bone and soft tissue.

1.6.1. Bone

Currently, the majority of scaffolds being used for tissue engineering bone applications are polymers, bioactive ceramics (glasses) and hybrids (composites) [92]. Naturally derived polymers that exhibit good biocompatibility, osteoconductivity and low immunogenicity are

those such as collagen, hyaluronic acid and chitosan [89], [90]. However, natural polymers possess disadvantages, the biggest being their low mechanical stability and degradation rate being difficult to control. Compared with other materials, bioceramics closely mimic bone tissue, providing greater osteoblast adherence and proliferation. Bioceramics are chemically similar to bone and show high compressive strength and low ductility. They provide a high resistance to deformation but are limited by their brittleness [95]. Due to the poor mechanical strength of pure collagen scaffolds, some kinds of bioceramics with a similar constituent to the intrinsic inorganic components of natural bone are widely used in conjunction with collagen scaffolds for bone regeneration. Along with the improvement of mechanical properties, they can also improve osteoconductive ability, and dimensional stability and increase the surface area for cell attachment [96].

Calcium phosphate (CaP) and its derivatives are commonly used ceramics that are combined with collagen for bone regeneration [97], as native bone is a combination of naturally occurring polymers and biological apatite. Recently, bioactive composite materials have emerged as a way to combine the advantages of two or more different materials. Composite materials improve the properties of scaffolds whilst allowing degradation to be controlled. By incorporating hydroxyapatite (HAp) into collagen scaffolds, the low compressive modulus of pure collagen scaffolds can be improved. Furthermore, the incorporation of HAp increases the surface area of collagen scaffolds and leads to incremental cellular adhesion [98]. Furthermore, apatite possesses the ability to form direct chemical bonds with the host bone tissue thus resulting in better and faster bone bonding between the scaffolds and neighbouring host bone tissue [99]. HAp/Collagen scaffolds are most commonly prepared by freeze-drying a suspension of collagen fibrils and HAp particles or precipitating HAp within a freeze-dried collagen scaffold. Kane *et al.* [96] prepared a HAp-reinforced collagen scaffold with improved

scaffold architecture (higher porosity and interconnected pores). Furthermore, they found that scaffolds reinforced with up to 60 vol% HAp exhibited fully recoverable elastic deformation upon loading to 50% compressive strain for 100,000 cycles.

1.6.2. Ligament

Type I collagen fibrils account for 60% of the dry mass of tendons and ligaments and are responsible for transferring forces between various musculoskeletal tissues. For this reason, collagen scaffolds have been widely studied to promote ligament repair. Dunn *et al* [100] and Bellincampi *et al* [101], have studied the use of type I collagen fibrils in potential ACL scaffolds. The grafts showed excellent biocompatibility and enhanced cell attachment, proliferation of the ACL fibroblasts adhered to these scaffolds, and produced an ECM. Along with naturally being a component of the native tissue, collagen can support ligament fibroblast growth under static tension and is therefore, a promising material to make scaffolds for ligament tissue engineering. Unfortunately, after 6 weeks the constructs were completely resorbed.

Goulet *et al* [102] reported on the decreasing mechanical strength of collagen scaffolds seeded with ACL fibroblasts. Furthermore, Murray *et al* [103], demonstrated that a collagen-glycosaminoglycan composite scaffold supported cell growth and the expression of fibroblast markers. Despite considerable efforts being made to try to improve the mechanical properties of collagen-based scaffolds, they have thus far not been able to mimic the strength of the natural ACL.

Some of the techniques that have been explored to improve the mechanical properties of collagen-based scaffolds have been to implore a special braid-twist design [100], [101], [106],

[107]. Altman *et al.*, [105] developed a tissue-engineered structure which is a matrix composed of twisted silk fibres. Bundles of silk fibres were wound into strands that were wound into cords and arranged to form the matrix. The twisted fibre architecture gave the scaffold mechanical properties similar to ACL (maximum load of $2337 \pm 72\text{N}$, elastic modulus $354 \pm 26\text{N/mm}$ and strain at failure $38.6 \pm 2.4\%$). Furthermore, the scaffolds demonstrated the three-phase mechanical behaviour seen in ligaments and tendons [105]. Furthermore, Walters *et al.* [104] were able to use 54 cross-linked reconstituted type I collagen fibres to create braid-twist scaffolds and chemically cross-linking their braid twists using zero-length cross-linker EDC (Figure 1.6). Several other studies developed ligament prostheses made of flexible composites consisting of fibres that have been woven or braided into structures [106], [108], [109], [107]. These designs enabled good control over the porosity of scaffolds through the combined changes in twisting angle, braiding angle, and filament diameter; overall scaffold porosity was found to alter the response of cells to the implant and implants were resistant to sudden rupture.

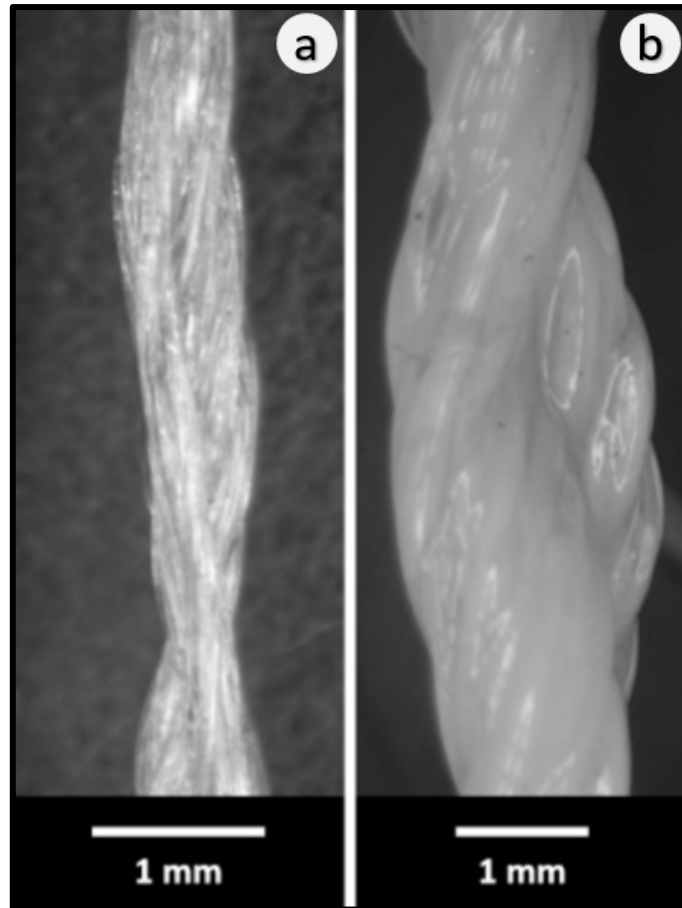


Figure 1.6: Picture of braid-twist type I collagen scaffold (a) dry braid, (b) wet braid [104].

Other groups have combined collagen with synthetic polymers in an effort to increase the mechanical strength of constructs. Panas-Perez *et al.* [110] combined Type I Collagen with silk fibres and showed that the constructs maintained their strength for 8 weeks after subcutaneous and intraarticular implantation in rabbits.

More recently, electrospinning has been used for the development of scaffolds for ligament tissue engineering [111]. This technique can be used to produce very thin fibres in the nanometer to micron range. Mizutani *et al.* [112] prepared a highly oriented ECM fibre scaffold by electrospinning and using elastin and collagen as the ECM proteins, trying to closely mimic the structure of the ligament. They observed cellular responses to these scaffolds and that the elastin scaffold hardly showed any TeM expression (known as a ligament marker) in ligament

cells relative to the cells on the collagen scaffold. However, ligament cells on the elastin scaffold showed higher ALP activity (known as an osteogenic marker) than cells on the collagen scaffold, suggesting that elastin scaffolds enabled osteogenic differentiation (differentiating ligament cells to osteoblasts). This indicates that they would be useful for reconstructing the interface region. Although elastin contributes 50% of elastic ligaments and can be found as parallel-oriented rope-like fibres throughout the length of the tissues [113], the use of elastin for tendon or ligament engineering has not been greatly explored.

1.6.3. Enthesis-multiphasic scaffolds

Kim *et al.* [114] were able to generate a tendon-to-bone construct using a combination of different biomaterials. The construct was composed of four layers: a tendon layer composed of collagen type I, a fibrocartilage layer composed of collagen cross-linked with chondroitin sulphate, a mineralised fibrocartilage layer composed of collagen and hydroxyapatite (low calcification) and a bone layer composed of collagen and hydroxyapatite (high calcification). Mechanical evaluations of the graded scaffold showed a change in mechanical properties through the structure: from the tendon layer to the bone there was an increase in Young's modulus paired with a decrease in elongation capacity. In addition, cell proliferation analysis of human fibroblasts, chondrocytes, and osteoblasts after 21 days of culture showed significantly increased proliferation of fibroblasts in the tendon layer, chondroblasts in the fibrocartilage layer, both chondroblasts and osteoblasts in the mineralised layer and osteoblasts in the bone layer. Their results showed how the mechanical properties of each layer are dependent on chemical composition and the importance of the matrix composition on cell survival and proliferation. Studies have shown that upon tensile deformation, the Young's modulus along the length of the scaffolds increased with an increasing mineral content whilst

elongation capacity decreased [115], [116], and osteogenesis of stem cells was promoted by increased mineral content [117].



Figure 1.7: Macroscopic image of multilayer collagen-based scaffold for tendon-to-bone tissue engineering, before cell culture [114].

The Lu group has developed a triphasic scaffold to mimic the fibrocartilaginous transition at the Tendon/Ligament-bone insertion site. [118], [119], [120]. The 3-layered scaffold was produced using polylactide-co-glycolide (PLGA) knitted sheets, PLGA microspheres seeded with and PLGA/bioactive glass microspheres to mimic the soft tissue, fibrocartilaginous, and bone regions respectively. The scaffolds were then seeded with cells, fibroblasts onto the PLGA sheets and osteoblasts onto the bioactive glass. Although cell migration into the central core was observed [119], there was no formation of a fibrocartilage-like region in the central core.

Another group successfully manufactured a continuous bone-soft tissue mimetic interface by seeding fibroblasts onto polymeric scaffolds containing a gradient of the transcription factor runt-related transcription factor 2 (Runx2) [121]. Runx2 is a bone-specific transcription factor that plays a critical role in bone development and osteoblast differentiation. By controlling the

spatial distribution of Runx2 within the scaffolds, osteoblastic differentiation and mineralised matrix deposition occurred in a graded fashion throughout the scaffold [122]. The mechanical properties of the scaffolds were examined by uniaxial tensile testing and increased mineral deposition resulted in increased construct stiffness, demonstrating the ability to produce a construct with graded mechanical properties with only one cell type, as opposed to the multicellular, multilayer constructs described elsewhere [118], [119], [120]. However, the fibrocartilaginous region was not present in these constructs therefore these constructs do not fully replicate the native bone-ligament insertion [121].

A study was conducted to evaluate the interaction of fibroblasts and osteoblast as would be found *in vivo* at the bone-ligament insertions. Although the interaction between the two cell types had a positive effect on the expression of matrix proteins present at the enthesis such as collagen type II, aggrecan and cartilage oligomeric matrix protein (COMP) [122], a fibrocartilaginous interface region was not formed in culture [123]. However, in subsequent studies, chondrocytes were included, and a fibrocartilage-like region was formed during triculture [120], highlighting the importance of chondrocytes for the successful formation of a fibrocartilaginous region.

1.7. Nanoindentation background

In cases where it is desirable to map the mechanical properties of a material and correlate the variations with changes in morphology or composition, micro/nano-mechanical contact techniques allow for region-specific mapping of material inhomogeneity [120], [121]. Therefore, nanoindentation is considered a powerful tool to conduct mechanical analyses of biomaterials, down to micro/nano-scale with nanometre depth and sub-nanonewton force resolution [9], [10]. While nanoindentation was initially developed to study stiff, elastic-plastic engineering materials, its use for examining viscoelastic material has become widespread [128], [129], [130], [131], [132], [133].

However, In a systematic study carried out by Rodriguez-Florez *et al.* [126] which compared mechanical properties of the same bone, using a broad range of testing parameters, it was concluded that the discrepancies in mechanical properties of bone measured using nanoindentation found in literature, should not only be attributed to the differences between the bones themselves but also to the testing and analysis protocols.

1.7.1. Nanoindentation of bone

To date, most of the indentation work on biological materials has focused on mineralised tissues, such as bone, tooth enamel and dentin [123], [130]–[138]. Nanoindentation techniques are more readily adapted to these systems because the materials are stiff, and the samples can be polished to present a flat surface to the indenter tip.

Bone behaves in a time-dependent manner due in part to its organic phase [139], [140] and its water content with corresponding poroelastic flow through the bone material [145]. Viscoelasticity in bone has been shown to correlate with hydration state [140], [142], [143] and

mineral content. The elastic modulus measured with nanoindentation has been shown to increase with degree of mineralisation (indentation results varied between ~24GPa and ~27GPa and were positively correlated to mineral content through quantitative backscattered electron imaging) [148], [149], [150].

The general approach for nanoindentation of bone has been to employ a range of techniques to eliminate creep effects. A long creep hold at maximum load can eliminate time-dependent behaviour on unloading and thus permits measurements of elastic properties [126], [147]. Whereas, a rapid unloading following no hold at peak load enables the determination of the unrelaxed modulus [152]. Another method eliminates viscoelastic effects by correlating the unloading stiffness [153] with the creep rate and contact depth during creep (measured during a hold at max load) [150], [151]. However, recent works have embraced nanoindentation as a tool to explore the time-dependent behaviour of biological materials [140], [152], [153]. Early studies on the viscoelasticity of dry bone, examined the role of loading rates or holding times [158]. The use of various loading rates demonstrated that elastic modulus was overestimated at “fast” indentation rates and that an extended hold time, of at least 30s, was required to eliminate the time-dependent plastic effect. Additional testing on dry bone specimens used a viscoelastic correction procedure to produce a corrected elastic modulus [155].

Hoffler *et al* [159], utilised nanoindentation techniques to measure microhardness and elastic modulus at the lamellar level of bone. The nanoindentation experiments produced measures of lamellar elastic moduli for human cortical bone (average 17.7 ± 4.0 GPa for osteons and 19.3 ± 4.7 GPa for interstitial bone tissue). Their preliminary results indicated that interstitial bone tissue has significantly higher modulus and hardness than osteonal bone tissue. Furthermore, the dependence of the elastic modulus in bone on the direction was shown by

Rho *et al* [127]. resulting in larger elastic moduli in the longitudinal direction than in the transverse (average 25.7 ± 1.7 GPa longitudinally and 16.6 ± 1.1 GPa for interstitial lamellae). This heterogeneity in elastic moduli of human bone at the lamellar level was observed also by other authors [160].

Preservation and processing are known to affect the physical and mechanical properties of mineralised tissues [142], [157], and indentation studies on bone have also reported significant increases in indentation moduli and hardness after dehydration (drying increased the elastic modulus between 9.7-15.4% and hardness by 12.2-17.6%) [162]. Despite this, the majority of published literature on bone indentation has been performed on dehydrated specimens embedded in epoxy or resin to facilitate sample preparation [162], [163], [164], [165]. In macroscopic dynamic mechanical tests, wet human cortical bone samples have been found to exhibit two or three times greater viscoelastic properties than their dry counterparts (average $\tan \delta$ $0.43 \pm 1.48 \times 10^{-1}$ for wet and $0.24 \pm 1.25 \times 10^{-1}$ for dry) [166], emphasising the need for testing samples that reflects bones natural hydrated states. Although dehydration and subsequent rehydration are known not to significantly affect the mechanical properties in the macroscale [167], [168], [169], the effect of such preparation techniques on the accuracy of sensitive surface probing techniques, such as nano-indentation is not yet known.

1.7.2. Nanoindentation of soft tissue

Over the past decade, nanoindentation has been used extensively to measure the elastic properties of calcified biological tissue, however, for compliant biological tissues, the application of this technique has proved to be much more challenging. However, despite these challenges nanoindentation has emerged in the tissue engineering community as a viable technique for the local mechanical assessment of healthy, diseased and replacement tissues,

owing to the in-situ tests and nondisruptive nature of nanoindentation techniques. Paired with histology findings, nanoindentation was used by Ebenstein *et al* [170], for the functional evaluation of cartilage repair tissue. By utilising a load-hold-unload profile and a small diameter spherical tip they were able to determine the inferior structural and mechanical properties of repair tissue. Furthermore, nanoindentation has been used to look at the influence of mineralisation in diseased arteries on the mechanical behaviour of the tissue, to generate input parameters for the computational models of diseased arteries [167], [168]. Healthy arteries have been characterised to serve as a baseline for comparison of diseased artery properties [173], [174], [175].

Other soft/compliant tissue studies have included quasi-static and dynamic mechanical analysis of the 20-40 micrometre thick stratum corneum layer of skin [176], the characterisation of viscoelastic properties of demineralised dentin [142], and the indentation of live fruit fly larvae at various developmental stages to investigate the role of the developmental stage on cuticle stiffness [177]. Also, the viscoelastic properties of liver tissues [178] and in vivo tests of skin [179].

Unlike calcified tissues, soft tissue specimens cannot be polished to provide a flat surface for testing. The resulting high surface roughness, in combination with a low elastic modulus, poses significant problems in data interpretation when using conventional nanoindentation testing methodologies that utilise a Berkovich indenter. To overcome the contact stiffness problem which can occur when using the sharp Berkovich indenter tips with soft tissue samples, flat punch and relatively large radius spherical indenter tips have been used [180], [181]. Ebenstein and Pruitt [180] presented a study of the indentation of gelatine and agarose gels, in which they concluded that the most reproducible results were obtained using conospherical indenter tips

with a radius of 100 μm . However, as the contact radius of the indenter tip increases, the achievable spatial resolution decreases. Therefore, large conospherical tips will measure the average mechanical properties of tissues rather than the micromechanical properties of individual micro-structural components.

Alternative methods used to negate contact stiffness problems have been to extend analyses beyond the widely adopted elastic models of Oliver and Pharr [128], [176], [177]. In such an analysis, Zhang *et al.* [128] were able to extract elastic-viscoelastic properties of polymeric materials using a sharp indenter tip by formulating an analytical solution based on the concepts proposed by Pharr [184] and Sakai [185].

1.7.3. Indentation across interfaces

There have been several studies that have mapped indentations across stiff-compliant tissue interfaces. The first studies to tackle these challenging interfaces were those focusing on the meniscal insertion site [182], [183]. It was found that the elastic moduli increase in a bi-linear fashion from the external ligamentous attachment to the subchondral bone (approximately 10Mpa to 900MPa, respectively) and that there is a gradient of stiffness from the superficial zones of the insertion close to the ligamentous attachment into the deeper zones of the bone.

Collagen distribution [33] and glycosaminoglycan (GAG) fraction [188] within the zones of the enthesis have been previously characterised, knowledge of the biomechanical characterisation of the zones may help in understanding how these zones assist in the strong fixation of the attachment of ACL to bone.

1.8. Influencing cell behaviour

Within a tissue, each cell is embedded in a mechanical environment and is constantly experiencing forces from its neighbours and the ECM. Adhesion to extracellular matrix and mechanical forces are known to modulate cell functions, including proliferation [189], [190], [191]. Each cell participates in the local force balance, either passively, by imposing a viscoelastic resistance to external force, or actively, via the constitutive tension of its cytoskeleton.

Cells are micro-sensitive; they can probe their mechanical environment, and sense forces exerted by the ECM through mechanosensitive activation of focal adhesion sites. This leads to an increase in the local actomyosin contractility, providing a way to sense the stiffness of the ECM. A basal contractile activity of the actomyosin connected to focal adhesions leads to a constant mechanical tug of war with the ECM, providing cells with a way to “feel” the compliance of their environment.

In vivo, cells are subjected to a complex array of biophysical and biochemical signals. These signals are processed and ‘integrated’ by the cells to regulate cell differentiation. Soluble factors, cell-matrix interactions and mechanical stimuli; which can be subdivided into ‘active’ and ‘passive’, affect cell differentiation. Soluble biochemicals such as growth factors, cytokines, and chemokines affect the morphogenesis of skeletal tissues. Furthermore, ECM proteins such as collagens, glycosaminoglycans, and proteoglycans provide instructive messages to cells that are transmitted across the cell membrane via the transmembrane receptors that recognise these ECM proteins. These cell-ECM interactions influence cell behaviour either directly or through crosstalk with growth factors [192], [193], [194], [195], [196]. Finally, cells have been shown to respond to external mechanical loading. For skeletal tissues, these ‘active’ external mechanical forces are required to maintain normal cell function

[197], [198], [199]. Physical characteristics of scaffolds such as roughness [200], [201], [202], micro- and nano- topography [201], [203] , surface energy [202] and porosity [204], [205], [206], [207] have been shown to influence cell behaviour. However, only recently has the stiffness of the ECM been appreciated as being a highly potent regulator of stem cell behaviour [208], [209], [210], which acts as a ‘passive’ mechanical cue [208].

It has been known for over a century that whereas soluble factors drive tissue development, mechanical forces dictate tissue pattern; in the case of bone, this governing principle by which bones can remodel following loading has the name, Wolff’s law. It states that bone in a healthy person or animal will adapt to the loads under which it is placed. If loading on a particular bone, increases, the bone will remodel itself over time to become stronger to resist that sort of loading. The remodelling of bone in response to loading is achieved via mechanotransduction, a process through which cells convert mechanical stimuli into a chemical response [211], [212]. Cells bind to a substrate with transmembrane molecules called integrins, which have an extracellular domain that attaches to the substrate and an intercellular domain that connects to the cytoskeleton. When cells bind to the ECM, integrins begin to cluster, which leads to the recruitment of structural and signalling proteins to form so-called focal adhesions at the site of integrin clustering. The formation and maturation of focal adhesions require the application of mechanical forces to these adhesions. Cells can actively generate these forces themselves using actin-myosin complexes which are part of the cytoskeleton. This is where substrate stiffness comes into play. On a hard substrate, cells generate large forces which leads to the formation of mature focal adhesions and a highly organised cytoskeleton with abundant stress fibres [209], [213]. In contrast, a soft substrate cannot provide enough resistance to counterbalance large, cell-generated forces. Therefore, on soft substrates, cells do not develop abundant stress fibres and generate smaller forces. In other words, a cell responds to differences in substrate

stiffness and adjusts its musculoskeletal system appropriately. Therefore, mechanical cues are recognised as central regulators of cell signalling and cell fate [214], [215], [216].

1.8.1. Scaffold Engineering

When focusing on the mechanical properties, a scaffold should be strong enough to withstand the loads that act on skeletal tissue at the defect site. Furthermore, the stiffness is important as it affects the strains acting on a cell while being attached to a scaffold [192], [193], [195], [217] [218]. If a scaffold is too stiff, it will not transfer external loads to cells as it will not deform under physiological loading conditions. However, the influence of mechanics on cell behaviour is not limited to external loading, even in the absence of external loads, the stiffness of a scaffold is important.

Current understanding suggests that to optimally promote cell growth, migration and differentiation, scaffold stiffness should match that of *in vivo* stiffness of the skeletal tissue under consideration. A scaffold should probably exhibit the stiffness of developing skeletal tissue, which is typically lower than the stiffness of mature tissue. However, in some cases, a stiffer scaffold may be more favourable to promote the invasion of the scaffold with cells from surrounding host tissue [219].

Since substrate stiffness and biochemical stimuli interact to determine cell behaviour, both parameters need to be controlled in scaffold design. Since natural polymers such as collagen provide ligands for cell binding, whilst synthetic polymers may require additional treatment to incorporate specific cell-binding sites, natural polymers are the more logical choice of scaffold material.

1.9. Aim

Most of our understanding of the enthesis is guided by studies that have focused on the tendon-bone enthesis. However, the force-elongation graph presented in Figure 1.5, illustrates that ligaments (ACL) and tendons (PT) present different mechanical behaviour and that a mismatch in laxity between grafts and the native tissue could cause graft failure. Therefore, a greater understanding of the native tissue is essential.

Tissue engineering strategies for enthesis development will focus on replicating the micro- and nano- structure of the native interface as well as its biochemical composition. The progressive multi-tissue transition observed at enthesis represents a significant challenge for scaffold design as the ideal scaffold should consist of several distinct yet continuous tissue regions such as the native insertion site. From literature, collagen-based scaffolds are identified as appealing products in tissue engineering strategies of bone, ligament and fibrocartilage tissues, as they are the main structural protein of most hard and soft tissue and play an important role in maintaining the biological and structural integrity of the extracellular matrix (ECM). However, collagen is most frequently used in its insoluble, naturally fibrillar form (polymeric collagen). The cross-links present in this form can lead to batch variations and limit tuneability of scaffolds. Furthermore, intact telopeptides have been linked with causing antigenicity [220]. Therefore, the soluble form of collagen, monomeric 'atelocollagen' was proposed in this research as an alternative material. While, to the author's knowledge, few studies have produced atelocollagen reconstituted fibrillar types I scaffolds with and without hydroxyapatite and type II with chondroitin sulphate incorporated, the combination of these to form multiphase scaffolds for the engineering of the complex ligament-bone enthesis is a novel approach.

Thus, the principal aims of this research are as follows:

1. To investigate the microstructure and chemical composition of native enthesis tissue along with the corresponding mechanical behaviour, to determine the optimum mechanical properties to be translated into the scaffold design.
2. To explore and evaluate the use of type I collagen fibre scaffolds for scaffold reinforcement.
3. To develop individual porous scaffold layers, corresponding to the 4 district regions within the ligament-bone enthesis, and multiphase scaffolds, by following the natural structure and composition of the interface, and using different types of collagen (type I and II) and other major components of this tissue (hydroxyapatite and chondroitin sulphate).
4. To investigate the properties of the fabricated porous scaffolds in terms of their microstructures and mechanical behaviour and to evaluate the stem cell response to these scaffolds.

1.10. References

- [1] R. Bahr and I. Holme, 'Risk factors for sports injuries--a methodological approach', *Br J Sports Med*, vol. 37, no. 5, pp. 384–392, 2003, doi: 10.1136/bjism.37.5.384.
- [2] N. G. Mohtadi, D. S. Chan, K. N. Dainty, and D. B. Whelan, 'Patellar tendon versus hamstring tendon autograft for anterior cruciate ligament rupture in adults', *Cochrane Database Syst Rev*, no. 9, p. CD005960, Sep. 2011, doi: 10.1002/14651858.CD005960.pub2.
- [3] K. P. Spindler and R. W. Wright, 'Anterior Cruciate Ligament Tear', *New England Journal of Medicine*, vol. 359, no. 20, pp. 2135–2142, Nov. 2008, doi: 10.1056/NEJMcp0804745.
- [4] C. C. Kaeding, B. Léger-St-Jean, and R. A. Magnussen, 'Epidemiology and Diagnosis of Anterior Cruciate Ligament Injuries', *Clinics in Sports Medicine*, vol. 36, no. 1, pp. 1–8, Jan. 2017, doi: 10.1016/j.csm.2016.08.001.
- [5] C. A. Thorstensson, L. S. Lohmander, R. B. Frobell, E. M. Roos, and R. Gooberman-Hill, 'Choosing surgery: patients' preferences within a trial of treatments for anterior cruciate ligament injury. A qualitative study', *BMC Musculoskelet Disord*, vol. 10, p. 100, Aug. 2009, doi: 10.1186/1471-2474-10-100.
- [6] T. Khan *et al.*, 'ACL and meniscal injuries increase the risk of primary total knee replacement for osteoarthritis: a matched case–control study using the Clinical Practice Research Datalink (CPRD)', *Br J Sports Med*, vol. 53, no. 15, pp. 965–968, Aug. 2019, doi: 10.1136/bjsports-2017-097762.
- [7] B. E. Øiestad, L. Engebretsen, K. Storheim, and M. A. Risberg, 'Knee osteoarthritis after anterior cruciate ligament injury: a systematic review', *Am J Sports Med*, vol. 37, no. 7, pp. 1434–1443, Jul. 2009, doi: 10.1177/0363546509338827.

- [8] L. M. Hernández, W. F. Micheo, and E. Amy, ‘Rehabilitation update for the anterior cruciate ligament injured patient: current concepts’, *Bol Asoc Med P R*, vol. 98, no. 1, pp. 62–72, 2006.
- [9] M. Abd Mutalib, M. A. Rahman, M. H. D. Othman, A. F. Ismail, and J. Jaafar, ‘Chapter 9 - Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray (EDX) Spectroscopy’, in *Membrane Characterization*, N. Hilal, A. F. Ismail, T. Matsuura, and D. Oatley-Radcliffe, Eds., Elsevier, 2017, pp. 161–179. doi: 10.1016/B978-0-444-63776-5.00009-7.
- [10] S. G. F. Abram, A. J. Price, A. Judge, and D. J. Beard, ‘Anterior cruciate ligament (ACL) reconstruction and meniscal repair rates have both increased in the past 20 years in England: hospital statistics from 1997 to 2017’, *Br J Sports Med*, vol. 54, no. 5, pp. 286–291, Mar. 2020, doi: 10.1136/bjsports-2018-100195.
- [11] S. S. Jameson, D. Dowen, P. James, I. Serrano-Pedraza, M. R. Reed, and D. Deehan, ‘Complications following anterior cruciate ligament reconstruction in the English NHS’, *Knee*, vol. 19, no. 1, pp. 14–19, Jan. 2012, doi: 10.1016/j.knee.2010.11.011.
- [12] L. Davies *et al.*, ‘Comparison of the clinical and cost effectiveness of two management strategies (rehabilitation versus surgical reconstruction) for non-acute anterior cruciate ligament (ACL) injury: study protocol for the ACL SNNAP randomised controlled trial’, *Trials*, vol. 21, no. 1, p. 405, May 2020, doi: 10.1186/s13063-020-04298-y.
- [13] S. Thomopoulos, G. M. Genin, and L. M. Galatz, ‘The development and morphogenesis of the tendon-to-bone insertion - what development can teach us about healing -’, *J Musculoskelet Neuronal Interact*, vol. 10, no. 1, pp. 35–45, Mar. 2010.
- [14] S. Font Tellado, E. R. Balmayor, and M. Van Griensven, ‘Strategies to engineer tendon/ligament-to-bone interface: Biomaterials, cells and growth factors’, *Adv. Drug Deliv. Rev.*, vol. 94, pp. 126–140, Nov. 2015, doi: 10.1016/j.addr.2015.03.004.

- [15] P. J. Yang and J. S. Temenoff, ‘Engineering orthopedic tissue interfaces’, *Tissue Eng Part B Rev*, vol. 15, no. 2, pp. 127–141, Jun. 2009, doi: 10.1089/ten.teb.2008.0371.
- [16] H. H. Lu and S. Thomopoulos, ‘Functional attachment of soft tissues to bone: development, healing, and tissue engineering’, *Annu Rev Biomed Eng*, vol. 15, pp. 201–226, 2013, doi: 10.1146/annurev-bioeng-071910-124656.
- [17] D. Wang *et al.*, ‘Kartogenin Enhances Collagen Organization and Mechanical Strength of the Repaired Enthesis in a Murine Model of Rotator Cuff Repair’, *Arthroscopy*, vol. 34, no. 9, pp. 2579–2587, Sep. 2018, doi: 10.1016/j.arthro.2018.04.022.
- [18] S. Thomopoulos, G. R. Williams, and L. J. Soslowsky, ‘Tendon to bone healing: differences in biomechanical, structural, and compositional properties due to a range of activity levels’, *J Biomech Eng*, vol. 125, no. 1, pp. 106–113, Feb. 2003, doi: 10.1115/1.1536660.
- [19] K. Messner and J. Gao, ‘The menisci of the knee joint. Anatomical and functional characteristics, and a rationale for clinical treatment’, *J. Anat.*, vol. 193 (Pt 2), pp. 161–178, Aug. 1998, doi: 10.1046/j.1469-7580.1998.19320161.x.
- [20] J. P. Spalazzi, A. L. Boskey, N. Pleshko, and H. H. Lu, ‘Quantitative mapping of matrix content and distribution across the ligament-to-bone insertion’, *PLoS ONE*, vol. 8, no. 9, p. e74349, 2013, doi: 10.1371/journal.pone.0074349.
- [21] A. Cahyanto, M. Maruta, K. Tsuru, S. Matsuya, and K. Ishikawa, ‘Fabrication of bone cement that fully transforms to carbonate apatite’, *Dental Materials Journal*, vol. 34, no. 3, pp. 394–401, 2015.
- [22] L. Rossetti *et al.*, ‘The microstructure and micromechanics of the tendon–bone insertion’, *Nature Materials*, vol. 16, no. 6, Art. no. 6, Jun. 2017, doi: 10.1038/nmat4863.

- [23] M. R. Buckley *et al.*, ‘Distributions of Types I, II and III Collagen by Region in the Human Supraspinatus Tendon’, *Connect Tissue Res*, vol. 54, no. 6, pp. 374–379, 2013, doi: 10.3109/03008207.2013.847096.
- [24] A. Hoffmann and G. Gross, ‘Tendon and ligament engineering in the adult organism: mesenchymal stem cells and gene-therapeutic approaches’, *Int Orthop*, vol. 31, no. 6, pp. 791–797, Dec. 2007, doi: 10.1007/s00264-007-0395-9.
- [25] S. L. Woo, K. Hildebrand, N. Watanabe, J. A. Fenwick, C. D. Papageorgiou, and J. H. Wang, ‘Tissue engineering of ligament and tendon healing’, *Clin. Orthop. Relat. Res.*, no. 367 Suppl, pp. S312–323, Oct. 1999, doi: 10.1097/00003086-199910001-00030.
- [26] M. Benjamin and J. R. Ralphs, ‘Fibrocartilage in tendons and ligaments--an adaptation to compressive load’, *J. Anat.*, vol. 193 (Pt 4), pp. 481–494, Nov. 1998, doi: 10.1046/j.1469-7580.1998.19340481.x.
- [27] M. Benjamin and J. R. Ralphs, ‘Entheses--the bony attachments of tendons and ligaments’, *Ital J Anat Embryol*, vol. 106, no. 2 Suppl 1, pp. 151–157, 2001.
- [28] G. M. Genin *et al.*, ‘Functional grading of mineral and collagen in the attachment of tendon to bone’, *Biophys. J.*, vol. 97, no. 4, pp. 976–985, Aug. 2009, doi: 10.1016/j.bpj.2009.05.043.
- [29] T. J. Lyons, R. W. Stoddart, S. F. McClure, and J. McClure, ‘The tidemark of the chondro-osseous junction of the normal human knee joint’, *J. Mol. Histol.*, vol. 36, no. 3, pp. 207–215, Mar. 2005, doi: 10.1007/s10735-005-3283-x.
- [30] E. J. Mackie, Y. A. Ahmed, L. Tatarczuch, K.-S. Chen, and M. Mirams, ‘Endochondral ossification: how cartilage is converted into bone in the developing skeleton’, *Int. J. Biochem. Cell Biol.*, vol. 40, no. 1, pp. 46–62, 2008, doi: 10.1016/j.biocel.2007.06.009.

- [31] J. Sanchez-Adams and K. A. Athanasiou, ‘The Knee Meniscus: A Complex Tissue of Diverse Cells’, *Cel. Mol. Bioeng.*, vol. 2, no. 3, p. 332, Jul. 2009, doi: 10.1007/s12195-009-0066-6.
- [32] M. Benjamin and J. R. Ralphs, ‘The cell and developmental biology of tendons and ligaments’, *Int. Rev. Cytol.*, vol. 196, pp. 85–130, 2000, doi: 10.1016/s0074-7696(00)96003-0.
- [33] J. Gao, ‘Immunolocalization of types I, II, and X collagen in the tibial insertion sites of the medial meniscus’, *Knee Surg Sports Traumatol Arthrosc*, vol. 8, no. 1, pp. 61–65, 2000, doi: 10.1007/s001670050013.
- [34] W. Petersen and B. Tillmann, ‘Structure and vascularization of the cruciate ligaments of the human knee joint’, *Anat. Embryol.*, vol. 200, no. 3, pp. 325–334, Sep. 1999, doi: 10.1007/s004290050283.
- [35] I.-N. E. Wang, S. Mitroo, F. H. Chen, H. H. Lu, and S. B. Doty, ‘Age-dependent changes in matrix composition and organization at the ligament-to-bone insertion’, *J. Orthop. Res.*, vol. 24, no. 8, pp. 1745–1755, Aug. 2006, doi: 10.1002/jor.20149.
- [36] T. Gong, J. Xie, J. Liao, T. Zhang, S. Lin, and Y. Lin, ‘Nanomaterials and bone regeneration’, *Bone Res*, vol. 3, p. 15029, 2015, doi: 10.1038/boneres.2015.29.
- [37] C. A. Moreira, D. W. Dempster, and R. Baron, ‘Anatomy and Ultrastructure of Bone – Histogenesis, Growth and Remodeling’, in *Endotext*, K. R. Feingold, B. Anawalt, A. Boyce, G. Chrousos, K. Dungan, A. Grossman, J. M. Hershman, G. Kaltsas, C. Koch, P. Kopp, M. Korbonits, R. McLachlan, J. E. Morley, M. New, L. Perreault, J. Purnell, R. Rebar, F. Singer, D. L. Trence, A. Vinik, and D. P. Wilson, Eds., South Dartmouth (MA): MDText.com, Inc., 2000. Accessed: Jun. 27, 2020. [Online]. Available: <http://www.ncbi.nlm.nih.gov/books/NBK279149/>

- [38] M. M. Giraud-Guille, 'Twisted plywood architecture of collagen fibrils in human compact bone osteons', *Calcif Tissue Int*, vol. 42, no. 3, pp. 167–180, May 1988, doi: 10.1007/BF02556330.
- [39] S. Weiner and W. Traub, 'Bone structure: from ångstroms to microns', *The FASEB Journal*, vol. 6, no. 3, pp. 879–885, 1992, doi: 10.1096/fasebj.6.3.1740237.
- [40] V. Ziv and S. Weiner, 'Bone crystal sizes: a comparison of transmission electron microscopic and X-ray diffraction line width broadening techniques', *Connect Tissue Res*, vol. 30, no. 3, pp. 165–175, 1994, doi: 10.3109/03008209409061969.
- [41] W. J. Landis, 'The strength of a calcified tissue depends in part on the molecular structure and organization of its constituent mineral crystals in their organic matrix', *Bone*, vol. 16, no. 5, pp. 533–544, May 1995, doi: 10.1016/8756-3282(95)00076-P.
- [42] M. J. Olszta *et al.*, 'Bone structure and formation: A new perspective', *Materials Science and Engineering: R: Reports*, vol. 58, no. 3–5, pp. 77–116, Nov. 2007, doi: 10.1016/j.mser.2007.05.001.
- [43] D. B. Burr, 'The contribution of the organic matrix to bone's material properties', *Bone*, vol. 31, no. 1, pp. 8–11, Jul. 2002, doi: 10.1016/s8756-3282(02)00815-3.
- [44] F. Gul-E-Noor, C. Singh, A. Papaioannou, N. Sinha, and G. S. Boutis, 'The Behavior of Water in Collagen and Hydroxyapatite Sites of Cortical Bone: Fracture, Mechanical Wear, and Load Bearing Studies', *J Phys Chem C Nanomater Interfaces*, vol. 119, no. 37, pp. 21528–21537, Sep. 2015, doi: 10.1021/acs.jpcc.5b06285.
- [45] C. Gao, S. Peng, P. Feng, and C. Shuai, 'Bone biomaterials and interactions with stem cells', *Bone Res*, vol. 5, no. 1, pp. 1–33, Dec. 2017, doi: 10.1038/boneres.2017.59.
- [46] L. Feng and I. Jasiuk, 'Multi-scale characterization of swine femoral cortical bone', *J Biomech*, vol. 44, no. 2, pp. 313–320, Jan. 2011, doi: 10.1016/j.jbiomech.2010.10.011.

- [47] Y. C. Chai *et al.*, ‘Current views on calcium phosphate osteogenicity and the translation into effective bone regeneration strategies’, *Acta Biomater*, vol. 8, no. 11, pp. 3876–3887, Nov. 2012, doi: 10.1016/j.actbio.2012.07.002.
- [48] H. A. Lowenstam and S. Weiner, *On Biomineralization*. Oxford, New York: Oxford University Press, 1989.
- [49] P. Fratzl, H. S. Gupta, E. P. Paschalis, and P. Roschger, ‘Structure and mechanical quality of the collagen–mineral nano-composite in bone’, *J. Mater. Chem.*, vol. 14, no. 14, pp. 2115–2123, Jul. 2004, doi: 10.1039/B402005G.
- [50] J. D. Currey, ‘How well are bones designed to resist fracture?’, *J. Bone Miner. Res.*, vol. 18, no. 4, pp. 591–598, Apr. 2003, doi: 10.1359/jbmr.2003.18.4.591.
- [51] J. D. Currey, *Bones: structure and mechanics*. 2002. Accessed: Jun. 28, 2020. [Online]. Available: <https://doi.org/10.1515/9781400849505>
- [52] J. Kastelic, A. Galeski, and E. Baer, ‘The Multicomposite Structure of Tendon’, *Connective Tissue Research*, vol. 6, no. 1, pp. 11–23, Jan. 1978, doi: 10.3109/03008207809152283.
- [53] V. Ottani, M. Raspanti, and A. Ruggeri, ‘Collagen structure and functional implications’, *Micron*, vol. 32, no. 3, pp. 251–260, Apr. 2001, doi: 10.1016/S0968-4328(00)00042-1.
- [54] C. B. Frank, D. A. Hart, and N. G. Shrive, ‘Molecular biology and biomechanics of normal and healing ligaments—a review’, *Osteoarthritis and Cartilage*, vol. 7, no. 1, pp. 130–140, Jan. 1999, doi: 10.1053/joca.1998.0168.
- [55] D. F. Bray, C. B. Frank, and R. C. Bray, ‘Cytochemical evidence for a proteoglycan-associated filamentous network in ligament extracellular matrix’, *J. Orthop. Res.*, vol. 8, no. 1, pp. 1–12, Jan. 1990, doi: 10.1002/jor.1100080102.

- [56] S. Zhang *et al.*, ‘Hierarchical ultrastructure: An overview of what is known about tendons and future perspective for tendon engineering’, *Bioactive Materials*, vol. 8, pp. 124–139, Feb. 2022, doi: 10.1016/j.bioactmat.2021.06.007.
- [57] C. J. Pendegrass, M. J. Oddy, S. R. Cannon, T. Briggs, A. E. Goodship, and G. W. Blunn, ‘A histomorphological study of tendon reconstruction to a hydroxyapatite-coated implant: Regeneration of a neo-enthesis in vivo’, *Journal of Orthopaedic Research*, vol. 22, no. 6, pp. 1316–1324, 2004, doi: 10.1016/j.orthres.2004.03.022.
- [58] F. R. Noyes, D. L. Butler, E. S. Grood, R. F. Zernicke, and M. S. Hefzy, ‘Biomechanical analysis of human ligament grafts used in knee-ligament repairs and reconstructions’, *J Bone Joint Surg Am*, vol. 66, no. 3, pp. 344–352, Mar. 1984.
- [59] K. G. Jones, ‘RECONSTRUCTION OF THE ANTERIOR CRUCIATE LIGAMENT. A TECHNIQUE USING THE CENTRAL ONE-THIRD OF THE PATELLAR LIGAMENT’, *J Bone Joint Surg Am*, vol. 45, pp. 925–932, Jul. 1963.
- [60] A. Weiler, G. Peters, J. Mäurer, F. N. Unterhauser, and N. P. Südkamp, ‘Biomechanical Properties and Vascularity of an Anterior Cruciate Ligament Graft can be Predicted by Contrast-Enhanced Magnetic Resonance Imaging: A Two-Year Study in Sheep*’, *Am J Sports Med*, vol. 29, no. 6, pp. 751–761, Nov. 2001, doi: 10.1177/03635465010290061401.
- [61] E. Kondo, K. Yasuda, T. Katsura, R. Hayashi, Y. Kotani, and H. Tohyama, ‘Biomechanical and histological evaluations of the doubled semitendinosus tendon autograft after anterior cruciate ligament reconstruction in sheep’, *Am J Sports Med*, vol. 40, no. 2, pp. 315–324, Feb. 2012, doi: 10.1177/0363546511426417.
- [62] S. Li, Y. Chen, Z. Lin, W. Cui, J. Zhao, and W. Su, ‘A systematic review of randomized controlled clinical trials comparing hamstring autografts versus bone-patellar tendon-bone autografts for the reconstruction of the anterior cruciate ligament’, *Arch Orthop*

- Trauma Surg*, vol. 132, no. 9, pp. 1287–1297, Sep. 2012, doi: 10.1007/s00402-012-1532-5.
- [63] K. B. Freedman, M. J. D’Amato, D. D. Nedeff, A. Kaz, and B. R. Bach, ‘Arthroscopic anterior cruciate ligament reconstruction: a metaanalysis comparing patellar tendon and hamstring tendon autografts’, *Am J Sports Med*, vol. 31, no. 1, pp. 2–11, Feb. 2003, doi: 10.1177/03635465030310011501.
- [64] L. S. Beasley *et al.*, ‘Anterior cruciate ligament reconstruction: A literature review of the anatomy, biomechanics, surgical considerations, and clinical outcomes’, *Operative Techniques in Orthopaedics*, vol. 15, no. 1, pp. 5–19, Jan. 2005, doi: 10.1053/j.oto.2004.11.003.
- [65] R. A. Marder, J. R. Raskind, and M. Carroll, ‘Prospective evaluation of arthroscopically assisted anterior cruciate ligament reconstruction. Patellar tendon versus semitendinosus and gracilis tendons’, *Am J Sports Med*, vol. 19, no. 5, pp. 478–484, Oct. 1991, doi: 10.1177/036354659101900510.
- [66] L. C. Almekinders, T. Moore, D. Freedman, and T. N. Taft, ‘Post-operative problems following anterior cruciate ligament reconstruction’, *Knee Surg Sports Traumatol Arthrosc*, vol. 3, no. 2, pp. 78–82, 1995, doi: 10.1007/BF01552379.
- [67] H. Cd and L. My, ‘Future of Allografts in Sports Medicine’, *Clinics in sports medicine*. Accessed: Jun. 01, 2020. [Online]. Available: <https://pubmed.ncbi.nlm.nih.gov/19306738/>
- [68] B. Br and J. Dl, ‘Allografts in Knee Surgery’, *Orthopedics*. Accessed: Jun. 01, 2020. [Online]. Available: <https://pubmed.ncbi.nlm.nih.gov/18019985/>
- [69] M. J. Gibbons, D. L. Butler, E. S. Grood, D. I. Bylski-Austrow, M. S. Levy, and F. R. Noyes, ‘Effects of gamma irradiation on the initial mechanical and material properties

- of goat bone-patellar tendon-bone allografts', *J. Orthop. Res.*, vol. 9, no. 2, pp. 209–218, Mar. 1991, doi: 10.1002/jor.1100090209.
- [70] G. A. Godette, J. A. Kopta, and D. M. Egle, 'Biomechanical effects of gamma irradiation on fresh frozen allografts in vivo', *Orthopedics*, vol. 19, no. 8, pp. 649–653, Aug. 1996.
- [71] L. Buhler, Y. Xu, W. Li, A. Zhu, and D. K. C. Cooper, 'An investigation of the specificity of induced anti-pig antibodies in baboons', *Xenotransplantation*, vol. 10, no. 1, pp. 88–93, Jan. 2003, doi: 10.1034/j.1399-3089.2003.01122.x.
- [72] P. Lavoie, J. Fletcher, and N. Duval, 'Patient satisfaction needs as related to knee stability and objective findings after ACL reconstruction using the LARS artificial ligament', *Knee*, vol. 7, no. 3, pp. 157–163, Jul. 2000, doi: 10.1016/s0968-0160(00)00039-9.
- [73] A. Ventura, C. Terzaghi, C. Legnani, E. Borgo, and W. Albisetti, 'Synthetic grafts for anterior cruciate ligament rupture: 19-year outcome study', *Knee*, vol. 17, no. 2, pp. 108–113, Mar. 2010, doi: 10.1016/j.knee.2009.07.013.
- [74] P. A. Indelicato, M. S. Pascale, and M. O. Huegel, 'Early experience with the GORE-TEX polytetrafluoroethylene anterior cruciate ligament prosthesis', *Am J Sports Med*, vol. 17, no. 1, pp. 55–52, 1989, doi: 10.1177/036354658901700109.
- [75] C.-Y. Wen, L. Qin, K.-M. Lee, and K.-M. Chan, 'The use of brushite calcium phosphate cement for enhancement of bone-tendon integration in an anterior cruciate ligament reconstruction rabbit model', *J. Biomed. Mater. Res. Part B Appl. Biomater.*, vol. 89, no. 2, pp. 466–474, May 2009, doi: 10.1002/jbm.b.31236.
- [76] C. Uggen, J. Dines, M. McGarry, D. Grande, T. Lee, and O. Limpisvasti, 'The effect of recombinant human platelet-derived growth factor BB-coated sutures on rotator cuff healing in a sheep model', *Arthroscopy*, vol. 26, no. 11, pp. 1456–1462, Nov. 2010, doi: 10.1016/j.arthro.2010.02.025.

- [77] Y. C. Tien, T. T. Chih, J. H. C. Lin, C. P. Ju, and S. D. Lin, 'Augmentation of tendon-bone healing by the use of calcium-phosphate cement', *J Bone Joint Surg Br*, vol. 86, no. 7, pp. 1072–1076, Sep. 2004, doi: 10.1302/0301-620x.86b7.14578.
- [78] W. J. Robertson, J. D. Hatch, and S. A. Rodeo, 'Evaluation of tendon graft fixation using alpha-BSM calcium phosphate cement', *Arthroscopy*, vol. 23, no. 10, pp. 1087–1092, Oct. 2007, doi: 10.1016/j.arthro.2007.04.007.
- [79] D. Kovacevic *et al.*, 'Calcium-phosphate matrix with or without TGF- β 3 improves tendon-bone healing after rotator cuff repair', *Am J Sports Med*, vol. 39, no. 4, pp. 811–819, Apr. 2011, doi: 10.1177/0363546511399378.
- [80] S. Yokoya, Y. Mochizuki, Y. Nagata, M. Deie, and M. Ochi, 'Tendon-bone insertion repair and regeneration using polyglycolic acid sheet in the rabbit rotator cuff injury model', *Am J Sports Med*, vol. 36, no. 7, pp. 1298–1309, Jul. 2008, doi: 10.1177/0363546508314416.
- [81] S. Sundar, C. J. Pendegrass, and G. W. Blunn, 'Tendon bone healing can be enhanced by demineralized bone matrix: a functional and histological study', *J. Biomed. Mater. Res. Part B Appl. Biomater.*, vol. 88, no. 1, pp. 115–122, Jan. 2009, doi: 10.1002/jbm.b.31157.
- [82] M. W. N. Wong, L. Qin, K. M. Lee, and K. S. Leung, 'Articular cartilage increases transition zone regeneration in bone-tendon junction healing', *Clin. Orthop. Relat. Res.*, vol. 467, no. 4, pp. 1092–1100, Apr. 2009, doi: 10.1007/s11999-008-0606-8.
- [83] L. V. Gulotta, D. Kovacevic, J. R. Ehteshami, E. Dagher, J. D. Packer, and S. A. Rodeo, 'Application of bone marrow-derived mesenchymal stem cells in a rotator cuff repair model', *Am J Sports Med*, vol. 37, no. 11, pp. 2126–2133, Nov. 2009, doi: 10.1177/0363546509339582.

- [84] M. Y. H. Soon, A. Hassan, J. H. P. Hui, J. C. H. Goh, and E. H. Lee, ‘An analysis of soft tissue allograft anterior cruciate ligament reconstruction in a rabbit model: a short-term study of the use of mesenchymal stem cells to enhance tendon osteointegration’, *Am J Sports Med*, vol. 35, no. 6, pp. 962–971, Jun. 2007, doi: 10.1177/0363546507300057.
- [85] G. Nourissat *et al.*, ‘Mesenchymal stem cell therapy regenerates the native bone-tendon junction after surgical repair in a degenerative rat model’, *PLoS ONE*, vol. 5, no. 8, p. e12248, Aug. 2010, doi: 10.1371/journal.pone.0012248.
- [86] L. V. Gulotta, D. Kovacevic, S. Montgomery, J. R. Ehteshami, J. D. Packer, and S. A. Rodeo, ‘Stem cells genetically modified with the developmental gene MT1-MMP improve regeneration of the supraspinatus tendon-to-bone insertion site’, *Am J Sports Med*, vol. 38, no. 7, pp. 1429–1437, Jul. 2010, doi: 10.1177/0363546510361235.
- [87] M. W. N. Wong, L. Qin, J. K. O. Tai, S. K. M. Lee, K. S. Leung, and K. M. Chan, ‘Engineered allogeneic chondrocyte pellet for reconstruction of fibrocartilage zone at bone-tendon junction--a preliminary histological observation’, *J. Biomed. Mater. Res. Part B Appl. Biomater.*, vol. 70, no. 2, pp. 362–367, Aug. 2004, doi: 10.1002/jbm.b.30049.
- [88] C. C. Kaeding *et al.*, ‘Allograft Versus Autograft Anterior Cruciate Ligament Reconstruction’, *Sports Health*, vol. 3, no. 1, pp. 73–81, Jan. 2011, doi: 10.1177/1941738110386185.
- [89] P. Lui, P. Zhang, K. Chan, and L. Qin, ‘Biology and augmentation of tendon-bone insertion repair’, *J Orthop Surg Res*, vol. 5, p. 59, Aug. 2010, doi: 10.1186/1749-799X-5-59.
- [90] Edward. D. Bonnevie and Robert. L. Mauck, ‘Physiology and Engineering of the Graded Interfaces of Musculoskeletal Junctions’, *Annual Review of Biomedical Engineering*, vol. 20, pp. 403–429, 2018.

- [91] D. F. Williams, ‘On the nature of biomaterials’, *Biomaterials*, vol. 30, no. 30, pp. 5897–5909, Oct. 2009, doi: 10.1016/j.biomaterials.2009.07.027.
- [92] F. Matassi, L. Nistri, D. Chicon Paez, and M. Innocenti, ‘New biomaterials for bone regeneration’, *Clin Cases Miner Bone Metab*, vol. 8, no. 1, pp. 21–24, 2011.
- [93] M. Jafari *et al.*, ‘Polymeric scaffolds in tissue engineering: a literature review’, *J Biomed Mater Res B Appl Biomater*, vol. 105, no. 2, pp. 431–459, Feb. 2017, doi: 10.1002/jbm.b.33547.
- [94] L. Gasperini, J. F. Mano, and R. L. Reis, ‘Natural polymers for the microencapsulation of cells’, *J R Soc Interface*, vol. 11, no. 100, p. 20140817, Nov. 2014, doi: 10.1098/rsif.2014.0817.
- [95] C. Gao *et al.*, ‘Current Progress in Bioactive Ceramic Scaffolds for Bone Repair and Regeneration’, *Int J Mol Sci*, vol. 15, no. 3, pp. 4714–4732, Mar. 2014, doi: 10.3390/ijms15034714.
- [96] R. J. Kane *et al.*, ‘Hydroxyapatite reinforced collagen scaffolds with improved architecture and mechanical properties’, *Acta Biomater*, vol. 17, pp. 16–25, Apr. 2015, doi: 10.1016/j.actbio.2015.01.031.
- [97] K. Rezwan, Q. Z. Chen, J. J. Blaker, and A. R. Boccaccini, ‘Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering’, *Biomaterials*, vol. 27, no. 18, pp. 3413–3431, Jun. 2006, doi: 10.1016/j.biomaterials.2006.01.039.
- [98] A. Sionkowska and J. Kozłowska, ‘Properties and modification of porous 3-D collagen/hydroxyapatite composites’, *International Journal of Biological Macromolecules*, vol. 52, pp. 250–259, Jan. 2013, doi: 10.1016/j.ijbiomac.2012.10.002.
- [99] D. Pasqui, P. Torricelli, M. D. Cagna, M. Fini, and R. Barbucci, ‘Carboxymethyl cellulose—hydroxyapatite hybrid hydrogel as a composite material for bone tissue

- engineering applications’, *Journal of Biomedical Materials Research Part A*, vol. 102, no. 5, pp. 1568–1579, 2014, doi: 10.1002/jbm.a.34810.
- [100] M. G. Dunn, J. B. Liesch, M. L. Tiku, and J. P. Zawadsky, ‘Development of fibroblast-seeded ligament analogs for ACL reconstruction’, *J. Biomed. Mater. Res.*, vol. 29, no. 11, pp. 1363–1371, Nov. 1995, doi: 10.1002/jbm.820291107.
- [101] L. D. Bellincampi, R. F. Closkey, R. Prasad, J. P. Zawadsky, and M. G. Dunn, ‘Viability of fibroblast-seeded ligament analogs after autogenous implantation’, *J. Orthop. Res.*, vol. 16, no. 4, pp. 414–420, Jul. 1998, doi: 10.1002/jor.1100160404.
- [102] F. Goulet, D. Rancourt, R. Cloutier, L. Gearmain, A. Poole, and F. Auger, ‘Tendons and ligaments’, in *Principles of Tissue Engineering*, London: Elsevier Academic Press, 2011, pp. 911–914.
- [103] M. M. Murray and M. Spector, ‘The migration of cells from the ruptured human anterior cruciate ligament into collagen-glycosaminoglycan regeneration templates in vitro’, *Biomaterials*, vol. 22, no. 17, pp. 2393–2402, Sep. 2001, doi: 10.1016/s0142-9612(00)00426-9.
- [104] V. I. Walters, A. L. Kwansa, and J. W. Freeman, ‘Design and Analysis of Braid-Twist Collagen Scaffolds’, *Connective Tissue Research*, vol. 53, no. 3, pp. 255–266, Jun. 2012, doi: 10.3109/03008207.2011.634532.
- [105] G. H. Altman *et al.*, ‘Silk matrix for tissue engineered anterior cruciate ligaments’, *Biomaterials*, vol. 23, no. 20, pp. 4131–4141, Oct. 2002, doi: 10.1016/s0142-9612(02)00156-4.
- [106] F. H. Silver, A. J. Tria, J. P. Zawadsky, and M. G. Dunn, ‘Anterior cruciate ligament replacement: a review’, *J Long Term Eff Med Implants*, vol. 1, no. 2, pp. 135–154, 1991.
- [107] E. J. Chung, M. J. Sugimoto, J. L. Koh, and G. A. Ameer, ‘A biodegradable tri-component graft for anterior cruciate ligament reconstruction’, *Journal of Tissue*

- Engineering and Regenerative Medicine*, vol. 11, no. 3, pp. 704–712, 2017, doi: 10.1002/term.1966.
- [108] J. W. Freeman, M. D. Woods, and C. T. Laurencin, ‘Tissue Engineering of the Anterior Cruciate Ligament Using a Braid-Twist Scaffold Design’, *J Biomech*, vol. 40, no. 9, pp. 2029–2036, 2007, doi: 10.1016/j.jbiomech.2006.09.025.
- [109] H. H. Lu *et al.*, ‘Anterior cruciate ligament regeneration using braided biodegradable scaffolds: in vitro optimization studies’, *Biomaterials*, vol. 26, no. 23, pp. 4805–4816, Aug. 2005, doi: 10.1016/j.biomaterials.2004.11.050.
- [110] T. Nau and A. Teuschl, ‘Regeneration of the anterior cruciate ligament: Current strategies in tissue engineering’, *World J Orthop*, vol. 6, no. 1, pp. 127–136, Jan. 2015, doi: 10.5312/wjo.v6.i1.127.
- [111] R. D. Cardwell, L. A. Dahlgren, and A. S. Goldstein, ‘Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage’, *J Tissue Eng Regen Med*, vol. 8, no. 12, pp. 937–945, Dec. 2014, doi: 10.1002/term.1589.
- [112] N. Mizutani, S. Kageyama, M. Yamada, M. Hasegawa, K. Miyamoto, and T. Horiuchi, ‘The behavior of ligament cells cultured on elastin and collagen scaffolds’, *J Artif Organs*, vol. 17, no. 1, pp. 50–59, Mar. 2014, doi: 10.1007/s10047-013-0736-y.
- [113] N. Annabi, S. M. Mithieux, G. Camci-Unal, M. R. Dokmeci, A. S. Weiss, and A. Khademhosseini, ‘Elastomeric Recombinant Protein-based Biomaterials’, *Biochem. Eng. J.*, vol. 77, pp. 110–118, Aug. 2013, doi: 10.1016/j.bej.2013.05.006.
- [114] B. S. Kim, E. J. Kim, J. S. Choi, J. H. Jeong, C. H. Jo, and Y. W. Cho, ‘Human collagen-based multilayer scaffolds for tendon-to-bone interface tissue engineering’, *Journal of Biomedical Materials Research - Part A*, vol. 102, no. 11, pp. 4044–4054, Nov. 2014, doi: 10.1002/jbm.a.35057.

- [115] C. Erisken, D. M. Kalyon, and H. Wang, 'Functionally graded electrospun polycaprolactone and beta-tricalcium phosphate nanocomposites for tissue engineering applications.', *Biomaterials.*, vol. 29, no. 30, pp. 4065–4073, Oct. 2008, doi: 10.1016/j.biomaterials.2008.06.022.
- [116] X. Li, J. Xie, J. Lipner, X. Yuan, S. Thomopoulos, and Y. Xia, 'Nanofiber scaffolds with gradations in mineral content for mimicking the tendon-to-bone insertion site', *Nano Lett.*, vol. 9, no. 7, pp. 2763–2768, Jul. 2009, doi: 10.1021/nl901582f.
- [117] W. Liu, J. Lipner, J. Xie, C. N. Manning, S. Thomopoulos, and Y. Xia, 'Nanofiber scaffolds with gradients in mineral content for spatial control of osteogenesis', *ACS Appl Mater Interfaces*, vol. 6, no. 4, pp. 2842–2849, Feb. 2014, doi: 10.1021/am405418g.
- [118] J. P. Spalazzi, E. Dagher, S. B. Doty, X. E. Guo, S. A. Rodeo, and H. H. Lu, 'In vivo evaluation of a tri-phasic composite scaffold for anterior cruciate ligament-to-bone integration', *Conf Proc IEEE Eng Med Biol Soc*, vol. 2006, pp. 525–528, 2006, doi: 10.1109/IEMBS.2006.259296.
- [119] J. P. Spalazzi, S. B. Doty, K. L. Moffat, W. N. Levine, and H. H. Lu, 'Development of controlled matrix heterogeneity on a triphasic scaffold for orthopedic interface tissue engineering', *Tissue Eng.*, vol. 12, no. 12, pp. 3497–3508, Dec. 2006, doi: 10.1089/ten.2006.12.3497.
- [120] J. P. Spalazzi, E. Dagher, S. B. Doty, X. E. Guo, S. A. Rodeo, and H. H. Lu, 'In vivo evaluation of a multiphased scaffold designed for orthopaedic interface tissue engineering and soft tissue-to-bone integration', *J Biomed Mater Res A*, vol. 86, no. 1, pp. 1–12, Jul. 2008, doi: 10.1002/jbm.a.32073.
- [121] J. E. Phillips, K. L. Burns, J. M. Le Doux, R. E. Guldberg, and A. J. García, 'Engineering graded tissue interfaces', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 105, no. 34, pp. 12170–12175, Aug. 2008, doi: 10.1073/pnas.0801988105.

- [122] J. Z. Paxton, L. M. Grover, and K. Baar, ‘Engineering an in vitro model of a functional ligament from bone to bone’, *Tissue Eng Part A*, vol. 16, no. 11, pp. 3515–3525, Nov. 2010, doi: 10.1089/ten.TEA.2010.0039.
- [123] I.-N. E. Wang *et al.*, ‘Role of osteoblast-fibroblast interactions in the formation of the ligament-to-bone interface’, *J. Orthop. Res.*, vol. 25, no. 12, pp. 1609–1620, Dec. 2007, doi: 10.1002/jor.20475.
- [124] J. Weickenmeier, R. de Rooij, S. Budday, P. Steinmann, T. C. Ovaert, and E. Kuhl, ‘Brain stiffness increases with myelin content’, *Acta Biomater*, vol. 42, pp. 265–272, Sep. 2016, doi: 10.1016/j.actbio.2016.07.040.
- [125] N. Bouchonville, M. Meyer, C. Gaude, E. Gay, D. Ratel, and A. Nicolas, ‘AFM mapping of the elastic properties of brain tissue reveals kPa μm^{-1} gradients of rigidity’, *Soft Matter*, vol. 12, no. 29, pp. 6232–6239, Jul. 2016, doi: 10.1039/C6SM00582A.
- [126] N. Rodriguez-Florez, M. L. Oyen, and S. J. Shefelbine, ‘Insight into differences in nanoindentation properties of bone’, *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 18, pp. 90–99, Feb. 2013, doi: 10.1016/j.jmbbm.2012.11.005.
- [127] J. Y. Rho, M. E. Roy, T. Y. Tsui, and G. M. Pharr, ‘Elastic properties of microstructural components of human bone tissue as measured by nanoindentation’, *J Biomed Mater Res*, vol. 45, no. 1, pp. 48–54, Apr. 1999, doi: 10.1002/(sici)1097-4636(199904)45:1<48::aid-jbm7>3.0.co;2-5.
- [128] C. Y. Zhang, Y. W. Zhang, K. Y. Zeng, and L. Shen, ‘Nanoindentation of Polymers with a Sharp Indenter’, *Journal of Materials Research*, vol. 20, no. 6, pp. 1597–1605, Jun. 2005, doi: 10.1557/JMR.2005.0200.
- [129] M. L. Oyen and R. F. Cook, ‘Load–displacement behavior during sharp indentation of viscous–elastic–plastic materials’, *Journal of Materials Research*, vol. 18, no. 1, pp. 139–150, Jan. 2003, doi: 10.1557/JMR.2003.0020.

- [130] B. J. Briscoe, L. Fiori, and E. Pelillo, 'Nano-indentation of polymeric surfaces', *J. Phys. D: Appl. Phys.*, vol. 31, no. 19, pp. 2395–2405, Oct. 1998, doi: 10.1088/0022-3727/31/19/006.
- [131] M. L. Oyen, 'Analytical techniques for indentation of viscoelastic materials', *Philosophical Magazine*, vol. 86, no. 33–35, pp. 5625–5641, Nov. 2006, doi: 10.1080/14786430600740666.
- [132] C. A. Tweedie and K. J. V. Vliet, 'Contact creep compliance of viscoelastic materials via nanoindentation', *Journal of Materials Research*, vol. 21, no. 6, pp. 1576–1589, Jun. 2006, doi: 10.1557/jmr.2006.0197.
- [133] C. Y. Zhang, Y. W. Zhang, and K. Y. Zeng, 'Extracting the mechanical properties of a viscoelastic polymeric film on a hard elastic substrate', *Journal of Materials Research*, vol. 19, no. 10, pp. 3053–3061, Oct. 2004, doi: 10.1557/JMR.2004.0382.
- [134] J. H. Kinney, M. Balooch, S. J. Marshall, G. W. Marshall, and T. P. Weihs, 'Atomic force microscope measurements of the hardness and elasticity of peritubular and intertubular human dentin', *J Biomech Eng*, vol. 118, no. 1, pp. 133–135, Feb. 1996, doi: 10.1115/1.2795939.
- [135] P. K. Zysset, X. Edward Guo, C. Edward Hoffler, K. E. Moore, and S. A. Goldstein, 'Elastic modulus and hardness of cortical and trabecular bone lamellae measured by nanoindentation in the human femur', *Journal of Biomechanics*, vol. 32, no. 10, pp. 1005–1012, Oct. 1999, doi: 10.1016/S0021-9290(99)00111-6.
- [136] C. E. Hoffler, K. E. Moore, K. Kozloff, P. K. Zysset, and S. A. Goldstein, 'Age, gender, and bone lamellae elastic moduli', *J Orthop Res*, vol. 18, no. 3, pp. 432–437, May 2000, doi: 10.1002/jor.1100180315.

- [137] C. E. Hoffler, K. E. Moore, K. Kozloff, P. K. Zysset, M. B. Brown, and S. A. Goldstein, 'Heterogeneity of bone lamellar-level elastic moduli', *Bone*, vol. 26, no. 6, pp. 603–609, Jun. 2000, doi: 10.1016/s8756-3282(00)00268-4.
- [138] J. G. Swadener, J. Y. Rho, and G. M. Pharr, 'Effects of anisotropy on elastic moduli measured by nanoindentation in human tibial cortical bone', *J Biomed Mater Res*, vol. 57, no. 1, pp. 108–112, Oct. 2001, doi: 10.1002/1097-4636(200110)57:1<108::aid-jbml148>3.0.co;2-6.
- [139] J. Y. Rho, P. Zioupos, J. D. Currey, and G. M. Pharr, 'Microstructural elasticity and regional heterogeneity in human femoral bone of various ages examined by nano-indentation', *J Biomech*, vol. 35, no. 2, pp. 189–198, Feb. 2002, doi: 10.1016/s0021-9290(01)00199-3.
- [140] F. Y. Lee, J. Y. Rho, R. Harten, J. R. Parsons, and F. F. Behrens, 'Micromechanical properties of epiphyseal trabecular bone and primary spongiosa around the physis: an in situ nanoindentation study', *J Pediatr Orthop*, vol. 18, no. 5, pp. 582–585, 1998, doi: 10.1097/00004694-199809000-00004.
- [141] J. L. Cuy, A. B. Mann, K. J. Livi, M. F. Teaford, and T. P. Weihs, 'Nanoindentation mapping of the mechanical properties of human molar tooth enamel', *Archives of Oral Biology*, vol. 47, no. 4, pp. 281–291, Apr. 2002, doi: 10.1016/S0003-9969(02)00006-7.
- [142] M. Balooch *et al.*, 'Viscoelastic properties of demineralized human dentin measured in water with atomic force microscope (AFM)-based indentation', *J. Biomed. Mater. Res.*, vol. 40, no. 4, pp. 539–544, Jun. 1998, doi: 10.1002/(sici)1097-4636(19980615)40:4<539::aid-jbm4>3.0.co;2-g.
- [143] N. Sasaki and M. Yoshikawa, 'Stress relaxation in native and EDTA-treated bone as a function of mineral content', *Journal of Biomechanics*, vol. 26, no. 1, pp. 77–83, Jan. 1993, doi: 10.1016/0021-9290(93)90615-L.

- [144] N. Sasaki and A. Enyo, 'Viscoelastic properties of bone as a function of water content', *J Biomech*, vol. 28, no. 7, pp. 809–815, Jul. 1995, doi: 10.1016/0021-9290(94)00130-v.
- [145] S. C. Cowin, 'Bone poroelasticity', *Journal of Biomechanics*, vol. 32, no. 3, pp. 217–238, Mar. 1999, doi: 10.1016/S0021-9290(98)00161-4.
- [146] J. Yamashita, B. R. Furman, H. R. Rawls, X. Wang, and C. M. Agrawal, 'The use of dynamic mechanical analysis to assess the viscoelastic properties of human cortical bone', *Journal of Biomedical Materials Research*, vol. 58, no. 1, pp. 47–53, 2001, doi: 10.1002/1097-4636(2001)58:1<47::AID-JBM70>3.0.CO;2-U.
- [147] A. K. Bembey, A. J. Bushby, A. Boyde, V. L. Ferguson, and M. L. Oyen, 'Hydration effects on the micro-mechanical properties of bone', *Journal of Materials Research*, vol. 21, no. 8, pp. 1962–1968, Aug. 2006, doi: 10.1557/jmr.2006.0237.
- [148] T. Hoc, L. Henry, M. Verdier, D. Aubry, L. Sedel, and A. Meunier, 'Effect of microstructure on the mechanical properties of Haversian cortical bone', *Bone*, vol. 38, no. 4, pp. 466–474, Apr. 2006, doi: 10.1016/j.bone.2005.09.017.
- [149] H. Gupta, U. Stachewicz, W. Wagermaier, P. Roschger, D. Wagner, and P. Fratzl, 'Mechanical modulation at the lamellar level in osteonal bone', *J. Mat. Res.*, vol. 21, pp. 1913–1921, Aug. 2006, doi: 10.1557/jmr.2006.0234.
- [150] T. Hofmann, F. Heyroth, H. Meinhard, W. Fränzel, and K. Raum, 'Assessment of composition and anisotropic elastic properties of secondary osteon lamellae', *J Biomech*, vol. 39, no. 12, pp. 2282–2294, 2006, doi: 10.1016/j.jbiomech.2005.07.009.
- [151] T. Chudoba and F. Richter, 'Investigation of creep behaviour under load during indentation experiments and its influence on hardness and modulus results', *Surface and Coatings Technology*, vol. 148, no. 2, pp. 191–198, Dec. 2001, doi: 10.1016/S0257-8972(01)01340-8.

- [152] Y.-T. Cheng and C.-M. Cheng, ‘Relationships between initial unloading slope, contact depth, and mechanical properties for conical indentation in linear viscoelastic solids’, *Journal of Materials Research*, vol. 20, no. 4, pp. 1046–1053, Apr. 2005, doi: 10.1557/JMR.2005.0141.
- [153] W. C. Oliver and G. M. Pharr, ‘An improved technique for determining hardness and elastic modulus using load and displacement sensing indentation experiments’, *Journal of Materials Research*, vol. 7, no. 6, pp. 1564–1583, Jun. 1992, doi: 10.1557/JMR.1992.1564.
- [154] B. Tang and A. H. W. Ngan, ‘Accurate measurement of tip–sample contact size during nanoindentation of viscoelastic materials’, *Journal of Materials Research*, vol. 18, no. 5, pp. 1141–1148, May 2003, doi: 10.1557/JMR.2003.0156.
- [155] G. Feng and A. H. W. Ngan, ‘Effects of Creep and Thermal Drift on Modulus Measurement Using Depth-sensing Indentation’, *Journal of Materials Research*, vol. 17, no. 3, pp. 660–668, Mar. 2002, doi: 10.1557/JMR.2002.0094.
- [156] A. K. Bembey, M. L. Oyen, A. J. Bushby, and A. Boyde, ‘Viscoelastic properties of bone as a function of hydration state determined by nanoindentation’, *Philosophical Magazine*, vol. 86, no. 33–35, pp. 5691–5703, Nov. 2006, doi: 10.1080/14786430600660864.
- [157] J. Zhang, G. L. Niebur, and T. C. Ovaert, ‘Mechanical property determination of bone through nano- and micro-indentation testing and finite element simulation’, *J Biomech*, vol. 41, no. 2, pp. 267–275, 2008, doi: 10.1016/j.jbiomech.2007.09.019.
- [158] Z. Fan and J.-Y. Rho, ‘Effects of viscoelasticity and time-dependent plasticity on nanoindentation measurements of human cortical bone’, *Journal of Biomedical Materials Research Part A*, vol. 67A, no. 1, pp. 208–214, 2003, doi: 10.1002/jbm.a.10027.

- [159] C. E. Hoffler, X. E. Guo, P. K. Zysset, and S. A. Goldstein, 'An Application of Nanoindentation Technique to Measure Bone Tissue Lamellae Properties', *Journal of Biomechanical Engineering*, vol. 127, no. 7, pp. 1046–1053, Aug. 2005, doi: 10.1115/1.2073671.
- [160] J. Y. Rho, P. Zioupos, J. D. Currey, and G. M. Pharr, 'Variations in the individual thick lamellar properties within osteons by nanoindentation', *Bone*, vol. 25, no. 3, pp. 295–300, Sep. 1999, doi: 10.1016/s8756-3282(99)00163-5.
- [161] J. J. Broz, S. J. Simske, A. R. Greenberg, and M. W. Luttges, 'Effects of rehydration state on the flexural properties of whole mouse long bones', *J Biomech Eng*, vol. 115, no. 4A, pp. 447–449, Nov. 1993, doi: 10.1115/1.2895510.
- [162] J.-Y. Rho and G. M. Pharr, 'Effects of drying on the mechanical properties of bovine femur measured by nanoindentation', *Journal of Materials Science: Materials in Medicine*, vol. 10, no. 8, pp. 485–488, Aug. 1999, doi: 10.1023/A:1008901109705.
- [163] M. E. Roy, J. Y. Rho, T. Y. Tsui, N. D. Evans, and G. M. Pharr, 'Mechanical and morphological variation of the human lumbar vertebral cortical and trabecular bone', *J Biomed Mater Res*, vol. 44, no. 2, pp. 191–197, Feb. 1999, doi: 10.1002/(sici)1097-4636(199902)44:2<191::aid-jbm9>3.0.co;2-g.
- [164] J.-Y. Rho, L. Kuhn-Spearing, and P. Zioupos, 'Mechanical properties and the hierarchical structure of bone', *Medical Engineering & Physics*, vol. 20, no. 2, pp. 92–102, Mar. 1998, doi: 10.1016/S1350-4533(98)00007-1.
- [165] J.-Y. Rho, T. Y. Tsui, and G. M. Pharr, 'Elastic properties of human cortical and trabecular lamellar bone measured by nanoindentation', *Biomaterials*, vol. 18, no. 20, pp. 1325–1330, Oct. 1997, doi: 10.1016/S0142-9612(97)00073-2.

- [166] J. Yamashita, X. Li, B. R. Furman, H. R. Rawls, X. Wang, and C. M. Agrawal, ‘Collagen and bone viscoelasticity: a dynamic mechanical analysis’, *J Biomed Mater Res*, vol. 63, no. 1, pp. 31–36, 2002, doi: 10.1002/jbm.10086.
- [167] P. Lucksanasombool, W. A. J. Higgs, R. J. E. D. Higgs, and M. V. Swain, ‘Fracture toughness of bovine bone: influence of orientation and storage media’, *Biomaterials*, vol. 22, no. 23, pp. 3127–3132, Dec. 2001, doi: 10.1016/S0142-9612(01)00062-X.
- [168] J. D. Currey, ‘The effects of drying and re-wetting on some mechanical properties of cortical bone’, *J Biomech*, vol. 21, no. 5, pp. 439–441, 1988, doi: 10.1016/0021-9290(88)90150-9.
- [169] A. Nazarian, B. J. Hermannsson, J. Muller, D. Zurakowski, and B. D. Snyder, ‘Effects of tissue preservation on murine bone mechanical properties’, *J Biomech*, vol. 42, no. 1, pp. 82–86, Jan. 2009, doi: 10.1016/j.jbiomech.2008.09.037.
- [170] D. M. Ebenstein, A. Kuo, J. J. Rodrigo, A. H. Reddi, M. Ries, and L. Pruitt, ‘A nanoindentation technique for functional evaluation of cartilage repair tissue’, *Journal of Materials Research*, vol. 19, no. 1, p. 27, Jan. 2004, doi: 10.1557/jmr.2004.19.1.273.
- [171] D. M. Ebenstein, D. Coughlin, J. Chapman, C. Li, and L. A. Pruitt, ‘Nanomechanical properties of calcification, fibrous tissue, and hematoma from atherosclerotic plaques’, *J Biomed Mater Res A*, vol. 91, no. 4, pp. 1028–1037, Dec. 2009, doi: 10.1002/jbm.a.32321.
- [172] D. M. Ebenstein, J. M. Chapman, C. Li, D. Saloner, J. Rapp, and L. A. Pruitt, ‘Assessing structure-property relations of diseased tissues using nanoindentation and FTIR’, in *Advanced Biomaterials-Characterization, Tissue Engineering and Complexity*, S. C. Moss, Ed., Warrendale: Materials Research Society, 2002, pp. 47–52. Accessed: Dec. 06, 2022. [Online]. Available: <https://www.webofscience.com/wos/woscc/full-record/WOS:000177402200007?SID=EUW1ED0F637751Kj8u4JYM40jxzeA>

- [173] J. A. Wahlquist *et al.*, ‘Indentation mapping revealed poroelastic, but not viscoelastic, properties spanning native zonal articular cartilage’, *Acta Biomaterialia*, vol. 64, pp. 41–49, Dec. 2017, doi: 10.1016/j.actbio.2017.10.003.
- [174] A. Lundkvist, E. Lilleodden, W. Siekhaus, J. Kinney, L. Pruitt, and M. Balooch, ‘Viscoelastic properties of healthy human artery measured in saline solution by AFM-based indentation technique’, in *Thin Films: Stresses and Mechanical Properties VI*, W. W. Gerberich, H. J. Gao, J. E. Sundgren, and S. P. Baker, Eds., Materials Research Society, 1997, pp. 353–358.
- [175] D. M. Ebenstein, ‘Nanoindentation of Soft Tissues and Other Biological Materials’, in *Handbook of Nanoindentation*, Jenny Stanford Publishing, 2011.
- [176] Y. Yuan and R. Verma, ‘Measuring microelastic properties of stratum corneum’, *Colloids and Surfaces B: Biointerfaces*, vol. 48, no. 1, pp. 6–12, Mar. 2006, doi: 10.1016/j.colsurfb.2005.12.013.
- [177] M. Kohane, A. Daugela, H. Kutomi, L. Charlson, A. Wyrobek, and J. Wyrobek, ‘Nanoscale in vivo evaluation of the stiffness of *Drosophila melanogaster* integument during development’, *Journal of Biomedical Materials Research Part A*, vol. 66A, no. 3, pp. 633–642, 2003, doi: 10.1002/jbm.a.10028.
- [178] D. Liu *et al.*, ‘Effect of ligation on the viscoelastic properties of liver tissues’, *Journal of Biomechanics*, vol. 76, pp. 235–240, Jul. 2018, doi: 10.1016/j.jbiomech.2018.05.018.
- [179] G. Boyer, L. Laquieze, A. Le Bot, S. Laquieze, and H. Zahouani, ‘Dynamic indentation on human skin in vivo: Ageing effects’, *Skin research and technology : official journal of International Society for Bioengineering and the Skin (ISBS) [and] International Society for Digital Imaging of Skin (ISDIS) [and] International Society for Skin Imaging (ISSI)*, vol. 15, pp. 55–67, Mar. 2009, doi: 10.1111/j.1600-0846.2008.00324.x.

- [180] D. M. Ebenstein and L. A. Pruitt, ‘Nanoindentation of soft hydrated materials for application to vascular tissues’, *J Biomed Mater Res A*, vol. 69, no. 2, pp. 222–232, May 2004, doi: 10.1002/jbm.a.20096.
- [181] M. R. VanLandingham, J. S. Villarrubia, W. F. Guthrie, and G. F. Meyers, ‘Nanoindentation of polymers: an overview’, *Macromolecular Symposia*, vol. 167, no. 1, pp. 15–44, 2001, doi: 10.1002/1521-3900(200103)167:1<15::AID-MASY15>3.0.CO;2-T.
- [182] F. Carrillo *et al.*, ‘Nanoindentation of polydimethylsiloxane elastomers: Effect of crosslinking, work of adhesion, and fluid environment on elastic modulus’, *Journal of Materials Research*, vol. 20, no. 10, pp. 2820–2830, Oct. 2005, doi: 10.1557/JMR.2005.0354.
- [183] S. Gupta, F. Carrillo, and L. Berkeley, ‘Simulated Soft Tissue Nanoindentation: A Finite Element Study’, *Journal of Materials Research*, vol. 20, no. 08, pp. 1979–1994, 2005.
- [184] G. M. Pharr and A. Bolshakov, ‘Understanding nanoindentation unloading curves’, *Journal of Materials Research*, vol. 17, no. 10, pp. 2660–2671, Oct. 2002, doi: 10.1557/JMR.2002.0386.
- [185] M. Sakai, ‘Elastic recovery in the unloading process of pyramidal microindentation’, *Journal of Materials Research*, vol. 18, no. 7, pp. 1631–1640, Jul. 2003, doi: 10.1557/JMR.2003.0224.
- [186] K. N. Hauch, M. L. Oyen, G. M. Odegard, and T. L. Haut Donahue, ‘Nanoindentation of the insertional zones of human meniscal attachments into underlying bone’, *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 339–347, Aug. 2009, doi: 10.1016/j.jmbbm.2008.10.005.
- [187] A. C. Abraham and T. L. Haut Donahue, ‘From meniscus to bone: A quantitative evaluation of structure and function of the human meniscal attachments’, *Acta*

- Biomaterialia*, vol. 9, no. 5, pp. 6322–6329, May 2013, doi: 10.1016/j.actbio.2013.01.031.
- [188] D. Villegas, T. Hansen, D. Liu, and T. Donahue, ‘A Quantitative Study of the Microstructure and Biochemistry of the Medial Meniscal Horn Attachments’, *Annals of biomedical engineering*, vol. 36, pp. 123–31, Feb. 2008, doi: 10.1007/s10439-007-9403-x.
- [189] S. Huang, C. S. Chen, and D. E. Ingber, ‘Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension’, *Mol. Biol. Cell*, vol. 9, no. 11, pp. 3179–3193, Nov. 1998, doi: 10.1091/mbc.9.11.3179.
- [190] S. Huang and D. E. Ingber, ‘The structural and mechanical complexity of cell-growth control’, *Nat. Cell Biol.*, vol. 1, no. 5, pp. E131-138, Sep. 1999, doi: 10.1038/13043.
- [191] R. K. Assoian and M. A. Schwartz, ‘Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression’, *Curr. Opin. Genet. Dev.*, vol. 11, no. 1, pp. 48–53, Feb. 2001, doi: 10.1016/s0959-437x(00)00155-6.
- [192] C. Streuli, ‘Extracellular matrix remodelling and cellular differentiation’, *Current Opinion in Cell Biology*, vol. 11, no. 5, pp. 634–640, Oct. 1999, doi: 10.1016/S0955-0674(99)00026-5.
- [193] F. M. Watt and B. L. M. Hogan, ‘Out of Eden: Stem Cells and Their Niches’, *Science*, vol. 287, no. 5457, pp. 1427–1430, Feb. 2000, doi: 10.1126/science.287.5457.1427.
- [194] ‘Multi-faceted regulation of cell differentiation by extracellular matrix. | The FASEB Journal’. Accessed: Jun. 17, 2020. [Online]. Available: <https://www.fasebj.org/doi/abs/10.1096/fasebj.7.9.8330681>

- [195] N. Datta, H. L. Holtorf, V. I. Sikavitsas, J. A. Jansen, and A. G. Mikos, 'Effect of bone extracellular matrix synthesized in vitro on the osteoblastic differentiation of marrow stromal cells', *Biomaterials*, vol. 26, no. 9, pp. 971–977, Mar. 2005, doi: 10.1016/j.biomaterials.2004.04.001.
- [196] 'Embryonic stem cell differentiation: The role of extracellular factors - Czyz - 2001 - Differentiation - Wiley Online Library'. Accessed: Jun. 17, 2020. [Online]. Available: <https://onlinelibrary.wiley.com/doi/full/10.1046/j.1432-0436.2001.680404.x>
- [197] 'Inhibition of human embryonic stem cell differentiation by mechanical strain - Saha - 2006 - Journal of Cellular Physiology - Wiley Online Library'. Accessed: Jun. 17, 2020. [Online]. Available: <https://onlinelibrary.wiley.com/doi/full/10.1002/jcp.20441>
- [198] D. Ingber, 'Mechanobiology and diseases of mechanotransduction', *Annals of Medicine*, vol. 35, no. 8, pp. 564–577, Jan. 2003, doi: 10.1080/07853890310016333.
- [199] S. Saha, L. Ji, J. J. de Pablo, and S. P. Palecek, 'TGF β /Activin/Nodal Pathway in Inhibition of Human Embryonic Stem Cell Differentiation by Mechanical Strain', *Biophysical Journal*, vol. 94, no. 10, pp. 4123–4133, May 2008, doi: 10.1529/biophysj.107.119891.
- [200] B. D. Boyan, C. H. Lohmann, D. D. Dean, V. L. Sylvia, D. L. Cochran, and Z. Schwartz, 'Mechanisms Involved in Osteoblast Response to Implant Surface Morphology', *Annual Review of Materials Research*, vol. 31, no. 1, pp. 357–371, 2001, doi: 10.1146/annurev.matsci.31.1.357.
- [201] T. P. Kunzler, T. Drobek, M. Schuler, and N. D. Spencer, 'Systematic study of osteoblast and fibroblast response to roughness by means of surface-morphology gradients', *Biomaterials*, vol. 28, no. 13, pp. 2175–2182, May 2007, doi: 10.1016/j.biomaterials.2007.01.019.

- [202] N. R. Washburn, K. M. Yamada, C. G. Simon, S. B. Kennedy, and E. J. Amis, ‘High-throughput investigation of osteoblast response to polymer crystallinity: influence of nanometer-scale roughness on proliferation’, *Biomaterials*, vol. 25, no. 7, pp. 1215–1224, Mar. 2004, doi: 10.1016/j.biomaterials.2003.08.043.
- [203] B. D. Boyan *et al.*, ‘Osteoblast-Mediated Mineral Deposition in Culture is Dependent on Surface Microtopography’, *Calcif Tissue Int*, vol. 71, no. 6, pp. 519–529, Dec. 2002, doi: 10.1007/s00223-001-1114-y.
- [204] ‘Effect of Pore Size and Void Fraction on Cellular Adhesion, Proliferation, and Matrix Deposition | Tissue Engineering’. Accessed: Jun. 17, 2020. [Online]. Available: <https://www.liebertpub.com/doi/abs/10.1089/107632701753213183>
- [205] V. Karageorgiou and D. Kaplan, ‘Porosity of 3D biomaterial scaffolds and osteogenesis’, *Biomaterials*, vol. 26, no. 27, pp. 5474–5491, Sep. 2005, doi: 10.1016/j.biomaterials.2005.02.002.
- [206] ‘Pore Morphology Effects on the Fibrovascular Tissue Growth in Porous Polymer Substrates - M. Conley Wake, Charles W. Patrick, Antonios G. Mikos, 1994’.
- [207] ‘Scaffold mesh size affects the osteoblastic differentiation of seeded marrow stromal cells cultured in a flow perfusion bioreactor - Holtorf - 2005 - Journal of Biomedical Materials Research Part A - Wiley Online Library’
- [208] A. J. Engler, S. Sen, H. L. Sweeney, and D. E. Discher, ‘Matrix Elasticity Directs Stem Cell Lineage Specification’, *Cell*, vol. 126, no. 4, pp. 677–689, Aug. 2006, doi: 10.1016/j.cell.2006.06.044.
- [209] ‘Tissue Cells Feel and Respond to the Stiffness of Their Substrate | Science’. Accessed: Jun. 17, 2020. [Online]. Available: <https://science.sciencemag.org/content/310/5751/1139>

- [210] F. Rehfeldt, A. J. Engler, A. Eckhardt, F. Ahmed, and D. E. Discher, ‘Cell responses to the mechanochemical microenvironment—Implications for regenerative medicine and drug delivery’, *Advanced Drug Delivery Reviews*, vol. 59, no. 13, pp. 1329–1339, Nov. 2007, doi: 10.1016/j.addr.2007.08.007.
- [211] N. Wang, J. P. Butler, and D. E. Ingber, ‘Mechanotransduction across the cell surface and through the cytoskeleton’, *Science*, vol. 260, no. 5111, pp. 1124–1127, May 1993, doi: 10.1126/science.7684161.
- [212] M. J. Dalby, ‘Topographically induced direct cell mechanotransduction’, *Medical Engineering & Physics*, vol. 27, no. 9, pp. 730–742, Nov. 2005, doi: 10.1016/j.medengphy.2005.04.005.
- [213] ‘Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion - Yeung - 2005 - Cell Motility - Wiley Online Library’. Accessed: Jun. 17, 2020. [Online]. Available: <https://onlinelibrary.wiley.com/doi/abs/10.1002/cm.20041>
- [214] C.-P. Heisenberg and Y. Bellaïche, ‘Forces in Tissue Morphogenesis and Patterning’, *Cell*, vol. 153, no. 5, pp. 948–962, May 2013, doi: 10.1016/j.cell.2013.05.008.
- [215] N. I. Petridou, Z. Spiró, and C.-P. Heisenberg, ‘Multiscale force sensing in development’, *Nature Cell Biology*, vol. 19, no. 6, Art. no. 6, Jun. 2017, doi: 10.1038/ncb3524.
- [216] V. Vogel and M. Sheetz, ‘Local force and geometry sensing regulate cell functions’, *Nature Reviews Molecular Cell Biology*, vol. 7, no. 4, Art. no. 4, Apr. 2006, doi: 10.1038/nrm1890.
- [217] C. Q. Lin and M. J. Bissell, ‘Multi-faceted regulation of cell differentiation by extracellular matrix’, *FASEB J*, vol. 7, no. 9, pp. 737–743, Jun. 1993, doi: 10.1096/fasebj.7.9.8330681.

- [218] E. Baas, J. H. Kuiper, Y. Yang, M. A. Wood, and A. J. El Haj, ‘In vitro bone growth responds to local mechanical strain in three-dimensional polymer scaffolds’, *J Biomech*, vol. 43, no. 4, pp. 733–739, Mar. 2010, doi: 10.1016/j.jbiomech.2009.10.016.
- [219] C.-M. Lo, H.-B. Wang, M. Dembo, and Y. Wang, ‘Cell Movement Is Guided by the Rigidity of the Substrate’, *Biophysical Journal*, vol. 79, no. 1, pp. 144–152, Jul. 2000, doi: 10.1016/S0006-3495(00)76279-5.
- [220] R. S. Walton, D. D. Brand, and J. T. Czernuszka, ‘Influence of telopeptides, fibrils and crosslinking on physicochemical properties of Type I collagen films’, *J Mater Sci: Mater Med*, vol. 21, no. 2, pp. 451–461, Feb. 2010, doi: 10.1007/s10856-009-3910-2.

Chapter 2 - Collagen

2.1. Introduction

Collagen is the most abundant structural protein in all animals and is the main insoluble fibrous protein in the extracellular matrix and connective tissue [1]. The name “collagen” is used to describe proteins forming a characteristic triple helix of three polypeptide chains. Each member of the collagen family forms this supramolecular structure in the ECM, although their size, functions and tissue distribution vary considerably [2], [3], [4]. Vertebrates have at least 27 genetically distinct collagen types [5], [6], [7]. Members of the collagen family can be sorted into different groups: fibril-forming collagens, fibril-associated collagens (FACIT), network-forming collagens, basement membranes collagens and others with unique functions.

This chapter will primarily focus on the most abundant and widespread fibril-forming family of collagens. It will in detail discuss the structure of collagen and routes of cross-linking these proteins. Furthermore, it will delve into the different sources for collagen extraction with some of their difference and identify the best ratio of chemical cross-linkers for the required degree of cross-linking.

2.2. Molecular and Sub-Molecular Structure of Collagen

The shape and structural properties of a native collagen molecule are established by its triple-helical α -domains. The fundamental structural unit of collagen is a rod about 300nm long and 1.5nm in diameter often referred to as tropocollagen [8]. These subunits of collagen are made up of three parallel polypeptide strands in a left-handed, polyproline type II helical conformation [9]. The proper folding of each of these chains mandates that in the polypeptide chain glycine (Gly) residue is present in every third position. For example, each α -chain is composed of multiple triplet sequences of Gly-X-Y (which is the predominating motif in fibril-forming collagens), in which X and Y can be any amino acid, but X is commonly found as proline and Y as hydroxyproline. It is thought that the presence of hydroxyproline in the Y position contributes to helical form stability [8]. Thus, repeat occurs in all types of collagen, however, it is disrupted at certain locations within the triple-helical domain of nonfibrillar collagens.

These three α -chains, staggered by one residue relative to each other, are then supercoiled around a central axis in a right-handed rope-like manner to produce the overall tightly packed triple-helical form of the molecule [10]. The α -chains assemble around a central axis in such a way that all the glycine residues are positioned in the centre of the triple helix, while the bulkier side chains of other amino acids occupy the outer positions, allowing for a close packing along the central axis of the molecule. The interactions of α -chains are stabilised via interchain hydrogen bonding, making the molecule fairly resistant to attack by other molecules. Each α -chain is surrounded by a hydrogen sphere which allows a hydrogen bonding network to be present between the water molecules and the peptide acceptor groups [8]. This hydrogen

bonding occurs when the amino group (NH) of the glycine residues forms a peptide bond with the carbonyl (C=O) of than adjacent residue [11].

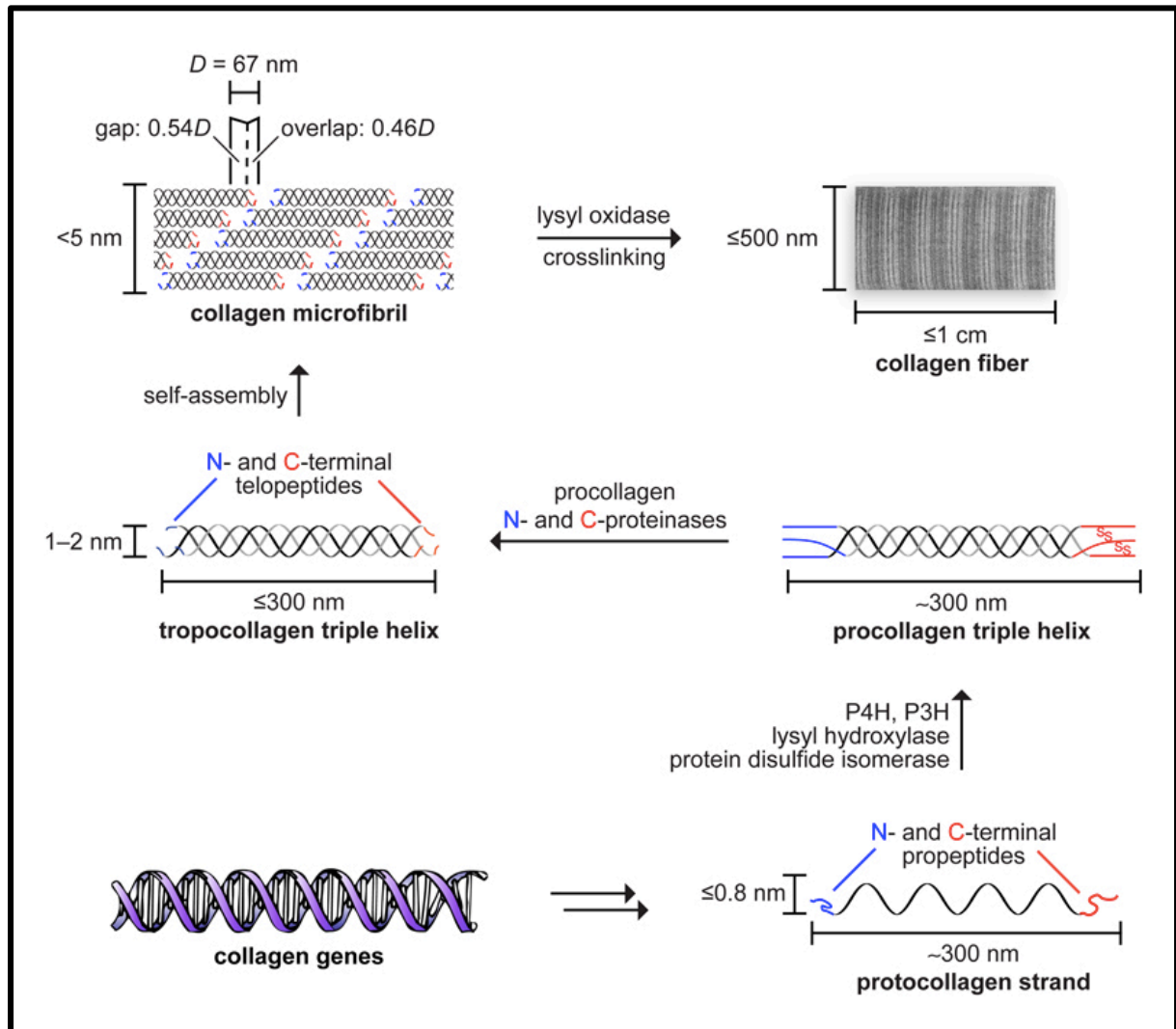


Figure 2.1: Biosynthesis route of collagen fibres [9].

Although the triple helix is a key feature of all collagens and represents the major part in fibril-forming collagens, this helical part is further flanked by short non-helical, non-collagenous domains, which are important structural components, the so-called telopeptides. The short non-helical N-terminal telopeptides (globular tail portion of the chains) (Figure 2.1) are involved in the covalent cross-linking of the collagen molecules as well as linking to other molecular structures of the surrounding matrix [12], and then the telopeptides (containing the cysteine

(Cys) and (Tyr) of pro-collagen) are shed leaving the rod-like ca. 3150 amino acids containing triple helix. These collagen rods assemble with a quarter-stagger to form the collagen fibre and the fibres are stabilised by further cross-links. The arrangement of the polypeptide chains and the variation of the terminals give distinction to the types of collagens, both fibrillar and non-fibrillar. These criteria are used to group collagens into their families.

2.2.1. Fibril forming collagens

Fibrillar collagens are the most abundant ECM proteins in vertebrates, providing stability, connectivity and form to tissues and organs. The classical fibril-forming collagens include types I, II, III, V and XI. These collagens are characterised by their ability to assemble into highly orientated supramolecular aggregates with a characteristic suprastructure.

Tropocollagens are staggered longitudinally and bilaterally by inter- and intra-molecular cross-links into microfibrils (4 to 8 tropocollagens) and further into fibrils. In the electron microscope, this typical quarter-staggered fibril-array with diameters between 25 and 400 nm is characterised by a gap of 40nm between succeeding collagen molecules and by a banding pattern with a periodicity of about 65-67nm, called the D-period (Figure 2.2) [13].

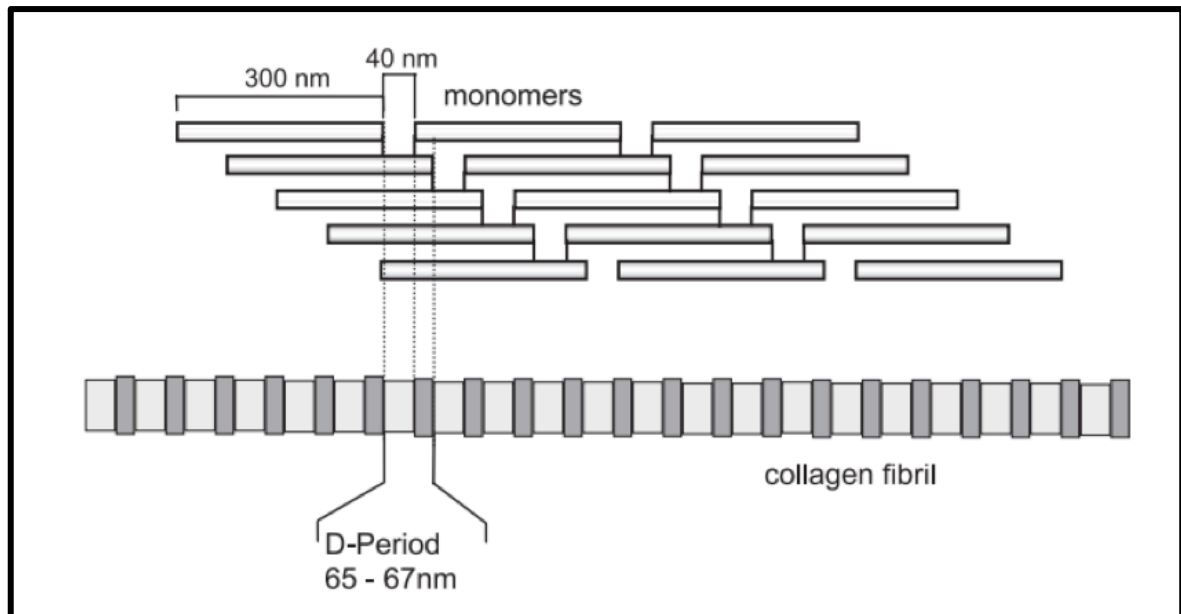


Figure 2.2: Schematic representation of the supramolecular assembly of the collagen fibrils in the characteristic quarter-staggered form. The monomers are 300-nm long and 40-nm gaps separate consecutive monomers causing the characteristic appearance of the collagen type I fibrils on the ultrastructural level [14].

Type I collagen is the most abundant accounting for more than 90% of the organic mass of bone and is the major collagen in ligaments and many interstitial connective tissues. The collagen type I triple helix is usually formed as a heterotrimeric co-polymer by two identical $\alpha 1(\text{I})$ -chains and one $\alpha 2(\text{I})$ -chain, containing approximately 1050 amino acids each. The molecule of this type of collagen consists of three domains shown in Figure 2.3: (A) amino non-triple helical (N-telopeptide), (B) central triple helical consisting of more than 300 repeat units and represents more than 95% of the polypeptide, and (C) carboxy-terminal non-triple helical (C-telopeptide) [15]. Besides the telopeptides, tropocollagens still contain N- and C-terminal propeptides sequences, called non-collagenous domains [11]. It is thought that the C-propeptide play a fundamental role in the initiation of triple helix formation, whereas the N-propeptide is thought to be involved in the covalent cross-linking of the collagen molecules as

well as linking to other molecular structures of the surrounding matrix [14]. The propeptides are removed before fibril formation and regulate the fibril formation process.

Whilst tropocollagen molecules consist of a superhelix of α chains with some non-helical portion of telopeptides at each end, atelocollagen is the name given to a low-immunogenic derivative of collagen obtained by removal of these non-helical telopeptides (Figure 2.3) . Telopeptides are removed by treatment of collagen with type I pepsin. The resulting atelocollagen (molecular weight of approximately 300kDa), has a rod-like structure, 300nm in length and 1.5nm in diameter [14], [16], [17].

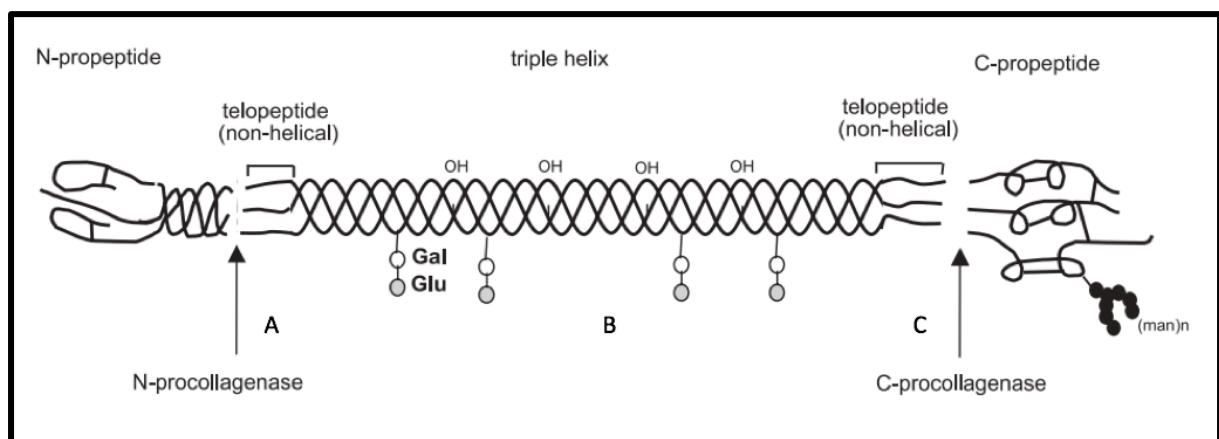


Figure 2.3: Molecular structure of fibrillar collagens with the various subdomains as well as the cleavage sites for N- and C-procollagenases (shown in the type I collagen molecule) [14].

The fibril-forming type II collagen is the characteristic and predominant component of hyaline cartilage where it accounts for about 80% of the total collagen content, however, it is not specifically restricted to cartilage [18]. The triple helix of type II collagen is composed of three α I(II)-chains forming a homotrimeric molecule similar in size and biomechanical properties to that of type I collagen [19]. Compared to type I collagen, type II collagen chains show a higher content of hydroxy-lysine as well as glucosyl and galactosyl residues which mediate the

interaction with proteoglycans, another typical component of the highly hydrated matrix of hyaline cartilage [20].

2.3. Crosslinking Routes

Native collagen possesses multiple inter- and intramolecular cross-links which occur enzymatically and provide sufficient mechanical strength and proteolytic resistance [21]. However, the lysyl oxidase-mediated cross-linking would not occur *in vitro* and therefore has impacts on relevant properties and biological response. To improve the structural stability and mechanics of collagenous materials several cross-linking approaches (physical, chemical and enzymatic) have been investigated through the years, giving different physical and chemical properties depending on the method undertaken [22]5/1/25 3:53:00 PM. A crosslink is a bond that connects the functional groups of a polymer chain to another through covalent bonding or supramolecular interactions such as ionic bonding, and hydrogen bonding [23]. Along with improving mechanical performance, a cross-linking agent should have no cytotoxic effects [24].

Physical cross-linking is the formation of a bond between polymer chains through weak physical interactions. These interactions include coordination bonding, hydrogen bonding, ionic interactions, and van der Waals interactions. The primary advantage of physical methods is that they do not introduce chemicals that may cause potential harm as there are no residues in this approach. Typical physical processes such as drying, heating and irradiation have been applied to collagen [25]. However, because of the weak interactions between polymer chains, their bonds have low durability, and in consequence are unstable mechanically and thermally. Furthermore, physical treatments generally result in a low crosslinking degree because the reaction occurs only at the surface of the materials.

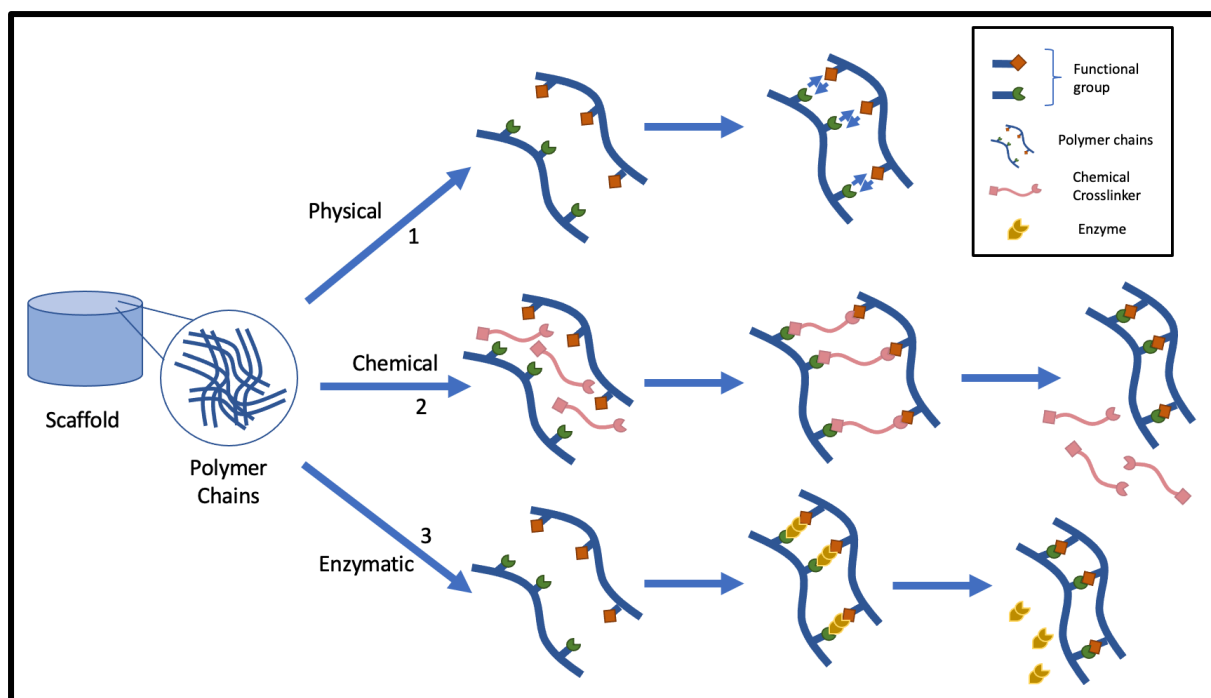


Figure 2.4: Mechanisms of physical, chemical and enzymatic methods of crosslinking 1) Physical crosslinking, non-covalent bond formed between polymer chains. 2) Chemical crosslinking, A. Chemical crosslink agent incorporated into the bond through polymer chains, B. Crosslinking agent leaves the reaction once crosslinking process is complete. 3) Enzymatic crosslinking, crosslinkers.

Chemical cross-linking refers to the process of forming inter- or intra- molecular covalent bonds between two or more molecules using reagents, referred to as “crosslinkers”. Present chemical cross-linking procedures often use bifunctional reagents containing reactive groups that form bridges between two amino acid side chains of adjacent protein molecules. The primary advantage of chemical cross-linking procedures is the strong bonds that are achieved which results in structures with greater stability, durability, and higher mechanical properties.

In addition to the prevalent chemical crosslinking reagents, enzymes can be employed as catalysts to promote the introduction of covalent bonds between protein molecules. A major

advantage relates to the mildness of the enzymatic reactions at normal physiological conditions; therefore this method is advantageous for natural polymers which cannot withstand harsh chemical conditions. Both physically and chemically crosslinked hydrogels can be formed via enzymatic reactions, depending on the activity of the enzyme. However, enzymatically cross-linked scaffolds yield lower hydrothermal stability and low elastic modulus [26], [27], which limits their applications to injectable hydrogels only.

A detailed review of all three cross-linking approaches and their most widely used methods was carried out. The full review can be found in supplementary data (Section S.1.4). The key advantages and disadvantages of each approach are presented in Table 2.1 below.

Crosslinking Method	Advantage	Disadvantage
Physical	-Do not introduce chemicals that cause potential harm -Inexpensive -Minimum tissue reaction after crosslinking process	-Bonds are weaker than chemical crosslinkers -More time needed for reaction -Lower degree of crosslinking - Potential denaturing of collagen molecules. - May destroy native structure of material - Lack of control over reaction kinetics of crosslinking
Chemical	-Very strong bonds -Higher degree of crosslinking than physical	-Needs washing to remove residue cross-linker -More expensive than physical crosslinkers -May alter material structure
Enzymatic	-Great control over degree of crosslinking through modification of temperature, pH, or ionic strength [28] -unwanted side reactions or toxicity are avoided due to substrate specificity of enzyme [29]. -Enzymatic reactions are mild at normal physiological conditions [29] (good for polymers that cannot withstand harsh chemical conditions)	-Most expensive crosslinker -Substrate specificity -Almost toxic for cells

Table. 2.1: Comparison of physical, chemical and enzymatic crosslinking techniques.

Most physical and enzymatic crosslinking cannot yield sufficient cross-linking efficiency for tissue engineering application demands. Consequently, chemical treatment methods are still necessary in most cases [30]. Therefore, chemical cross-linking would be the most appropriate route to meet the demands of these studies. The advantages and disadvantages of the most commonly used chemical cross-linkers are outlined in Table 2.2.

Method	Advantages	Disadvantages
Gluteraldehyde	-Inexpensive -Easily available -Reduce the immunogenicity of scaffolds -Water-soluble reagent	-High cell toxicity -Hazardous material for public health -Needs to be washed with specific solutions after crosslinking -The colour of crosslinked scaffolds changes to yellow
Carbodiimide [31]	-Less toxic than GTA -Can be washed away with DI water after crosslinking -Thermal stability and resistance to collagenase resistance comparable to GTA	-Expensive -The degree of crosslinking is lower than GTA -Low mechanical properties and rapid degradation compared with GTA
Acyl-azides [31]	-Thermal stability and resistance to collagenase resistance comparable to GTA -Better biocompatibility than GTA	-The degree of crosslinking is lower than GTA -less resistant to proteolytic attack -potencies calcification
Formaldehyde	-Reversible -Easy to wash away after cross-linking -Very cheap and commercially available -Fast cross-linking and	-inefficient -use limited due to small size
Genipin	-Biocompatibility -The least toxic chemical cross-linker -Reduce the immunogenicity of xenogeic matrices , even more than GA	-Very expensive -The colour of the crosslinked scaffolds changes to blue

Table 2.2: Advantages and disadvantages of Chemical crosslinkers

Previous efforts have shown that the use of zero-length crosslinker carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS) as an effective route for the cross-linking of both porous scaffolds and extruded collagen fibres [34], and because of the advantages of this route outlined in Table 2.2, It has been chosen as the method for cross-linking in these studies.

EDC/NHS crosslinking involves the activation of carboxylic groups in the peptide chain and the formation of O-acylisourea intermediate which can be nucleophilic attacked by free amino groups of lysine and hydroxyl lysine residues thus forming an amide bond (Figure 2.5) [35]. Because no portion of their chemical structure becomes part of the final bond between conjugated molecules, they are considered zero-length crosslinkers.

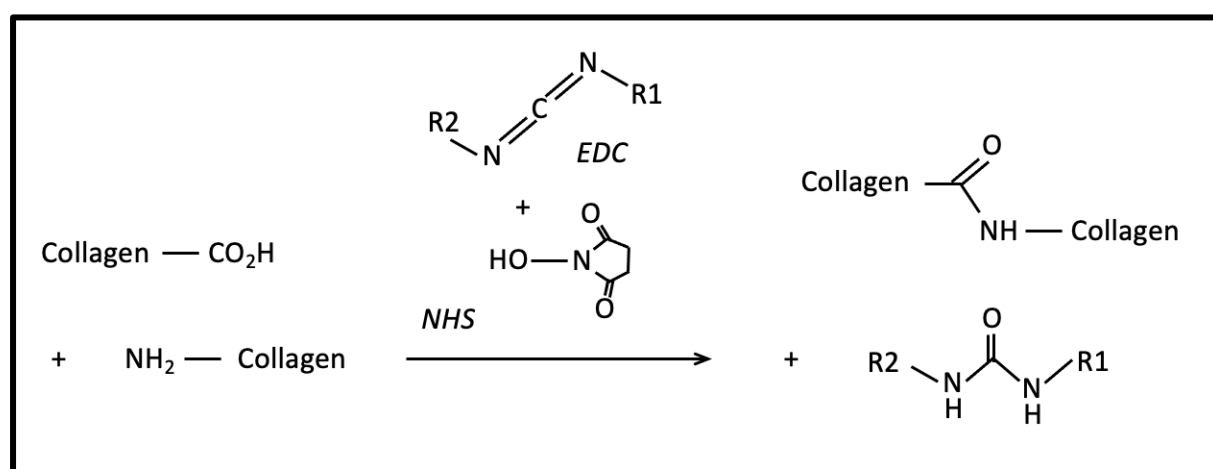


Figure 2.5: Cross-linking route of chemical cross-linkers EDC and NHS.

2.4. Collagen extraction and processing

Collagen for biomaterial production must first be sourced, extracted, and purified. Collagen may be extracted from animals and humans (cadavers, placenta, amniotic sac) [36] or synthesised using recombinant techniques [37]. Isolated collagen triple helices are “monomeric”, whereas extractions of collagen with a retained fibrillar structure are “polymeric”. The majority of collagen scaffolds for tissue engineering are fabricated using polymeric collagen due to the ease and low cost of its extraction. However, the polymeric form of collagen is insoluble and is composed of naturally cross-linked tropocollagen. As cross-linking has been found to impact scaffold porosity, mechanical properties and cell interactions, the presence of these native cross-links can produce batch-to-batch variability and limit our ability to control the final product.

For extraction of monomeric collagen, two different methods are used: pepsin digestion or acid solubilisation, obtaining atelocollagen and tropocollagen, respectively. Most collagens are extracted from animal tissues which may induce issues with immunogenicity. Atelocollagen is devoid of both immunogenic determinants due to the lack of associated cross-species antigenicity of the p-determinant located in the telopeptides [38] and native cross-links. For this reason, atelocollagen is the preferred form of collagen for tissue engineering work.

2.4.1. Monomeric collagen extraction

During the extraction process, it is critical to eliminate all non-collagenous proteins, cells and cell remnants as these can cause an immunological response and lead to the rejection of the graft. To remove all non-collagenous elements, the tissue from which the collagen is to be extracted is extensively washed with distilled water, frozen and then minced. This minced

mixture is then washed three more times in buffers and salt solutions before being suspended in ethanoic acid to extract the acid-soluble portion of the collagen (tropocollagen). The acid-soluble collagen is filtered off, repeatedly purified by salt precipitation and centrifugation, and finally dialysed against 0.01 M ethanoic acid. To create the less immunogenic form of collagen (atelocollagen), the telopeptides are cleaved off by re-suspending the insoluble collagen in pepsin.

2.4.2. Fibre formation

Atelocollagen can self-assemble *in vitro*, where fibril formation and diameter depend on the type of collagen [39], method of extraction [40] and processing conditions [41]. The ability of collagen molecules to form highly ordered fibrillar structures in the extracellular space is an important feature. This self-assembly, which can also occur *in vitro* is mainly controlled by the primary structure of collagen and both polar and hydrophobic interactions are involved in the ordered aggregation of molecules. Studies have indicated that pH, temperature, ionic strength, ion species, surfactants, saccharides, and the removal of the non-helical ends of collagen have a strong influence on modifying collagen formation [45]. Collagen fibrillogenesis is a thermally driven process that is favoured by a large positive entropy contribution due to the displacement of structured water around collagen molecules [46].

2.4.3. Sources of collagen

Collagen as a biomaterial for tissue engineering endeavours is often extracted from tissues that are rich in the desired collagen type. It has been found that the source from which the collagen is extracted influences its properties.

Throughout this work, collagen extracted from bovine skin (BS) and amnionic membrane (AM) were utilised and some differences were noted. These differences can be explained through studies previously carried out by others.

In a study by Sang-Hyug *et al* [47]. BS collagens showed a more prominent amide A band at 3337 cm^{-1} during FTIR analysis, suggesting greater involvement of hydrogen bonding compared to AM collagen. This implies that AM collagens are composed of more complex intermolecular structures with less possibility to form triple helix structures than BS collagens. This was also observed through the expression of their amino acids. The amino acid compositions were expressed as residues per 1000 total residues and differences between AM and BS collagens were observed. AM collagens were found to contain higher amounts of lysine residues, which is an indication of the extent of inter-collagen cross-linking. Whereas BS collagens had higher amounts of proline and hydroxyproline residues. Hydroxyproline residue is known to increase helix stability as the hydrogen bonds of hydroxyproline hold the three α -chains together. Furthermore, AM collagen presented higher zeta potential values than BS collagen, indicating the good gelation properties of AM collagen.

Hydrophobic interactions between non-polar regions of adjacent molecules are the predominant effect governing collagen fibril formation due to the negative temperature coefficient of collagen solubility and the endothermic nature of *in vitro* fibrillogenesis [46], [48]. Collagen molecules being largely hydrophobic, the assembly of the collagen molecules into fibrils is a process driven by the loss of solvent molecules from its surface. Polar groups tend to cluster together in a polar liquid, like water, to minimise the surface between groups of different polarity, resulting in assemblies with a circular cross-section [49], [50].

During preliminary work, collagen fibres were produced from both AM and BS collagen (the process of fabrication will be detailed later in Section 2.5). When extruded, collagen fibres formed from AM collagen had a more even and circular diameter in comparison to BS collagen fibres (Figure 2.6), thus suggesting that overall AM collagens are more polar than BS collagens which is a difference likely caused by the variations in amino acid compositions discussed.

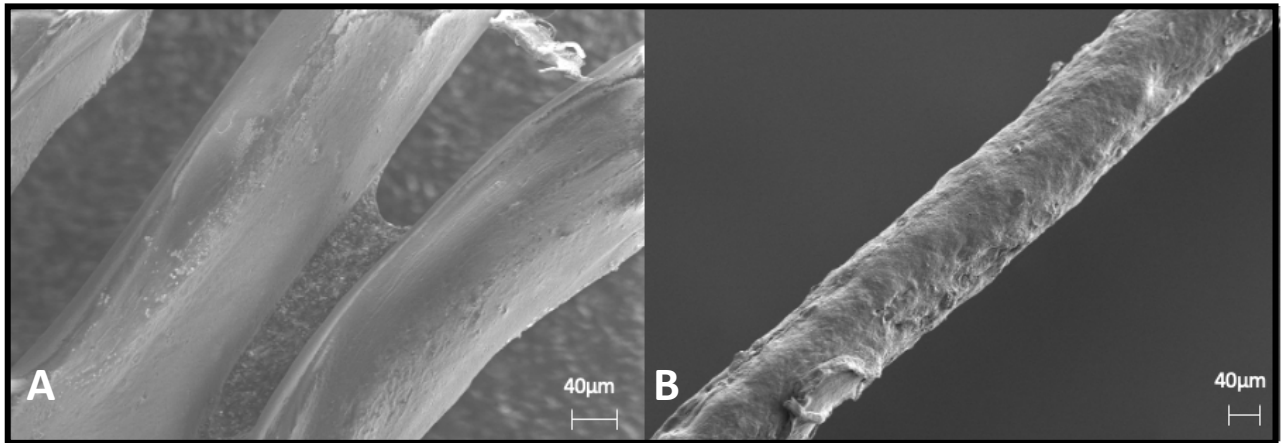


Figure 2.6: (A) SEM of BS collagen fibres, (B) SEM of AM collagen fibres

2.5. Degree of crosslinking- 2,4,6-trinitro-benzene-sulphonic acid

Assay

Literature reports suggest that crosslinking carried out with 60 mM EDC and NHS (24 mM NHS in 100ml of an aqueous solution for a molar ratio of EDC/NHS/COO⁻(col) =5/2/1 is one of the best conditions to achieve the highest degree of mechanical and material stabilisation for collagen type matrices and was defined as the standard condition (100%) [51], [52].

However, previous works from members of our lab found that a 60mM concentration of EDC produced lyophilised collagen scaffolds with the highest mechanical and thermal properties [53], [54], [55]. For these studies the ratio of EDC:NHS was fixed at 2:1 similar to other EDC/NHS studies [56].

The goal was to find out which ratio of cross-linking agents achieved the highest degree of cross-linking. Furthermore, partial cross-linking of collagen could be utilised when infiltrating collagen with elastin, therefore, the effects of halving the cross-linking agents on the degree of cross-linking were also observed.

2.5.1. Method

2.5.1.1. Collagen suspension production

A 1% wt/v monomeric collagen suspension was prepared by dissolving Type I lyophilised monomeric atelocollagen from amnion (AM) (1g), in acetic acid solution (100 ml, pH=3.2). The suspension was homogenised with a conventional blender to create a uniform dispersion. The homogenised suspension was centrifuged (Thermo, IEC CL10, UK) at room temperature for 5 mins at 3000 rpm to remove entrapped air bubbles, 5ml of the suspension was syringed

into a bath containing a fibre formation buffer (FFB, 30mM Tris Buffer Solution and 30mM Phosphate Buffer Solution) and cross-linking agents EDC/NHS at various ratios at pH 7.5. The ratios of cross-linking agents added to 100ml of FFB are outlined in Table 2.3. The syringed collagen was incubated at 37°C for 4 hours to self-assemble, and crosslinked as desired, before being taken out and washed with 0.1 M Disodium Phosphate (Na_2HPO_4) followed by deionised water. After washing the collagen was left to air dry for 24hrs.

	EDC (g)	NHS (g)	EDC/NHS crosslinker conc. (mM)
1	1.150	0.276	60/24
2	1.150	0.350	60/30
3	0.575	0.138	30/12
4	0.058	0.014	15/6
U	0	0	-

Table 2.3: Labels used for fibres produced using different EDC/NHS ratios.

EDC molecular weight: 191.70 g/mol [57]

NHS molecular weight: 115.09 g/mol [58]

NOTE: As 1g of collagen is present in 100ml of suspension, there is approximately 0.05g of collagen present in 5ml of the extruded suspension into the 100ml of FFB solution. Therefore, for a molar ratio of 5/2/1 for 0.05g of collagen, crosslinking should be carried out with 0.0575g EDC and 0.0138g NHS.

2.5.1.2. Amine group content and degree of crosslinking

The content of free primary amine groups present in the initial non-crosslinked samples and those crosslinked with different EDC and NHS concentrations were determined using a 2,4,6-

trinitro-benzene-sulfonic acid (TNBS) assay [52], [59], [60], [61]. For each sample (15mg), 1.5 ml of a 4% (w/v) sodium bicarbonate (NaHCO₃) solution (pH 8.5) and 1.5 ml of a freshly prepared solution of 0.5% (w/v) TNBS solution (prepared in di-water) were added. After 2hrs in a 40°C water bath at 150rpm, 4.5 ml of 6 M HCl was added to the samples and they were further hydrolysed at 60°C for 90 min. The reaction was diluted with 7.5 ml of di-water to bring the total solution volume to 15 ml and cooled to room temperature. Samples were transferred to a spectrometer (UV-vis, Agilent Cary 5000) and the absorbance at 345 nm was measured. Controls (blank samples) were prepared using the same protocol, except that HCl was added, before introducing the TNBS solution, to prohibit any reaction of TNBS with the amine groups. Three replicates were used for each determination.

The degree of crosslinking was calculated as follows:

$$\text{The extent of crosslinking} = 1 - \left(\frac{\text{absorbance}_s}{\text{mass}_s} \right) * \left(\frac{\text{absorbance}_{ncl}}{\text{mass}_{ncl}} \right)^{-1}$$

(Equation 2.1)

Where the subscripts s and ncl stand for the sample and non-crosslinked collagen, respectively.

2.5.1.3. Statistical Analysis

Results have been expressed as mean $\pm$ standard deviation. The results were compared with one-way analysis of variance (ANOVA) with a Tukey's HSD post-hoc test For values of $p < 0.05$, the difference was considered statistically significant.

2.5.2. Results

The extent of cross-linking of the collagen fibres with various concentrations of EDC and NHS was measured, and the values are listed in Table 2.4. When comparing samples 1 and 2, results show that the extent of cross-linking was greater for a ratio of 5:2 compared to 2:1, however, there was no significant statistical difference between these values.

Label	EDC/NHS ratio	Mean Absorbance at 345nm	Extent of XL (%)	SD of %
1	5:2	0.29	85.91	0.63
2	2:1	0.37	80.64	0.63
3	5:2	0.36	81.66	0.09
4	5:2	1.52	21.27	3.59
U (control)	-	1.94	0	-

Table 2.4: Degree of cross-linking of fibres calculated after treatment with different EDC/NHS concentrations.

When observing the results for samples the 3 samples that had a ratio of 5:2, Figure 2.8, results show that the extent of cross-linking follows a logarithmic relationship and that an EDC content greater than 60mM is unlikely to significantly increase the extent of cross-linking. and that halving the EDC content does not half the extent of cross-linking. There was no significant statistical difference between values for samples 1 and 3 and therefore it can be assumed that complete cross-linking was approached at an EDC concentration of 30mM.

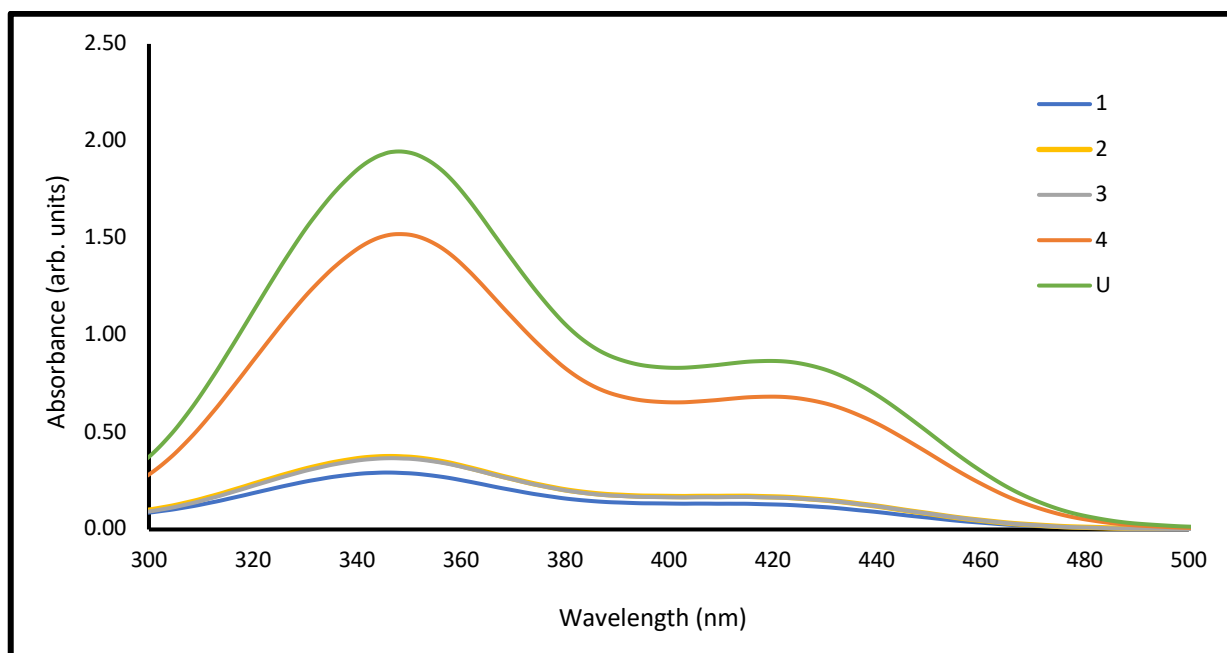


Figure 2.7: UV-vis spectrum for TNBS study of collagen fibres with various EDC/NHS ratios. Labels for cross-linkers presented in table 2.4.

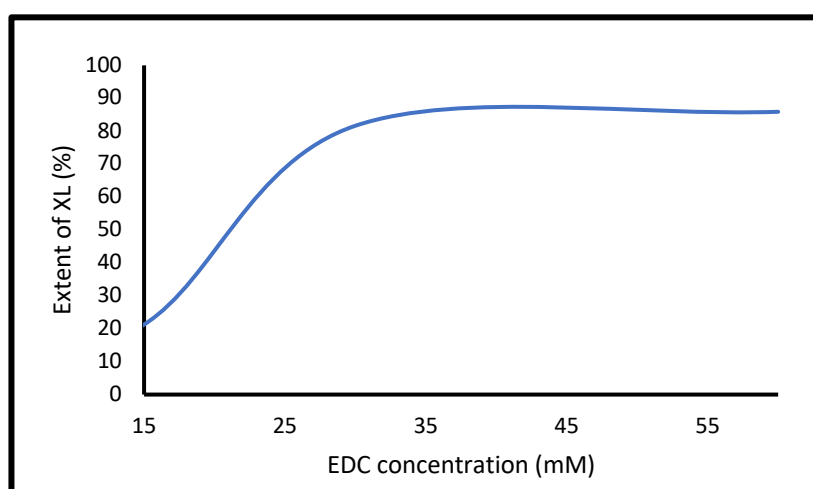


Figure 2.8: Extent of cross-linking vs EDC concentration for samples with EDC/NHS ratio of 5:2

2.5.3. Discussion

As mentioned in Section 3.4.3, the assembly of collagen molecules into fibres is driven by hydrophobic interactions. Upon extruding Type I, atelocollagen into FFB solution with no cross-linkers, collagen fibres sunk to the bottom of the bath and fibres formed were flat (Figure 2.6 A). Suggesting that the hydrophobic interaction between the FFB solution and collagen suspension was not great enough for fibre formation. On the other hand, with even the smallest traces of cross-linking agents present, the fibres formed were uniformly cylindrical and remained on the surface of the FFB solution.

Furthermore, when trying to extract uncrosslinked fibres, they broke before being transferred into isopropanol for dehydration. Water reduces any secondary binding present between fibrils as oxygen is present in collagen molecules which would be forming the interfibrillar hydrogen bonds are being bonded to hydrogen in water molecules instead (Figure 2.9). Therefore, once hydrated fibrils are less stiff and structurally stable. Thus, it is necessary to introduce intra-fibrillar cross-links to restore the rigidity and stability of hydrated collagen fibres (Figure 2.10). The presence of EDC/NHS was enough to provide stability to the formed fibres and extraction was possible.

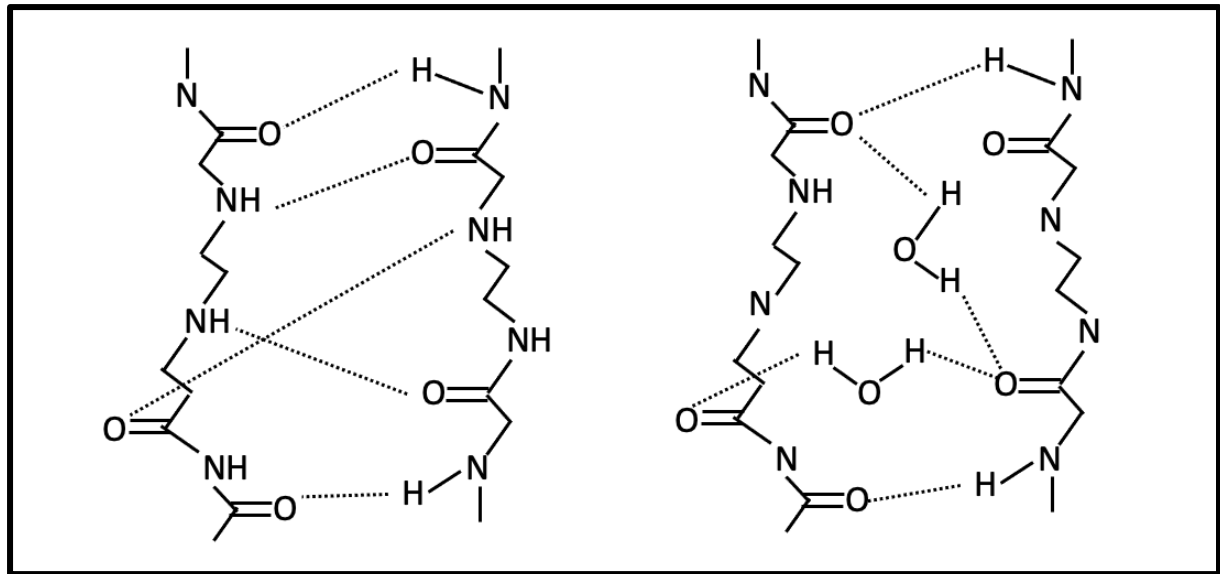


Figure 2.9: hydrogen bonds between collagen fibrils in fibres. Left: dry collagen fibrils with interfibrillar hydrogen bonding, right: wet collagen fibrils bonded to water molecules.

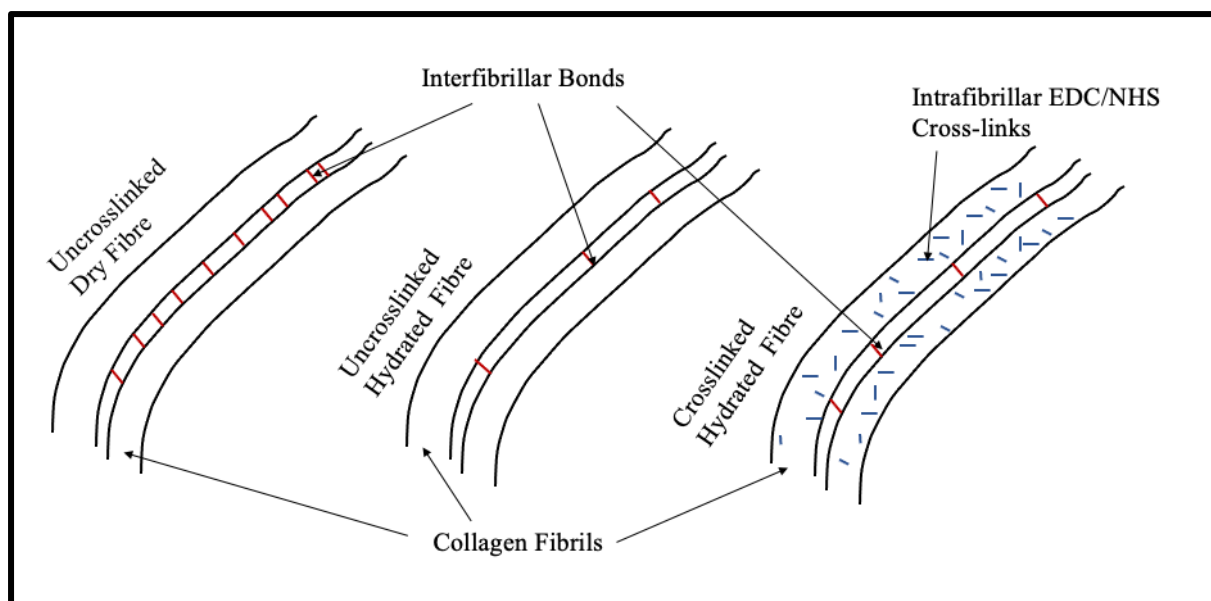


Figure 2.10: Interfibrillar bonds and intrafibrillar cross-links and the effects of hydration.

There was no significant difference between samples 1 and 2, suggesting that there is no significance between using cross-linkers EDC:NHS at a ratio of 5:2 or 2:1. However, by utilising the 5:2 ratio we use less NHS and therefore save on consumables. Furthermore, although not significant, the 5:2 ratio did produce greater cross-linking.

During EDC/NHS cross-linking, bonds are formed between primary amines and carboxylic acids. The TNBS study only detects free amine groups present in collagen, however, carboxylic acids are also needed for cross-linking. Although the maximum degree of cross-linking achieved was ~85%, we can assume that complete cross-linking was achieved as there was no statistical difference between samples 1 and 3. Meaning, the addition of more cross-linking agents did not result in the significant formation of more cross-links. Therefore, although there are still free amine groups present in collagen, there are likely no more carboxylic acids for them to bond with.

2.6. Summary

In summary, the structural properties of collagen molecules are established by their triple helical α -domains, and differences or changes in this structure from the molecular to the sub-molecular level result in differences in their chemical and mechanical behaviour. By using this we can engineer their structures through cross-linking to give us properties that better suit our needs for tissue regeneration endeavours. Differences between sources of collagen were noted. Bovine skin was found to not be a suitable source for collagen fibre fabrication due to its inability to form uniform fibres. Furthermore, it was decided that cross-linking agents at a ratio of 5:2 EDC:NHS would be utilised moving forward.

2.7. References

- [1] C. M. Kielty and M. E. Grant, 'The Collagen Family: Structure, Assembly, and Organization in the Extracellular Matrix', in *Connective Tissue and Its Heritable Disorders*, John Wiley & Sons, Ltd, 2002, pp. 159–221. doi: 10.1002/0471221929.ch2.
- [2] K. E. Kadler, C. Baldock, J. Bella, and R. P. Boot-Handford, 'Collagens at a glance', *Journal of Cell Science*, vol. 120, no. 12, pp. 1955–1958, Jun. 2007, doi: 10.1242/jcs.03453.
- [3] S. Ricard-Blum, 'The Collagen Family', *Cold Spring Harb Perspect Biol*, vol. 3, no. 1, p. a004978, Jan. 2011, doi: 10.1101/cshperspect.a004978.
- [4] P. Bruckner, 'Suprastructures of extracellular matrices: paradigms of functions controlled by aggregates rather than molecules', *Cell Tissue Res*, vol. 339, no. 1, pp. 7–18, Jan. 2010, doi: 10.1007/s00441-009-0864-0.
- [5] K. Von der mark, 'CHAPTER 1 - Structure, Biosynthesis and Gene Regulation of Collagens in Cartilage and Bone', in *Dynamics of Bone and Cartilage Metabolism (Second Edition)*, M. J. Seibel, S. P. Robins, and J. P. Bilezikian, Eds., Burlington: Academic Press, 2006, pp. 3–40. doi: 10.1016/B978-012088562-6/50002-9.
- [6] J. Myllyharju and K. I. Kivirikko, 'Collagens, modifying enzymes and their mutations in humans, flies and worms', *Trends in Genetics*, vol. 20, no. 1, pp. 33–43, Jan. 2004, doi: 10.1016/j.tig.2003.11.004.
- [7] J. Myllyharju and K. I. Kivirikko, 'Collagens and collagen-related diseases', *Ann Med*, vol. 33, no. 1, pp. 7–21, Feb. 2001, doi: 10.3109/07853890109002055.
- [8] J. Bella, M. Eaton, B. Brodsky, and H. M. Berman, 'Crystal and molecular structure of a collagen-like peptide at 1.9 Å resolution', *Science*, vol. 266, no. 5182, pp. 75–81, Oct. 1994, doi: 10.1126/science.7695699.

- [9] M. D. Shoulders and R. T. Raines, ‘Collagen Structure and Stability’, *Annu. Rev. Biochem.*, vol. 78, no. 1, pp. 929–958, Jun. 2009, doi: 10.1146/annurev.biochem.77.032207.120833.
- [10] R. D. Fraser, T. P. MacRae, and E. Suzuki, ‘Chain conformation in the collagen molecule’, *J. Mol. Biol.*, vol. 129, no. 3, pp. 463–481, Apr. 1979, doi: 10.1016/0022-2836(79)90507-2.
- [11] S. Gorgieva and V. Kokol, ‘Collagen- vs. Gelatine-Based Biomaterials and Their Biocompatibility: Review and Perspectives’, *Biomaterials Applications for Nanomedicine*, Nov. 2011, doi: 10.5772/24118.
- [12] J. Rossert and B. de Crombrughe, ‘Chapter 12 - Type I Collagen: Structure, Synthesis, and Regulation’, in *Principles of Bone Biology (Second Edition)*, J. P. Bilezikian, L. G. Raisz, and G. A. Rodan, Eds., San Diego: Academic Press, 2002, pp. 189–XVIII. doi: 10.1016/B978-012098652-1.50114-1.
- [13] D. J. Hulmes and A. Miller, ‘Molecular packing in collagen’, *Nature*, vol. 293, no. 5829, pp. 239–234, Sep. 1981, doi: 10.1038/293239c0.
- [14] K. Gelse, E. Pöschl, and T. Aigner, ‘Collagens--structure, function, and biosynthesis’, *Adv. Drug Deliv. Rev.*, vol. 55, no. 12, pp. 1531–1546, Nov. 2003, doi: 10.1016/j.addr.2003.08.002.
- [15] M. Yamauchi, M. Terajima, and M. Shiiba, ‘Lysine Hydroxylation and Cross-Linking of Collagen’, *Methods Mol Biol*, vol. 1934, pp. 309–324, 2019, doi: 10.1007/978-1-4939-9055-9_19.
- [16] A. Sorushanova, I. Skoufos, A. Tzora, A. M. Mullen, and D. I. Zeugolis, ‘The influence of animal species, gender and tissue on the structural, biophysical, biochemical and biological properties of collagen sponges’, *J Mater Sci: Mater Med*, vol. 32, no. 1, p. 12, Jan. 2021, doi: 10.1007/s10856-020-06485-4.

- [17] R. Schrieber and H. Gareis, *Gelatine Handbook: Theory and Industrial Practice*. John Wiley & Sons, 2007.
- [18] M. J. Seibel, S. P. Robins, and J. P. Bilezikian, *Dynamics of Bone and Cartilage Metabolism: Principles and Clinical Applications*. Elsevier, 2006.
- [19] P. Bruckner and M. van der Rest, ‘Structure and function of cartilage collagens’, *Microsc. Res. Tech.*, vol. 28, no. 5, pp. 378–384, Aug. 1994, doi: 10.1002/jemt.1070280504.
- [20] R. Mayne, ‘Cartilage collagens. What is their function, and are they involved in articular disease?’, *Arthritis Rheum.*, vol. 32, no. 3, pp. 241–246, Mar. 1989, doi: 10.1002/anr.1780320302.
- [21] D. I. Zeugolis, G. R. Paul, and G. Attenburrow, ‘Cross-linking of extruded collagen fibers—A biomimetic three-dimensional scaffold for tissue engineering applications’, *Journal of Biomedical Materials Research Part A*, vol. 89A, no. 4, pp. 895–908, 2009, doi: 10.1002/jbm.a.32031.
- [22] K. Weadock, R. M. Olson, and F. H. Silver, ‘Evaluation of Collagen Crosslinking Techniques’, *Biomaterials, Medical Devices, and Artificial Organs*, vol. 11, no. 4, pp. 293–318, Jan. 1983, doi: 10.3109/10731198309118815.
- [23] H. Daemi, S. Rajabi-Zeleti, H. Sardon, M. Barikani, A. Khademhosseini, and H. Baharvand, ‘A robust super-tough biodegradable elastomer engineered by supramolecular ionic interactions’, *Biomaterials*, vol. 84, pp. 54–63, Apr. 2016, doi: 10.1016/j.biomaterials.2016.01.025.
- [24] F. Esfandiari *et al.*, ‘Microparticle-Mediated Delivery of BMP4 for Generation of Meiosis-Competent Germ Cells from Embryonic Stem Cells’, *Macromol Biosci*, vol. 17, no. 3, Mar. 2017, doi: 10.1002/mabi.201600284.

- [25] T. Miyata, T. Taira, and Y. Noishiki, 'Collagen engineering for biomaterial use', *Clin Mater*, vol. 9, no. 3–4, pp. 139–148, 1992, doi: 10.1016/0267-6605(92)90093-9.
- [26] R. Jin, L. S. Moreira Teixeira, P. J. Dijkstra, C. A. van Blitterswijk, M. Karperien, and J. Feijen, 'Enzymatically-crosslinked injectable hydrogels based on biomimetic dextran–hyaluronic acid conjugates for cartilage tissue engineering', *Biomaterials*, vol. 31, no. 11, pp. 3103–3113, Apr. 2010, doi: 10.1016/j.biomaterials.2010.01.013.
- [27] N. Raia, B. Partlow, M. McGill, E. Kimmerling, C. Ghezzi, and D. Kaplan, 'Enzymatically crosslinked silk-hyaluronic acid hydrogels', *Biomaterials*, vol. 131, pp. 58–67, Mar. 2017, doi: 10.1016/j.biomaterials.2017.03.046.
- [28] S. J. Sofia, A. Singh, and D. L. Kaplan, 'PEROXIDASE-CATALYZED CROSSLINKING OF FUNCTIONALIZED POLYASPARTIC ACID POLYMERS', *Journal of Macromolecular Science, Part A*, vol. 39, no. 10, pp. 1151–1181, Jan. 2002, doi: 10.1081/MA-120014843.
- [29] L. S. Moreira Teixeira, J. Feijen, C. A. van Blitterswijk, P. J. Dijkstra, and M. Karperien, 'Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering', *Biomaterials*, vol. 33, no. 5, pp. 1281–1290, Feb. 2012, doi: 10.1016/j.biomaterials.2011.10.067.
- [30] K. Yamauchi, T. Goda, N. Takeuchi, H. Einaga, and T. Tanabe, 'Preparation of collagen/calcium phosphate multilayer sheet using enzymatic mineralization', *Biomaterials*, vol. 25, no. 24, pp. 5481–5489, Nov. 2004, doi: 10.1016/j.biomaterials.2003.12.057.
- [31] E. Jorge-Herrero *et al.*, 'Influence of different chemical cross-linking treatments on the properties of bovine pericardium and collagen', *Biomaterials*, vol. 20, no. 6, pp. 539–545, Mar. 1999, doi: 10.1016/s0142-9612(98)90205-8.

- [32] J. H. Shepherd, S. Ghose, S. J. Kew, A. Moavenian, S. M. Best, and R. E. Cameron, 'Effect of fiber crosslinking on collagen-fiber reinforced collagen–chondroitin-6-sulfate materials for regenerating load-bearing soft tissues', *Journal of Biomedical Materials Research Part A*, vol. 101A, no. 1, pp. 176–184, 2013, doi: <https://doi.org/10.1002/jbm.a.34317>.
- [33] S. J. Kew *et al.*, 'Synthetic collagen fascicles for the regeneration of tendon tissue', *Acta Biomaterialia*, vol. 8, no. 10, pp. 3723–3731, Oct. 2012, doi: [10.1016/j.actbio.2012.06.018](https://doi.org/10.1016/j.actbio.2012.06.018).
- [34] D. Enea *et al.*, 'Collagen fibre implant for tendon and ligament biological augmentation. In vivo study in an ovine model', *Knee Surg Sports Traumatol Arthrosc*, vol. 21, no. 8, pp. 1783–1793, Aug. 2013, doi: [10.1007/s00167-012-2102-7](https://doi.org/10.1007/s00167-012-2102-7).
- [35] L. H. Olde Damink, P. J. Dijkstra, M. J. van Luyn, P. B. van Wachem, P. Nieuwenhuis, and J. Feijen, 'Cross-linking of dermal sheep collagen using a water-soluble carbodiimide', *Biomaterials*, vol. 17, no. 8, pp. 765–773, Apr. 1996, doi: [10.1016/0142-9612\(96\)81413-x](https://doi.org/10.1016/0142-9612(96)81413-x).
- [36] M. Spira, B. Liu, Z. Xu, R. Harrell, and H. Chahadeh, 'Human amnion collagen for soft tissue augmentation--biochemical characterizations and animal observations', *J Biomed Mater Res*, vol. 28, no. 1, pp. 91–96, Jan. 1994, doi: [10.1002/jbm.820280112](https://doi.org/10.1002/jbm.820280112).
- [37] D. Olsen *et al.*, 'Recombinant collagen and gelatin for drug delivery', *Adv Drug Deliv Rev*, vol. 55, no. 12, pp. 1547–1567, Nov. 2003, doi: [10.1016/j.addr.2003.08.008](https://doi.org/10.1016/j.addr.2003.08.008).
- [38] R. S. Walton, D. D. Brand, and J. T. Czernuszka, 'Influence of telopeptides, fibrils and crosslinking on physicochemical properties of Type I collagen films', *J Mater Sci: Mater Med*, vol. 21, no. 2, pp. 451–461, Feb. 2010, doi: [10.1007/s10856-009-3910-2](https://doi.org/10.1007/s10856-009-3910-2).

- [39] D. E. Birk and F. H. Silver, 'Collagen fibrillogenesis in vitro: comparison of types I, II, and III', *Arch Biochem Biophys*, vol. 235, no. 1, pp. 178–185, Nov. 1984, doi: 10.1016/0003-9861(84)90266-2.
- [40] B. R. Williams, R. A. Gelman, D. C. Poppke, and K. A. Piez, 'Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results', *J Biol Chem*, vol. 253, no. 18, pp. 6578–6585, Sep. 1978.
- [41] Y. Li, A. Asadi, M. R. Monroe, and E. P. Douglas, 'pH effects on collagen fibrillogenesis in vitro: Electrostatic interactions and phosphate binding', *Materials Science and Engineering: C*, vol. 29, no. 5, pp. 1643–1649, Jun. 2009, doi: 10.1016/j.msec.2009.01.001.
- [42] D. L. Helseth Jr. and A. Veis, 'Collagen self-assembly in vitro. Differentiating specific telopeptide-dependent interactions using selective enzyme modification and the addition of free amino telopeptide.', *Journal of Biological Chemistry*, vol. 256, no. 14, pp. 7118–7128, 1981.
- [43] G. C. Wood and M. K. Keech, 'The formation of fibrils from collagen solutions 1. The effect of experimental conditions: kinetic and electron-microscope studies*', *Biochemical Journal*, vol. 75, no. 3, pp. 588–598, Jun. 1960, doi: 10.1042/bj0750588.
- [44] T. Hayashi and Y. Nagai, 'Factors affecting the interactions of collagen molecules as observed by in vitro fibril formation: II. effects of species and concentration of anions', *Journal of Biochemistry*, vol. 74, no. 2, pp. 253–262, 1973.
- [45] T. Hayashi and Y. Nagai, 'Factors affecting the interactions of collagen molecules as observed by in Vitro fibril formation: III. non-helical regions of the collagen molecules', *Journal of Biochemistry*, vol. 76, no. 1, pp. 177–186, 1974, doi: 10.1093/oxfordjournals.jbchem.a130543.

- [46] A. Cooper, 'Thermodynamic studies of the assembly in vitro of native collagen fibrils', *Biochem J*, vol. 118, no. 3, pp. 355–365, 1970.
- [47] S.-H. Park *et al.*, 'Comparative analysis of collagens extracted from different animal sources for application of cartilage tissue engineering', *Int. J. Precis. Eng. Manuf.*, vol. 13, no. 11, pp. 2059–2066, Nov. 2012, doi: 10.1007/s12541-012-0271-4.
- [48] J. M. Cassel, 'Collagen aggregation phenomena', *Biopolymers*, vol. 4, no. 9, pp. 989–997, 1966, doi: 10.1002/bip.1966.360040904.
- [49] M. Schauperl, M. Podewitz, B. J. Waldner, and K. R. Liedl, 'Enthalpic and Entropic Contributions to Hydrophobicity', *J. Chem. Theory Comput.*, vol. 12, no. 9, pp. 4600–4610, Sep. 2016, doi: 10.1021/acs.jctc.6b00422.
- [50] K. E. Kadler, D. F. Holmes, J. A. Trotter, and J. A. Chapman, 'Collagen fibril formation', *Biochem J*, vol. 316 (Pt 1), pp. 1–11, May 1996, doi: 10.1042/bj3160001.
- [51] L. H. Olde Damink, P. J. Dijkstra, M. J. van Luyn, P. B. van Wachem, P. Nieuwenhuis, and J. Feijen, 'Cross-linking of dermal sheep collagen using a water-soluble carbodiimide', *Biomaterials*, vol. 17, no. 8, pp. 765–773, Apr. 1996, doi: 10.1016/0142-9612(96)81413-x.
- [52] N. Davidenko *et al.*, 'Control of crosslinking for tailoring collagen-based scaffolds stability and mechanics', *Acta Biomater*, vol. 25, pp. 131–142, Oct. 2015, doi: 10.1016/j.actbio.2015.07.034.
- [53] Yang Xu, 'Development of Tri-layered Biomimetic Atelocollagen Scaffolds with Interfaces for Osteochondral Tissue Engineering', University of Oxford, 2022.
- [54] Sonia Iftekhar, 'Isolation of Fibrillar Elastin Gel (FEG) and its Application in Heart Valve Tissue Engineering', University of Oxford, 2020.

- [55] Rabia Nazir, ‘Development and Characterization of Collagen type I and Hyaluronic acid (HA) based Interlaced Scaffolds for Tissue Engineered Heart Valves (TEHVs)’, University of Oxford, 2017.
- [56] D. V. Shepherd, J. H. Shepherd, S. Ghose, S. J. Kew, R. E. Cameron, and S. M. Best, ‘The process of EDC-NHS Cross-linking of reconstituted collagen fibres increases collagen fibrillar order and alignment’, *APL Mater*, vol. 3, no. 1, Jan. 2015, doi: 10.1063/1.4900887.
- [57] ‘N-(3-Dimethylaminopropyl)-N -ethylcarbodiimide crystalline 25952-53-8’. Accessed: Sep. 20, 2023. [Online]. Available: <https://www.sigmaaldrich.com/GB/en/product/sial/e6383>
- [58] ‘N-Hydroxysuccinimide 98 6066-82-6’. Accessed: Sep. 20, 2023. [Online]. Available: <http://www.sigmaaldrich.com/>
- [59] M.-T. Sheu, J.-C. Huang, G.-C. Yeh, and H.-O. Ho, ‘Characterization of collagen gel solutions and collagen matrices for cell culture’, *Biomaterials*, vol. 22, no. 13, pp. 1713–1719, Jul. 2001, doi: 10.1016/S0142-9612(00)00315-X.
- [60] R. B. Sashidhar, A. K. Capoor, and D. Ramana, ‘Quantitation of epsilon-amino group using amino acids as reference standards by trinitrobenzene sulfonic acid. A simple spectrophotometric method for the estimation of hapten to carrier protein ratio’, *J Immunol Methods*, vol. 167, no. 1–2, pp. 121–127, Jan. 1994, doi: 10.1016/0022-1759(94)90081-7.
- [61] I. Ofner Clyde M. and W. A. Bubnis, ‘Chemical and Swelling Evaluations of Amino Group Crosslinking in Gelatin and Modified Gelatin Matrices’, *Pharm Res*, vol. 13, no. 12, pp. 1821–1827, Dec. 1996, doi: 10.1023/A:1016029023910.

Chapter 3 - Biological

3.1. Introduction

Traditional conservative or surgical repair techniques for the enthesis are unable to achieve enthesis healing and regeneration effectively to recapitulate the complex transition between ligament and bone. Although enthesis regeneration has increasingly become a subject of intense interest within the field of orthopaedic and tissue engineering research, the development of a successful alternative to highly invasive traditional techniques has been hindered by a lack of understanding of enthesis structure and composition.

The fibrocartilage is said to play a key role in absorbing mechanical loads. When force is applied, the unmineralised zone protects the soft tissue and the mineralised zone protects the bone [1]. However, the most prominent component in the extracellular matrix (ECM) of all four regions is collagen, therefore, it is important to understand the differences which allow for the absorption of mechanical loads and the prevention of damage. Furthermore, it has been suggested that the mechanical feature of natural tissues is their nonlinear properties, which have been strongly linked to their degree of mineralisation [2]-[3].

Given the important role of the structure-function relationship, the following chapter will evaluate the mechanical and histological properties of the ligament-bone interface to understand what should be considered in the scaffold design of this complex region.

3.2. Methods

3.2.1. Dissection of Pig Knees

Pig knees were obtained from a local butcher in Oxford (Alder's Butchers). Sacrificed animals had been stored at +4°C for 2-3 days before collection. The pig knees were dissected and the anterior cruciate ligament (ACL) to the tibia insertion site was extracted and sectioned into 1cm thick pieces running parallel to the direction of ligament fibres (Figure 3.1).

3.2.2. Polished Samples

The dissected sections were first left in PBS overnight to allow for the bones to release any excess blood. Samples were then dehydrated stepwise in increasing concentrations of ethanol starting at 70%, followed by 80%, 95% and finishing with 100% EtOH for 1hr and left to air dry. Pieces of the knee were then embedded in a cold-curing epoxy resin (Epofix, Struers). This embedding medium was selected to avoid infiltration of the polymer in the tissue pores. Once the epoxy had completely cured, samples were gradually rehydrated in preparation for polishing. Surfaces were polished using finer grades of carbide papers (P800 P1200, P2500 and P4000) under deionised water. Although finer grades of carbide paper could be used, ultrastructural mineral aggregated (100µm spheroids) ultimately limit the bone specimens' surface quality [4].

3.2.3. Sectioned Samples

Dissected knee samples were fixed with 10% formalin for 24hrs at room temperature, and cryo-protected in 30% sucrose for 2 hours at 4°C. Samples were then placed in dry ice for 1min before mounting on a cryostat holder with optimal cutting temperature compound (OCT, Sakura Tissue Tek, 4583). Samples were cryo-sectioned using a cryostat (Bright 5040) at -15°C into 20µm sections according to the Kawamoto method [5]. Briefly, the adhesive film

(Cryofilm) was cut so that the adhesive area was the same dimensions as the frozen block surface. The adhesive film was mounted onto the specimen's surface and cut slowly at a constant speed. The sections were taped to a glass slide and stored at +4°C until the staining procedure. The cryostat was provided by histology lab facilities in the Department of Physiology, Anatomy and Genetics (DPAG).

All samples were left to reach room temperature before being hydrated in an ethanol gradient (Abs. EtOH, 96%, 90%, 70%, and DI water respectively for 3 mins each), stained, dehydrated (70%, 90%, 96%, and Abs. EtOH respectively for 3 mins each) and left to air dry in preparation for applying the coverslip. The slides were cleared with Histo-Clear II and covered with a non-aqueous mounting agent. Histology staining was performed in the Department of Materials. Samples were examined under a Nikon Eclipse Ci microscope in DPAG at various magnifications.

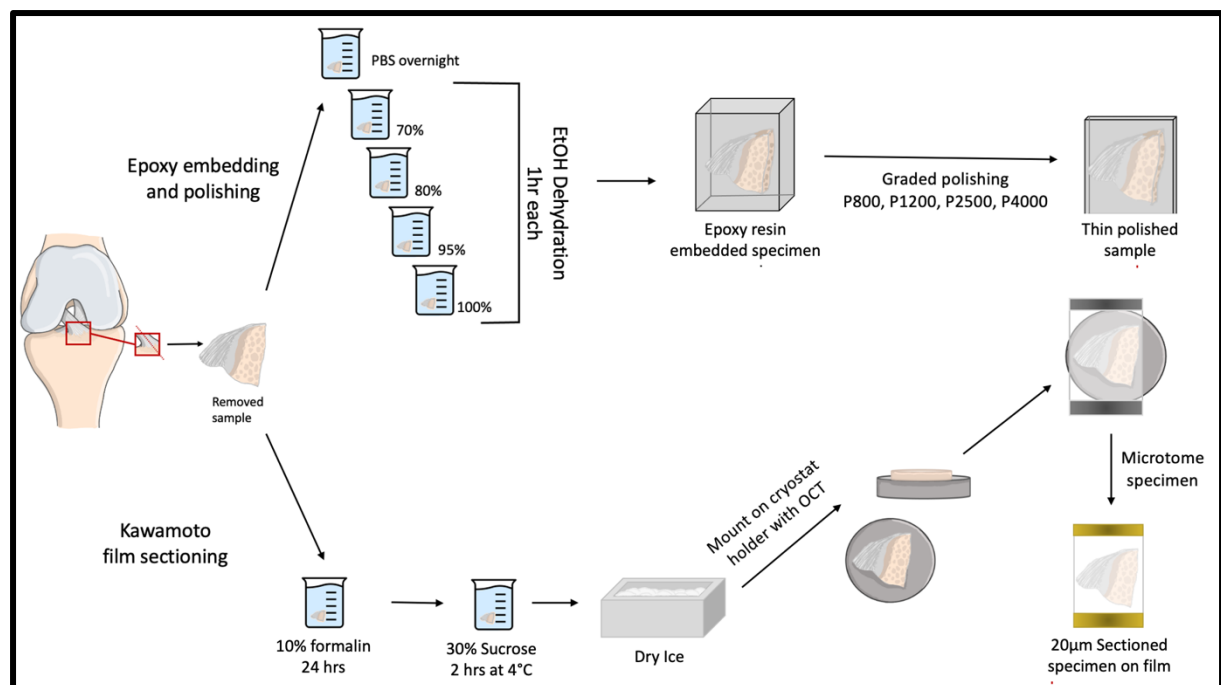


Figure 3.1: Representation of specimen preparation for polished and sectioned samples.

3.2.4. Histology Staining

3.2.4.1. Haematoxylin and Eosin (H&E) Staining

Enthesis slides were stained using Haematoxylin solution according to Mayer (Sigma-Aldrich, UK) and Eosin Y solution 1% alcoholic for microscopy (Sigma-Adrich, UK), according to the standard procedures. Briefly, slides were re-hydrated, as described in Section 3.2.3. The hydrated slides were stained with haematoxylin for 7 mins to counterstain the nuclei. The slides were rinsed under running water for 5 mins before being stained with Eosin for 13 mins. The slides were then immediately dehydrated and mounted as described in Section 3.2.3.

3.2.4.2. Safranin O Staining

Enthesis slides were stained using 0.1% safranin O (Merck, UK) according to the standard protocol. Briefly, slides were re-hydrated, as described in Section 3.2.3. The hydrated slides were stained in Weigert's iron haematoxylin working solution for 10 mins before being rinsed under running water for a further 10 mins. The samples were then stained with 0.2% fast green for 5 mins and quickly rinsed with 1% acetic acid solution for no more than 10-15 secs before being stained in the safranin O solution for 5 mins and were then immediately dehydrated and mounted as described in Section 3.2.3.

3.2.4.3. Alcian Blue Staining

To stain the acidic glycosaminoglycans (GAGs), including chondroitin sulphate, entheses slides were stained using Alcian Blue (AB) solution (Merck, Germany) 1% in 3% acetic acid, pH 2.5, according to the standard procedure. Briefly, slides were hydrated as described in Section 3.2.3. The hydrated slides were stained in AB solution for 15 mins and rinsed under running water for 5 mins. The slides were then rinsed in distilled water before being

counterstained with a Neutral red solution (MP Biomedicals, USA) for 1 min. Samples were dehydrated and mounted as described in Section 3.2.3.

3.2.4.4. von Kossa Staining

Enthesis slides were treated with silver nitrate using the standard procedures to examine the calcium deposition within the tissue. Briefly, slides were hydrated as described in Section 3.2.3, before being immersed in 1% silver nitrate (Sigma-Aldrich, UK) and incubated for 20 mins under UV light. The slides were then rinsed in several changes of distilled water before using 5% thiosulphate for 5 mins to remove any unreacted silver. The slides were rinsed before being counterstained with 0.1% neutral red solution (MP Biomedicals, UK). Samples were rinsed 1 last time before being dehydrated and mounted as described in Section 3.2.3.

3.2.5. Mechanical Testing

Mechanical testing of biological samples was carried out using an INSTRON 3360 series with BlueHill Universal system and a 10kN load cell.

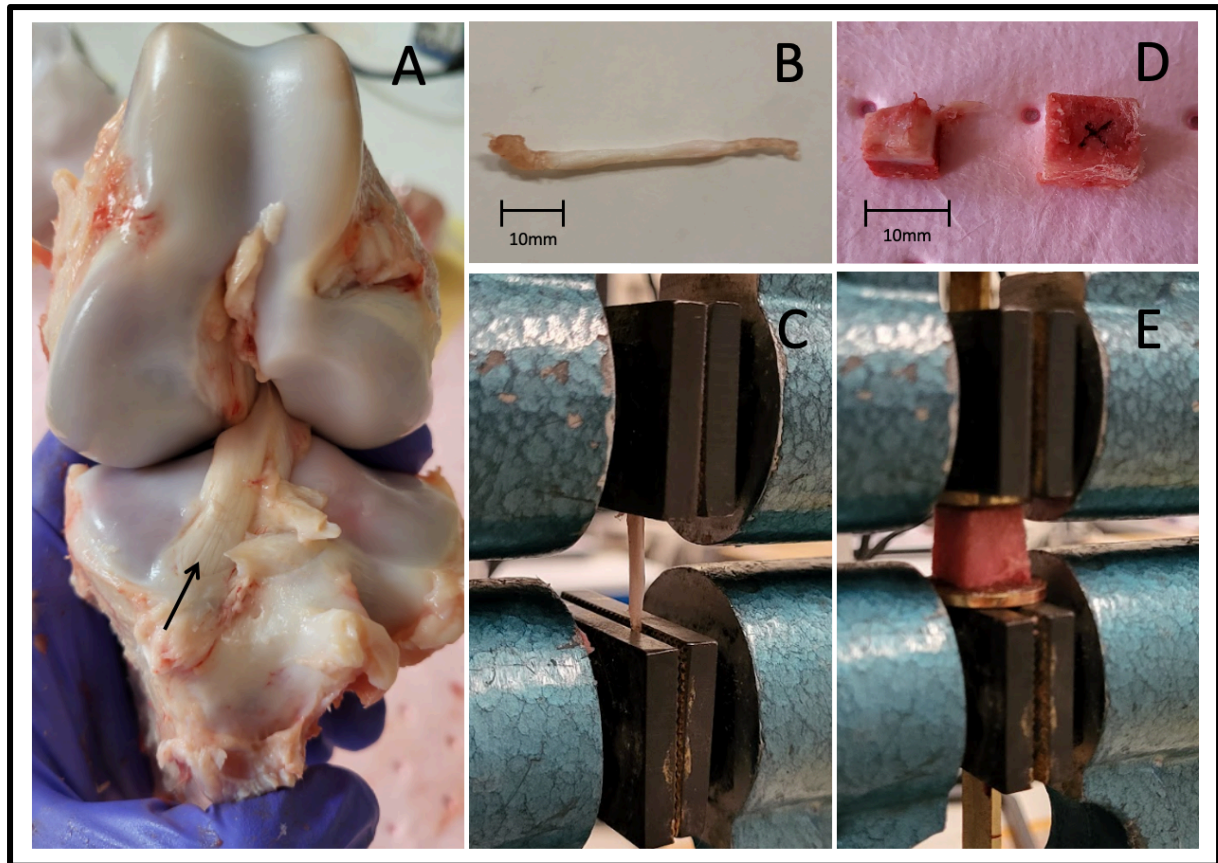


Figure 3.2: Pig knee dissection and sample preparation for tensile and compression testing. (A) Pig Knee during dissection. The arrow is pointing at the ACL still attached to the tibia. (B) ligament sample prepped for tensile testing with ends that have been air-dried. (C) Ligament sample mounted in tensile testing setup. (D) Samples were prepped for compression testing. Left is a fibrocartilage/bone sample, right is a bone sample. (E) Bone sample mounted in compression testing setup. Scale bars are 10mm

3.2.5.1. Tensile

The pig knees were dissected, and the ACL was removed. The ACL was longitudinally sliced into thinner sections (1-2mm diameter), being careful to not disrupt any of the individual fibres. The sectioned slices were then wrapped in parafilm with the ends left out to dry. Once the ends had dried, the samples were ready to be mounted. The dried ends ensured that the samples did

not split out of the clamps. Tests were run at 500N/min with a preload of 25N using at 10kN load cell. Samples were tested longitudinally in the direction of fibres.

3.2.5.2. Compression

The pig knees were dissected, and the tibia was removed. 10x10x10mm cubes of cortical bone were sectioned out. A couple of the samples were taken from the ACL-tibia enthesis region. Flat plates were prepared for the samples to be positioned on during compression testing which were lined with abrasive paper to prevent the wet samples from slipping. Tests were run at 500N/min with a preload of 25N using a 10kN load cell. Samples were compressed along the axis which they would naturally be compressed during movement. The top surface of the specimen ran parallel to the outside surface of the bone. Illustrated in Figure 3.3.

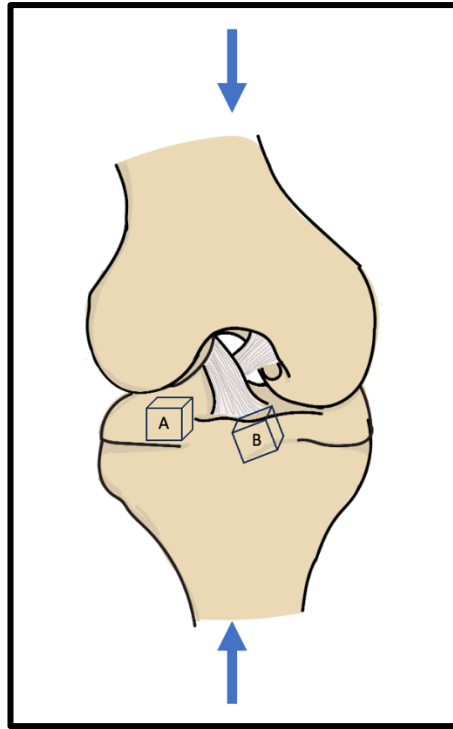


Figure 3.3: Representation of the orientation of specimen obtained for compression testing. Arrows represent the axial load applied to the knee during standing. A: orientation of bone samples parallel to direction of loading. B: orientation of enthesis samples parallel to direction of ligament fibres.

3.2.6. Scanning Electron Microscopy/ Energy Dispersive X-Ray Spectroscopy

The morphology and microstructure of the enthesis were studied using scanning electron microscopy (SEM). Polished samples, prepared as described in section 3.2.2, were adhered to aluminium stubs using carbon tabs, and sputter coated with platinum (thickness 6nm) using the EM ACE600 (Leica Microsystems, Germany). Samples were imaged using the Zeiss EVO[®] MA10 SEM (Zeiss, Germany) with a vacuum of 1×10^{-5} - 1×10^{-6} mbar. Images were generated using the secondary electron mode at a 20-35 mm working distance, operated at an accelerating voltage of 10 kV, and a probe current of 100pA.

Samples were analysed using energy dispersive X-ray spectroscopy (EDX) at an accelerating voltage of 20kV to determine the relative distribution of mineral content (calcium and phosphate) throughout the enthesis. The higher accelerating voltage is used for EDX as sufficient energy is required to excite the K and L transitions within the atoms. These samples were sputter-coated with carbon (thickness 6nm) instead of platinum.

Instruments were supplied by the David Cockayne Centre for Electron Microscopy, University of Oxford. Images were analysed using ImageJ[®] (National Institutes of Health, USA).

3.2.7. Nanoindentation

The mechanical properties through the enthesis were measured using the Hysitron TI Premier Nanoindenter (Bruker, USA). A Berkovich diamond three-sided pyramid tip, max. ID 10 μ m was used. Indentations were performed for each region of the enthesis at least 5 times. A load-controlled trapezoidal load function with a peak load of 1000 μ N and a 3-second hold. Whilst wet samples were tested using displacement controlled trapezoidal load function with a peak displacement of 1000nm and a 3-second hold. A pre-load of 5 μ N was set with a lift height of 35nm.

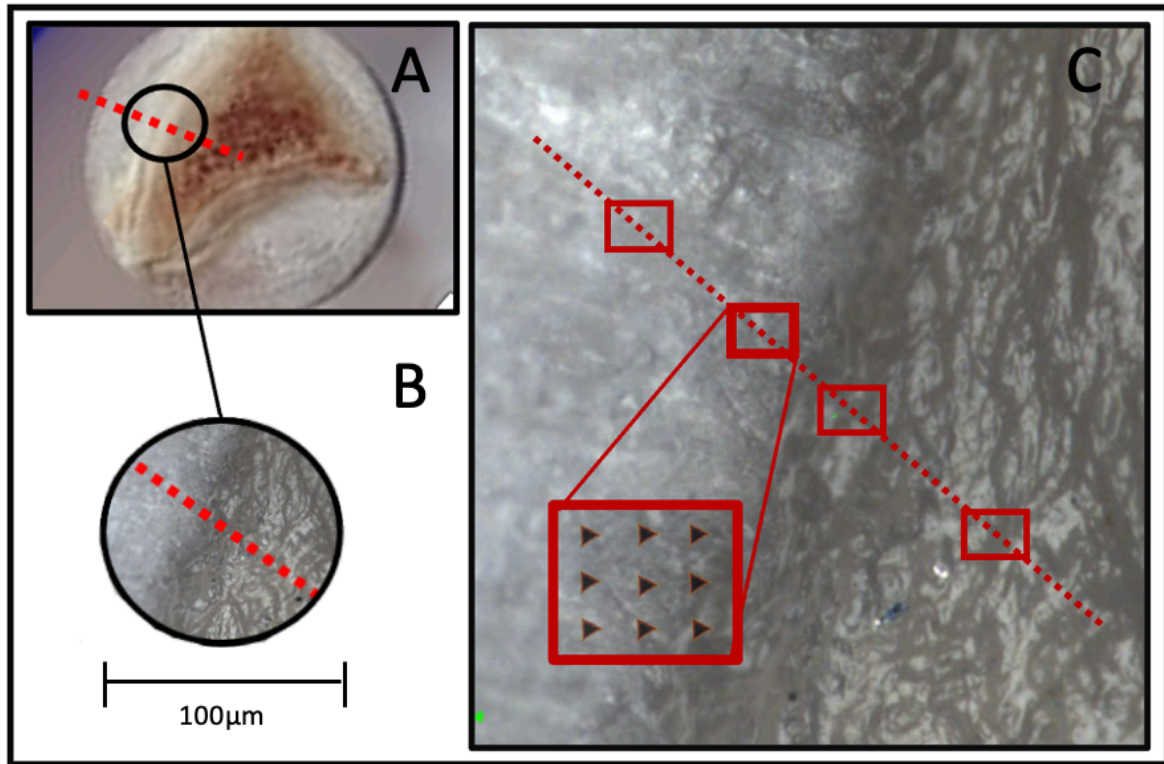


Figure 3.4: Nanoindentation sample and experimentation setup. Dotted lines run through the entire enthesis, from ligament to bone. (A) Polished sample of ACL-Tibia mounted onto stub. with four regions in which indents were performed. (B) Microscopic image of the enthesis with scale bar 100µm. (C) Microscopic image of enthesis with the 4 regions in which results were obtained for each distinct zone of the enthesis shown within rectangles.

3.2.8. Statistical Analysis

Results were expressed as mean $\pm$ standard deviation. The results were compared with one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test. For values of $p < 0.05$, the difference was considered statistically significant.

3.3. Techniques

3.3.1. Scanning Electron Microscopy/ Energy Dispersive X-Ray

Spectroscopy

Scanning Electron Microscopy (SEM) is an electron microscope that uses a finely focused electron beam to scan the surface of a sample to relay compositionally and topographical information. The interaction between incident electrons and atoms on the sample surface (and a limited sub-surface volume) leads to the emission of secondary electrons, backscattering of high energy primary electrons and creation of element-specific X-rays, which are then detected with respective detectors and finally displayed on the monitor. The main components in a typical SEM include the electron source, a column that contains electromagnetic lenses, the electron detector, the sample chamber, and the computer display (Figure 3.4).

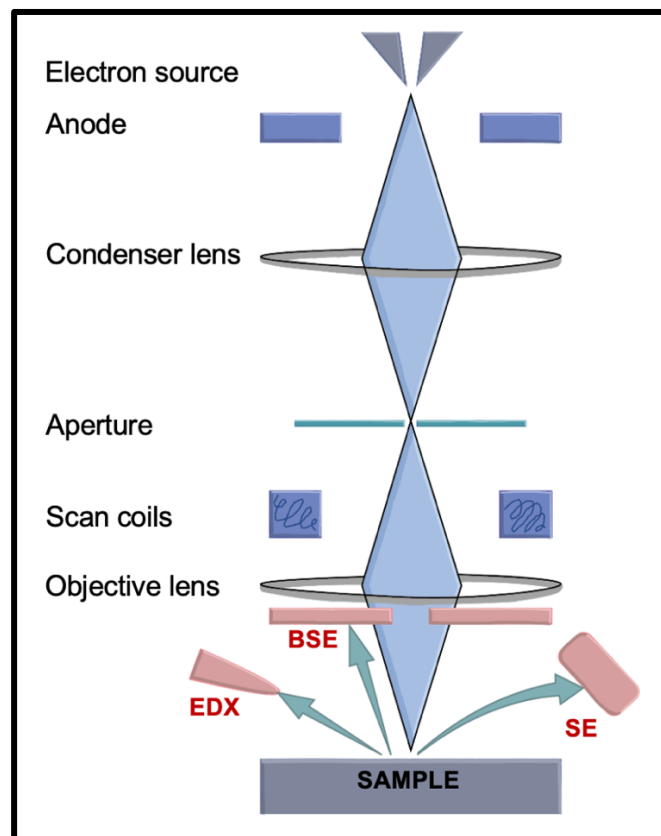


Figure 3.5: Parts of a scanning electron microscope.

Secondary Electrons (SE) mode is the most common type of signal used for SEM image processing. SEs are low-energy electrons ejected from the inner shells of the atoms in the sample. Because of inelastic scattering interactions with the incident electrons, SEs typically originate from within a few nanometres of the sample surface. Therefore, SE is relatively accurate to produce the topological contrast of the sample such as surface texture and roughness.

Backscattered Electrons (BSE) are high-energy electrons (higher than 50eV) of the incident beam that are deflected back by very high angles due to elastic scattering interactions with atomic nuclei. The elastic collision of the electron with a higher than 90 degrees directional change will result in almost half of the electrons bouncing back to the direction of their origin and retaining most of their initial energy [18]. BSE Z- (atomic number) contrast enables distinguishing between regions on the sample surface having different average atomic numbers. Higher atomic number elements (high Z) will deflect more electrons than lighter elements (low Z) due to a high number of positive ions on its nucleus, thus will appear brighter in the image.

As most biological materials are non-conductive, it can cause an accumulation of static electric charges on the specimen surface, causing what is known as the charging effect. This static charge influences the electron signals and deteriorates the image information. Therefore, these non-conductive samples must be rendered electrically conductive, for which a variety of strategies are available: The use of less accelerating voltage, impregnation of the samples with heavy metals, application of thin conductive coatings (Au, Pt, Pd, C), and use of room

temperature ionic liquids. A conventional SEM is operated at high vacuum conditions that require samples to be clean, dry, and electrically conductive.

The gold standard method of reducing charge build-up, a conductive film, works by passing developed charges to the ground via the conductive layer, thus negative charges are not accumulated over the specimen surface. Without a conductive coating, these negative charges are left to build up on the surface of the specimen and therefore, the number of secondary electrons emitted from the specimen may decrease due to the decrease of incident electrons landing on the specimen and hence the image quality is deteriorated (Figure 3.6).

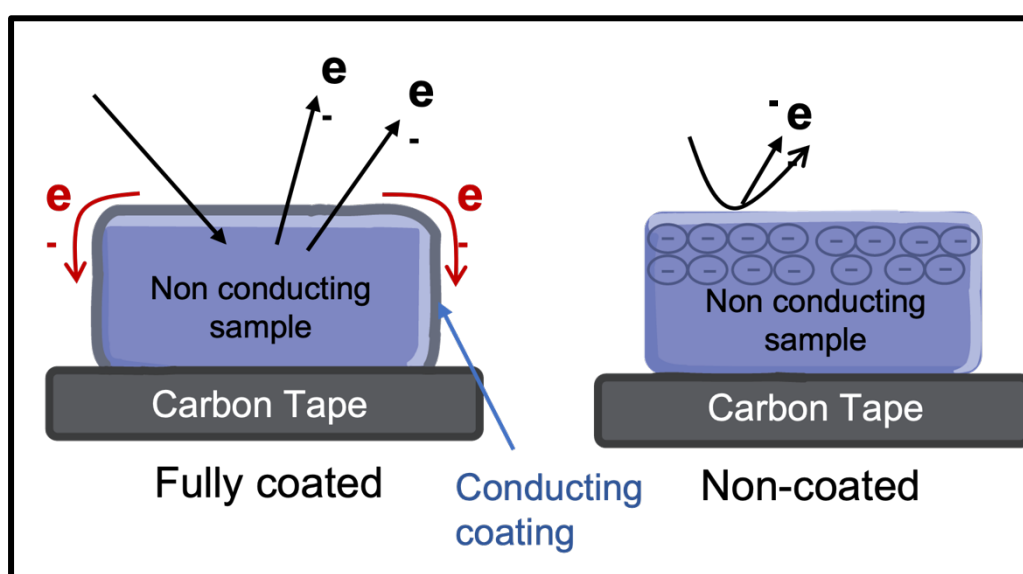


Figure 3.6: Schematic illustration of secondary electron emission during SEM analysis of non-conductive specimens depending on the coating conditions.

Electron Dispersive X-Ray (EDX) spectroscopy is involved in the detection of elemental composition of substances by using SEM. EDX can detect elements that possess an atomic number higher than that of boron. Upon collision with the electron beam in typical SEM, samples interact with the beam and produce characteristic X-rays. Due to the principle that

none of the elements have the same X-ray emission spectrum, they can be differentiated and measured for their concentration, of at least 0.1%, in the sample.

The X-ray is the result of the primary beam of electron interaction with the nucleus of the sample atom. A primary electron beam will excite the electron in the nucleus of an atom, ejecting it from the nucleus and creating a core hole. An electron from an outer shell transitions into the core hole, generating a characteristic X-ray. Although no elements share the same X-ray emission spectrum, many elements will have overlapping X-ray emission peaks. Therefore, if EDX analysis is required, SEM users typically coat their samples with carbon because carbon's X-ray peak does not conflict with the peak of any other element.

3.3.2. Brightfield Microscope

Bright-field microscopy is the most basic type of optical microscopy technique. It utilises light rays to produce dark images against a bright background. It is the standard microscope used in biology studies to observe cells, tissue, and other biological samples. The microscope is used to view specimens that have been stained with basic stains which gives a contrast between the image and the background. The stained specimens will have a refractive index that distinguishes them from their surroundings through both absorption and refractive contrast. The sample which is placed on a transparent stage is illuminated from below by a light source such as a bulb or LED lamp. The light passes through the sample and is focused onto the objective lens by a condenser lens. The objective lenses magnify the image, and the ocular lenses magnify the image for the observer.

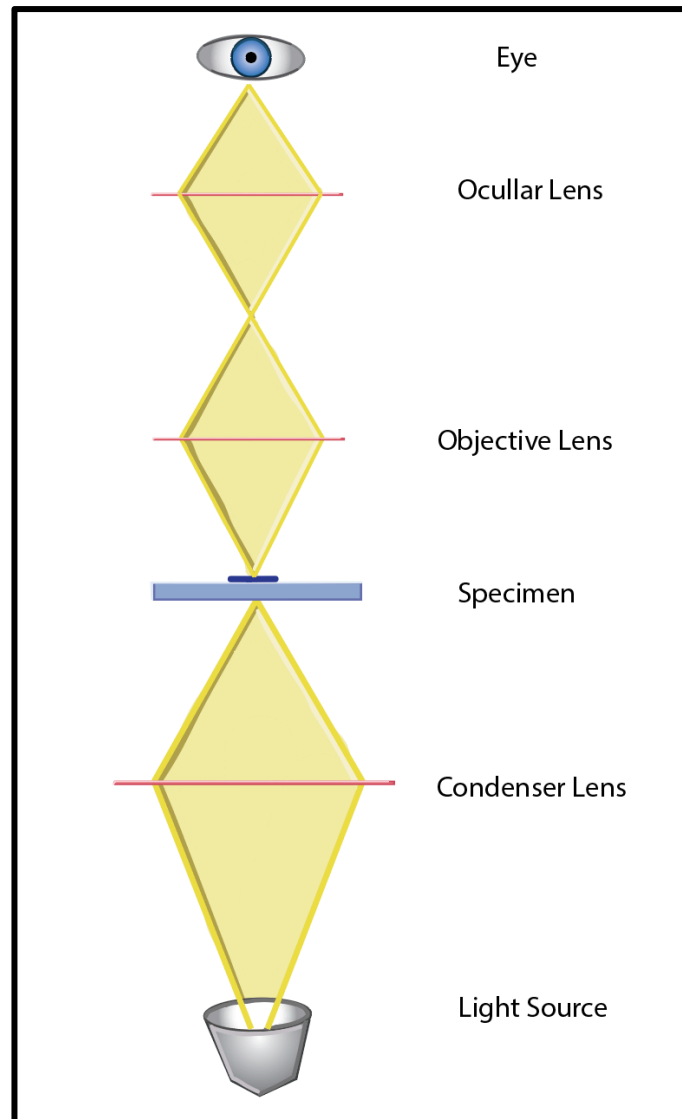


Figure 3.7: Parts of a brightfield microscope.

3.3.3. Nanoindentation

Nanoindentation is a technique used to measure the modulus and hardness of a material based on its contact with another known material, most commonly diamond. The major benefit of nanoindentation over traditional hardness testing is that, during macro- or micro-indentation, a hard tip, is pressed into a sample with a known load and after a set period, the load is removed. The area of the residual indentation in the sample is measured and the hardness is calculated.

However, to determine the indent area, a powerful microscope is needed, or valuable time-dependent information will be lost.

A Quasi-static nanoindentation test is performed by applying and removing a load to a sample in a controlled manner with a geometrically well-defined probe of known material. During nanoindentation testing, a force is applied by the transducer and the depth of penetration of the tip is subsequently measured during the process. The indentation depth is used to calculate the area of the tip that is in contact during the indentation which is used to measure the hardness of the material.

Hardness (H) is a measure of a material's resistance to deformation by surface indentation.

To measure hardness, H, the equation is:

$$H = \frac{P_{max}}{A(h_c)}$$

(Equation 3.1)

Where $A(h_c)$ is the indentation contact area as a function of depth, h_c . P_{max} is the indentation load.

$$h_c = h_{max} - \epsilon \frac{P_{max}}{S}$$

(Equation 3.2)

The maximum depth recorded by the indenter is h_{max} . The constant ϵ accounts for edge effects and deflection at the contact perimeter, taken as 0.75.

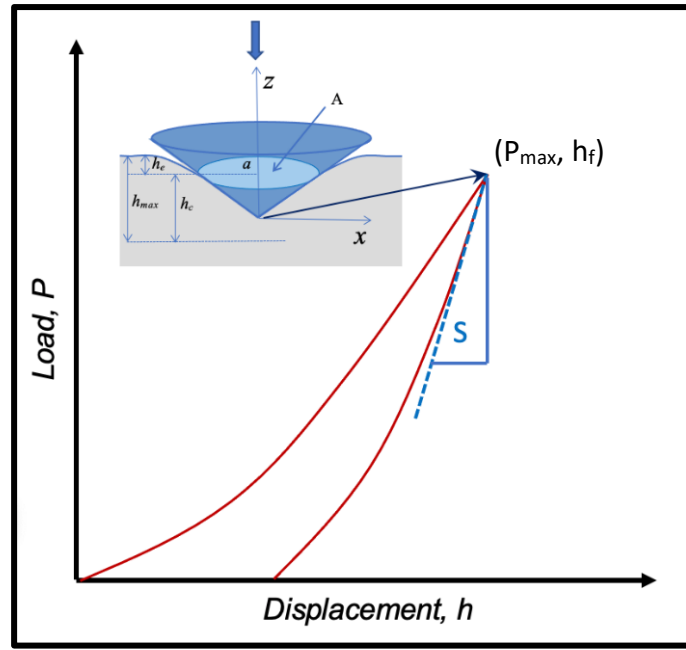


Figure 3.8: Representation of a loading-unloading curve for a nano-indentation measurement; P_{max} is the load at maximum indentation, h_{max} is the indenter displacement at peak load, h_f is the final depth of the contact impression after unloading and S is the initial unloading stiffness.

On the other hand, Oliver and Pharr utilises the specimen's unloading response, which is assumed to be purely elastic, to analyse the stiffness of the material. This method is based on Hertz' theory for elastic contact, which states that the stiffness $S = dP/dh$ of the contact between two elastic spheres (P = contact force, h = relative movement of the centres of the spheres) is given by:

$$E_r = \left(\frac{\sqrt{\pi}}{2}\right)\left(\frac{S}{\sqrt{A_c}}\right)$$

(Equation 3.3)

Where $E_r = 1/\left[\frac{1-v_1^2}{E_1} + \frac{1-v_2^2}{E_2}\right]$ is an effective elastic modulus of the two spheres, E_1 and E_2 are their Young's moduli, v_1 and v_2 are their Poisson's ratio and A_c the projected area of contact. In the Oliver–Pharr method, the tip and the sample are modelled as two such contacting bodies. Diamond tips have $E_2=1140$ GPa and $v_2=0.07$, and so if E_r is known, the quantity

$(1 - \nu_1^2)/E_1$ for the sample can be obtained.

The Poisson's ratio for:

Cortical bone: 0.3 [6], [7]

Calcified fibrocartilage: 0.35 [8]

Uncalcified fibrocartilage: 0.42 [6], [8]

Ligament: 0.49 [6]

It is known that almost all biomaterials are viscoelastic, exhibiting time-dependent behaviour.

The main effect of viscoelasticity on indentation is creep, which means that the tip continues sinking into the sample even under constant load.

In cases where loading is followed directly by unloading, displacement increases slightly in the initial part of the unloading phase, as the creep rate of the material is initially higher than the imposed unloading rate. With this phenomenon, it is not possible to use the method outlined above, as a negative slope is observed in the initial unloading region. Therefore, the typical triangular (load-unload) load function is replaced by a trapezoidal load function (load-hold-unload) which has been found to allow the material to approach equilibrium and eliminate this effect [9].

3.4. Results

It is very important to correlate biochemical results to tissue structures in biological research. Histology, a type of staining, is generally carried out for this correlation. Hard tissue sections are often prepared from tissues, which are chemically fixed and decalcified, to allow for easy cutting. As the distribution of calcium through the enthesis is of interest, it is best to prepare frozen sections from undecalcified tissues. However, section preparation from completely mineralised bone is extremely difficult.

3.4.1. Histology

3.4.1.1. H&E

Figure 3.9 A shows ACL is a hypocellular ligament with collagen fibres oriented in a parallel manner and elongated cells which follow the alignment of the fibres. In Figure 3.9 B, rounder fibrocartilage cells in the zone of uncalcified fibrocartilage continue alignment and run between the parallel bundles of collagen fibres. However, as the cells near the basophilic line separating the fibrocartilage zones, they become less populated and begin to depart from alignment. There is a very visible colour transition separating the two fibrocartilage zones.

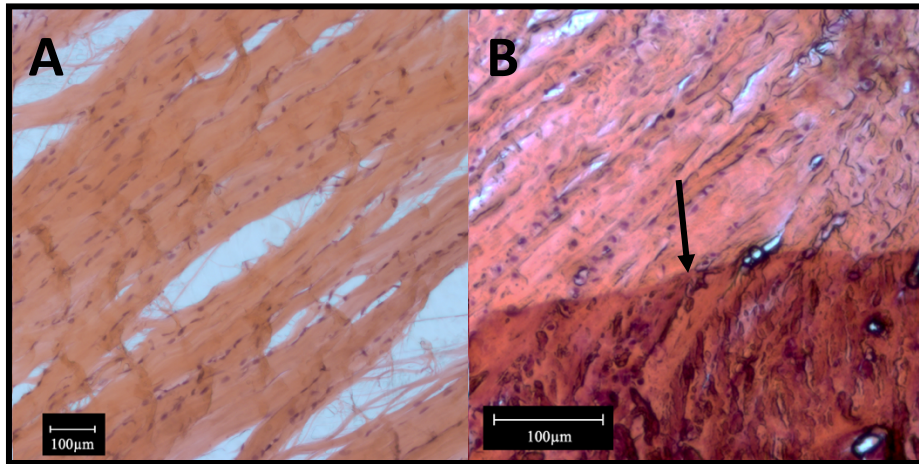


Figure 3.9: Histological sections dissected from pig, stained with H&E of ACL to tibia enthesis. (A) ligament (B) ligament to the bone with an arrow indicating the basophilic line separating the two fibrocartilage zones.

3.4.1.2. Safranin O

The safranin O stain works by binding to acidic proteoglycans in cartilaginous tissues with a high affinity, forming a reddish-orange complex. Cytoplasm appears blueish-green whilst cell nuclei appear deep purple. Both fibrocartilage regions were stained a reddish orange, Figure 3.10. There is a gradual transition in the orange dye from the ligament tissue to the uncalcified fibrocartilage. The border between the two fibrocartilage regions is where the tissue has been stained the deepest red. Similar to H&E stained samples, it can be seen through safranin O staining that collagen fibres and cells are aligned and parallel within the ligament region [10], and that fibrocartilage cells in the zone of UFC follow the arranged longitudinal rows of the ligament section but that the cells become rounder. However, moving towards the CFC, although the purple staining of the cell nuclei becomes harder to visualise with the increasing intensity of red staining, they are less populated and in an array without a distinguishable alignment. In these images, it is also very clear to see the crimp pattern of fibre bundles.

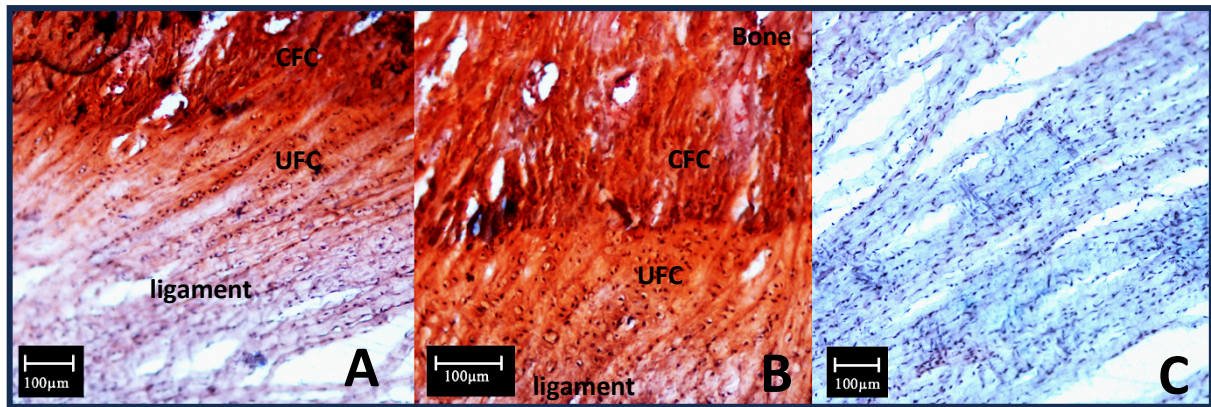


Figure 3.10: Histological sections dissected from pig, stained with safranin O of ACL to tibia enthesis (A) Ligament – CFC (B) Ligament- Bone, with roughly aligned round cells present in the UFC region, and unaligned cells present in the CFC region (C) Ligament, with elongated aligned nuclei of cells within aligned blue stained cytoplasm.

3.4.1.3. Alcian Blue

The stain molecules carry a positive charge and are attracted to compounds containing anionic groups such as acidic polysaccharides such as glycosaminoglycans in cartilaginous tissue. Figure 3.11 shows how the AB stain is concentrated in the fibrocartilage region, both calcified and uncalcified. As the tissue transitions from the UFC to CFC, the intensity of the blue staining increases.

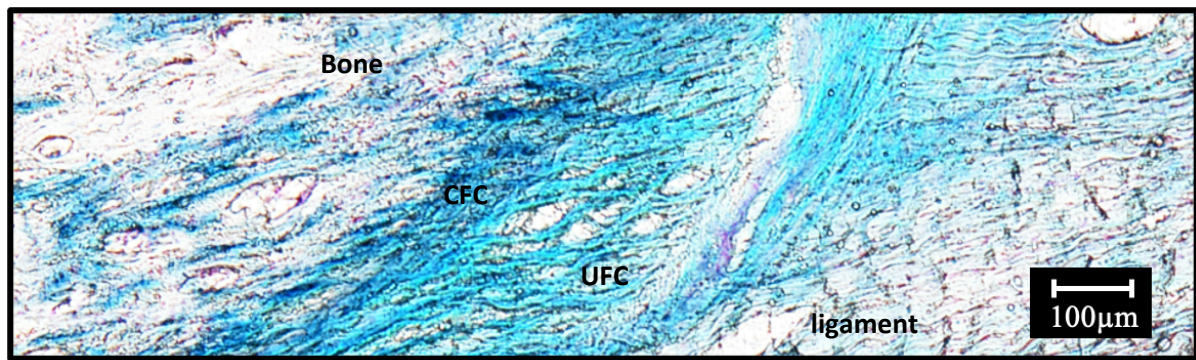


Figure 3.11: Histological sections dissected from pig, stained with Alcian Blue of the tibia (left) to Anterior Cruciate Ligament (right) enthesis. Areas containing glycosaminoglycans in blue.

3.4.1.4. Von Kossa

The von Kossa histological stain is used to observe calcification in the ligament-bone interface. Calcium, which is present as carbonates and phosphates, is replaced by silver ions, which are subsequently reduced to metallic silver in the presence of light. Therewith, mineral deposits within the tissue appear black whilst nuclei and cytoplasm appear red and pink, respectively. Figure 3.12 shows the calcium content through the enthesis, and the line of calcification “tidemark” is indicated by the arrow. The crimped nature of ligament fibres can easily be seen.

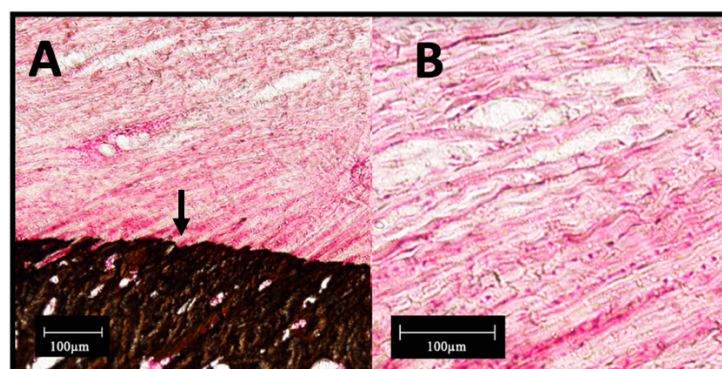


Figure 3.12: Histological sections dissected from pig, stained with von Kossa of ACL to tibia enthesis. Arrow shows the line of calcification.

3.4.2. Scanning Electron Microscopy/ Energy Dispersive X-Ray

Spectroscopy

SEM was used to study the microstructure of the ligament to bone enthesis. The microstructure of the enthesis varies drastically from the aligned ligament to the unaligned bone tissue, as illustrated in Figure 3.13. However, the transition between the two tissues is very smooth and indistinguishable with an absence of sharp boundaries. The red arrow shows the direction of the ligament fibres as they connect to the bone. They attach to the bone at roughly a 15° angle.

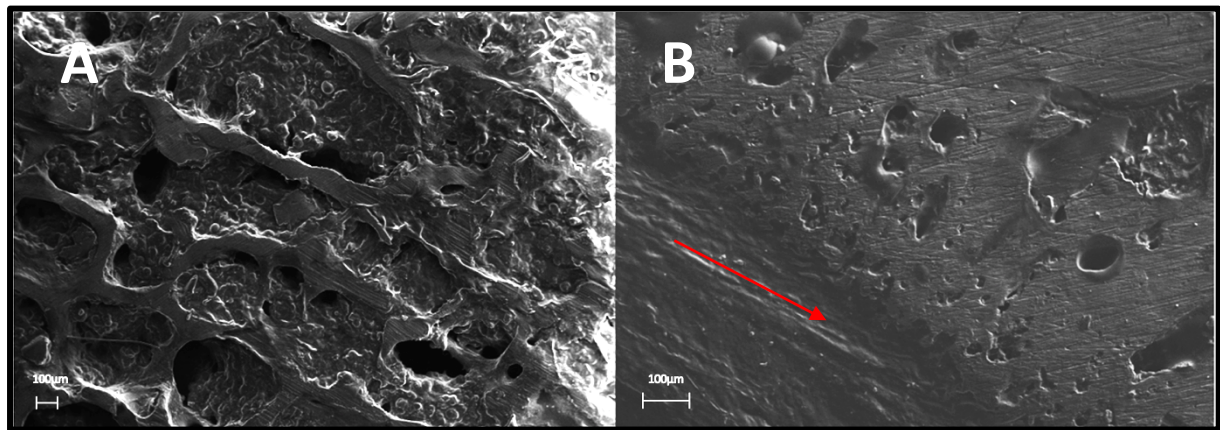


Figure 3.13: Electron microscope view (A) bone trabecular in the femur, (B) Enthesis of ACL-Tibia attachment.

Calcium phosphate presence across the interface was characterised by EDX analysis. The Ca and P peaks associated with mineral presence were only detectable in the calcified fibrocartilage (CFC) and bone regions. By scanning points within these regions, the mean wt% of Ca and P present was identified (Table 3.1). The calcium and phosphorus content in CFC was 11.25 wt% and 6.77 wt%, respectively. Whilst calcium and phosphorus content in bone was 2-folds higher at 23.36 wt% and 13.77 wt%, respectively. The ratio of Ca/P is very similar at 1.66 and 1.7 for CFC and bone, respectively.

	Ca	P	Ca/P Ratio
CFC	11.25 ± 0.03	6.77 ± 0.00	1.66 ± 0.00
Bone	23.36 ± 0.05	13.77 ± 0.09	1.7 ± 0.00

Table 3.1: Mean concentrations in wt% of Ca and P in mineralised bone and mineralised fibrocartilage. (Mean ± SD).

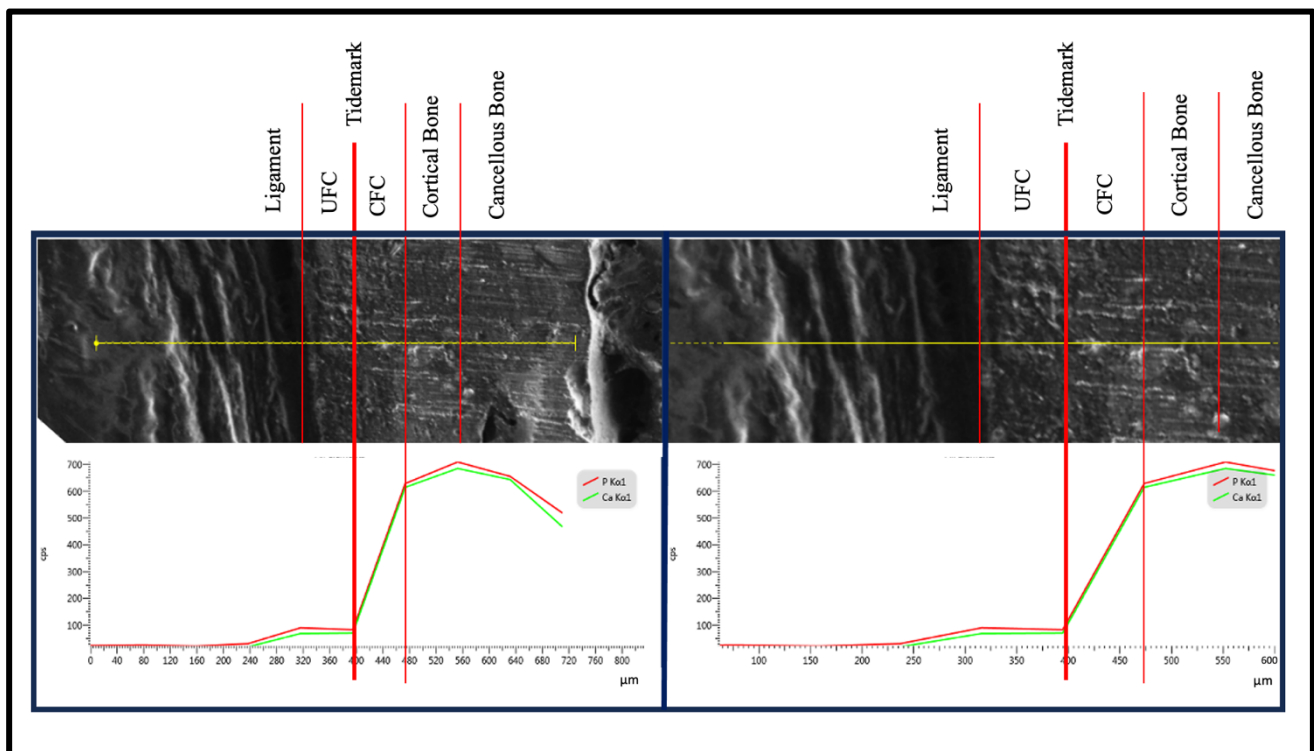


Figure 3.14: EDX line scans through enthesis from ligament to bone. Top, SEM images of ACL to Tibia enthesis with yellow line highlighting the line which was scanned, bottom, corresponding readings for calcium and phosphorus through region.

Moving from ligament to bone there is a definitive point in which the mineral content increases significantly, illustrated by the red vertical lines in Figure 3.14, which is an EDX line scan across the entire enthesis from ligament to bone. This point marks the start of the calcified fibrocartilage. When the mineral content plateaus, the calcified fibrocartilage has transitioned

into bone tissue. Beyond this point, the drop in mineral content is likely caused by the increase in porosity of the bone as it transitions from dense cortical bone to more porous cancellous bone. The small traces of Ca and P before the CFC are likely caused by contamination during the sample preparation process. By using the SEM images and EDX line scans, it can be seen that the entirety of the ligament-bone enthesis is approximately 500µm with fibrocartilage tissue (both calcified and uncalcified) covering around 200µm of that region.

3.4.3. Mechanical Testing

3.4.3.1. Tensile

The force-displacement behaviour of ligament samples being stretched longitudinally is plotted in Figure 3.15. Values for tensile testing can be found in Table 3.2. Mean Young's modulus values for hydrated ligament segments were 183.0 ± 13.7 MPa. After ultimate tensile strength, ligaments failed gradually before ultimate failure was reached.

Sample	Diameter (mm)	Length (mm)	Modulus (MPa)
1	1.72	14.23	199.81
2	1.91	14.23	170.40
3	2.10	14.23	173.44
4	1.20	14.23	188.73

Table 3.2: Tensile testing results for sections of ACL dissected from pig specimen.

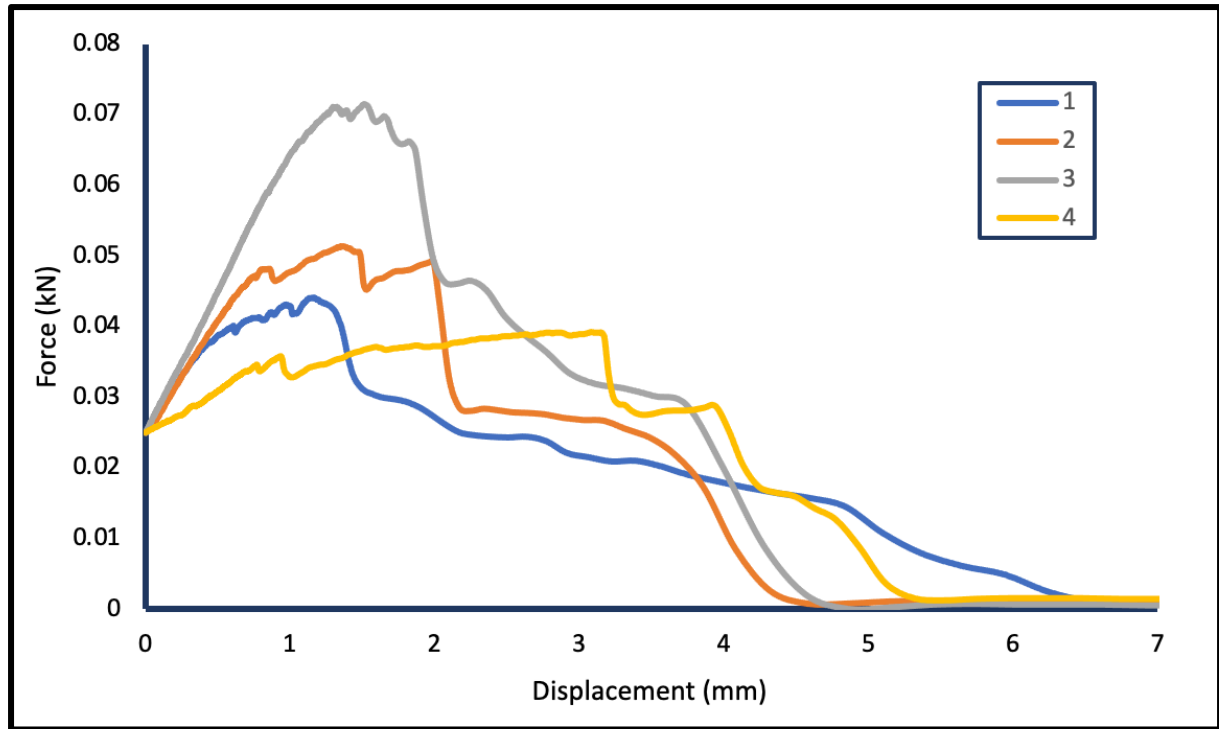


Figure 3.15: Tensile force-displacement plot of an isolated ACL section dissected from pig specimen. $n=4$.

3.4.3.2. Compression

The force-displacement behaviour of the samples being compressed is shown in Figure 3.16. Bone and enthesis samples show different characteristics from one another. Values for compression testing can be found in Table 3.3.

The load-displacement curve for compression testing can be divided into elastic and plastic deformation regions the slope of the linear portion is taken by many researchers as the elastic modulus or Young's modulus. Although this linear region is easy to locate with bone specimens, this region is harder to determine for enthesis samples. The stiffness of enthesis samples was 2-fold lower at 43.45 ± 1.3 MPa, whilst the stiffness of bone samples was 81.75 ± 13.8 MPa. Furthermore, failure occurred much sooner, and ultimate strengths only reached roughly 0.3kN whilst bone sample values ranged between 0.7-1.2kN.

Sample	Width (mm)	Thickness (mm)	Height (mm)	Modulus (MPa)
B1	10.20	11.55	11.89	72.34
B2	10.33	12.66	9.90	67.79
B3	11.66	12.07	11.66	90.64
B4	11.48	11.20	12.47	96.22
E1	9.61	10.63	9.90	44.41
E2	9.95	10.64	11.53	42.49

Table 3.3: Compressive testing results for sections of tibia (B1-4, n=4) and enthesis between ACL and tibia (E1-2, n=2) dissected from pig specimen.

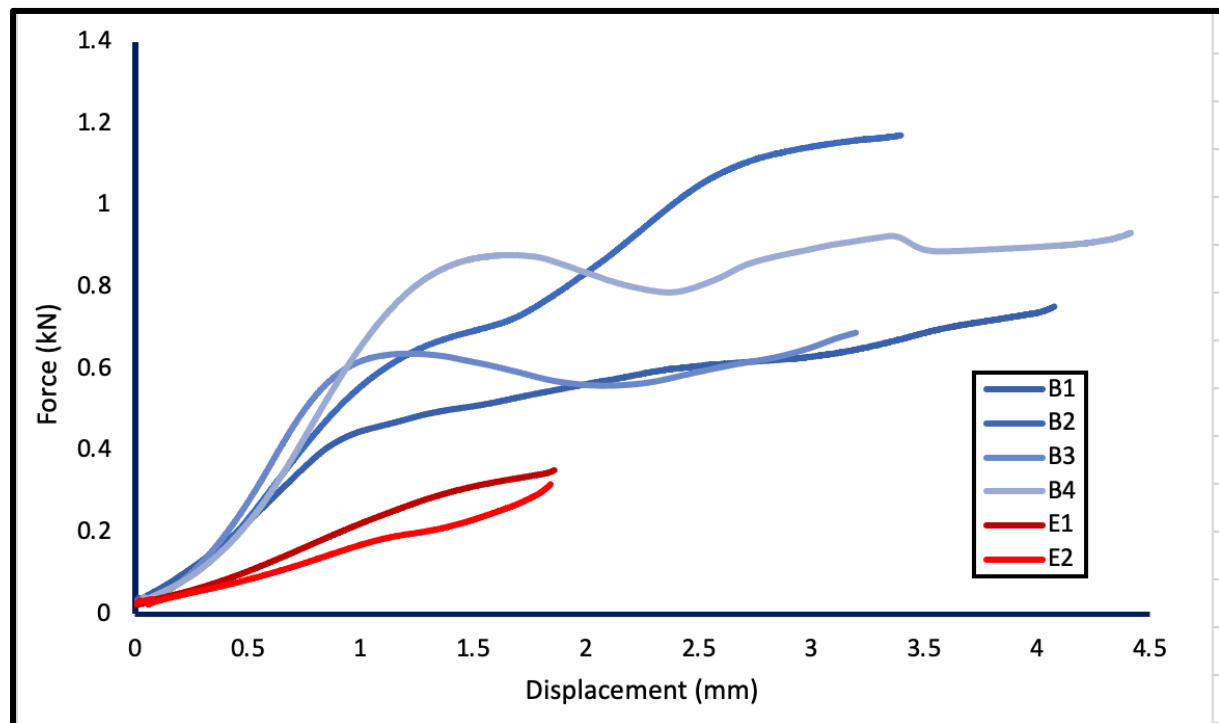


Figure 3.16: Compressive force-displacement plot of isolated tibia (B1-4, n=4) and enthesis between ACL and tibia (E1-2, n=2) dissected from pig specimen.

3.4.4. Nanoindentation

The enthesis length scale is around 500-800µm [11]–[13]. Therefore, nanoindentation was used to characterise the mechanical properties of each region.

Mean modulus and hardness results for each region of the enthesis tested both wet and dry are summarised in Table 3.4 and visualised as bar charts in Figures 3.18 and 3.20.

Dry testing produced moduli measurements of 2.23GPa for ligament, 6.57GPa for UFC, 7.49GPa for CFC and 10.56GPa for bone. Wet testing produced moduli results 800-fold lower for ligament at 2.69MPa, 15-fold lower for UFC at 433.31MPa, and 5-fold lower for CFC and bone at 1540MPa and 2063MPa, respectively.

Dry testing produced hardness measurements of 72.79MPa for ligament, 199.74 MPa for UFC, 252.96 MPa for CFC and 291.25 MPa for bone. Wet testing produced hardness results 560-fold lower for ligament at 0.13MPa, 10-fold lower for UFC at 20 MPa, and CFC and bone measurements at 157.71 MPa and 238.66 MPa, respectively.

Moduli and hardness results show that when samples are hydrated, each zone within the enthesis has a significantly different modulus. However, once samples lose their water content there is no significant difference in modulus between the two fibrocartilage layers.

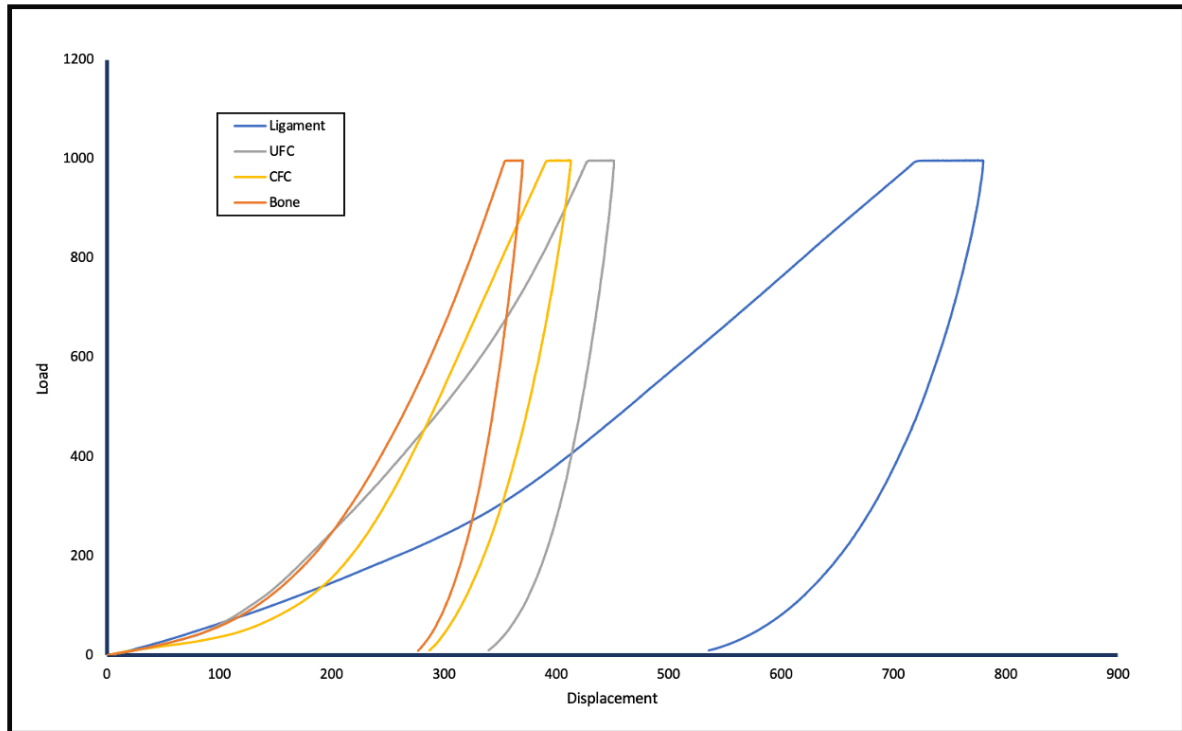


Figure 3.17: Examples of load-displacement plot of nanoindented dry enthesis samples, loaded trapezoidally to a maximum load of $1000\mu\text{N}$ and held for 3 seconds.

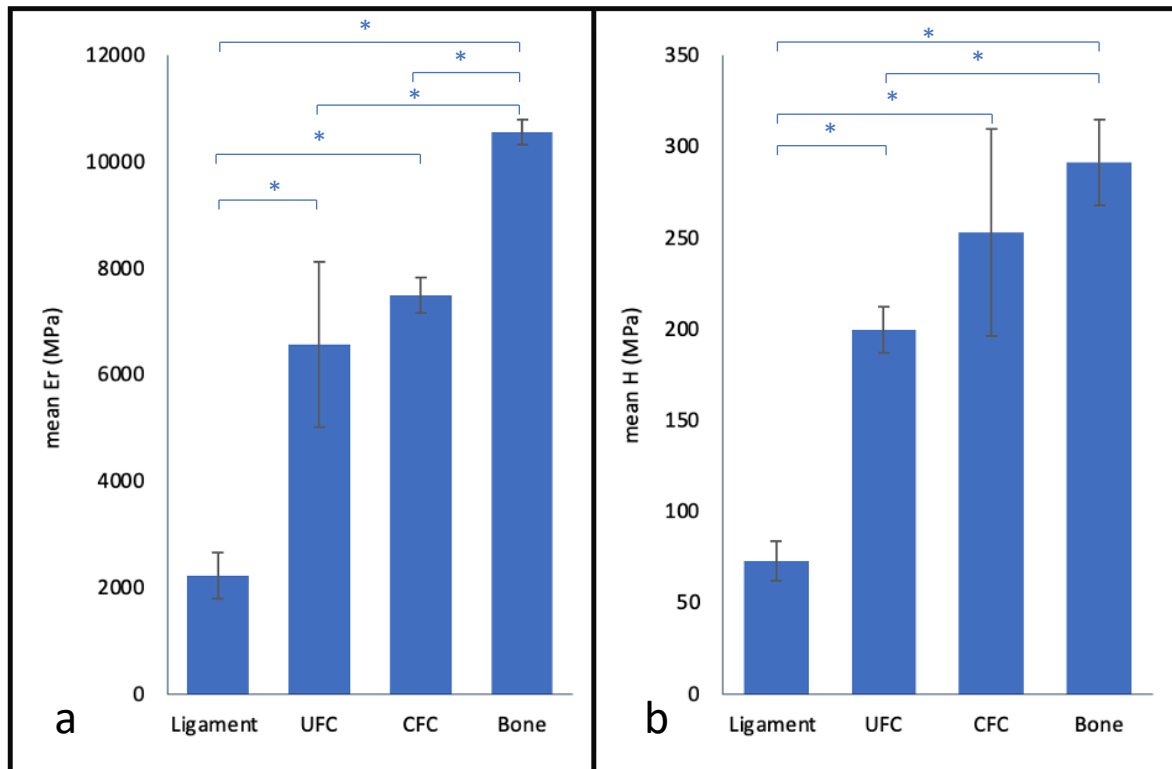


Figure 3.18: Bar charts showing the mean value and standard deviation of reduced modulus (Er, a) and hardness (H, b) of dry enthesis (N=5, * $p<0.05$).

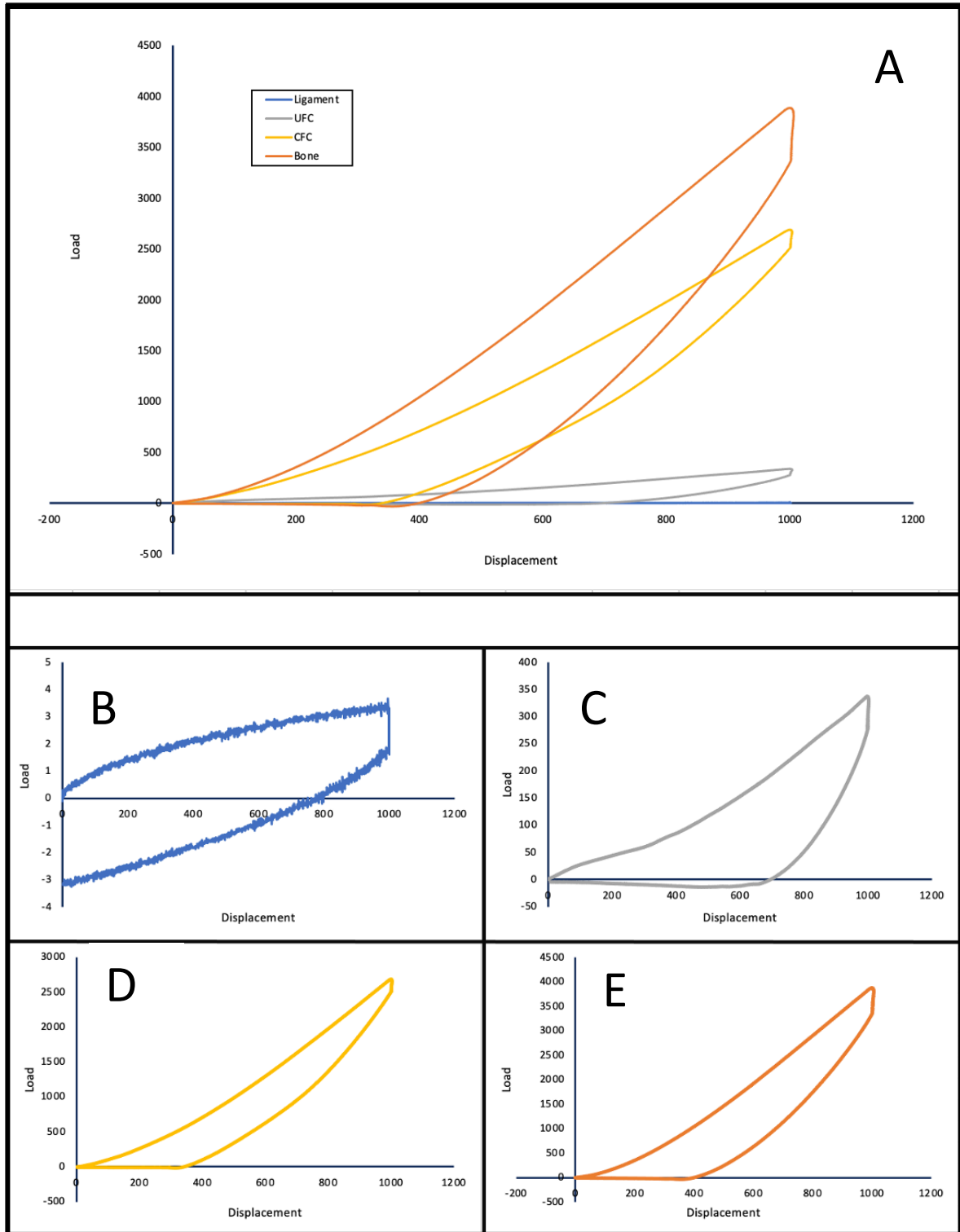


Figure 3.19: Examples of load-displacement plot of nanoindented wet enthesis samples, loaded trapezoidally to a maximum depth of 1000nm and held for 3 seconds. (A) all four zones plotted on the same graph, (B) ligament, (C) Uncalcified fibrocartilage (D) Calcified fibrocartilage, (E) Bone.

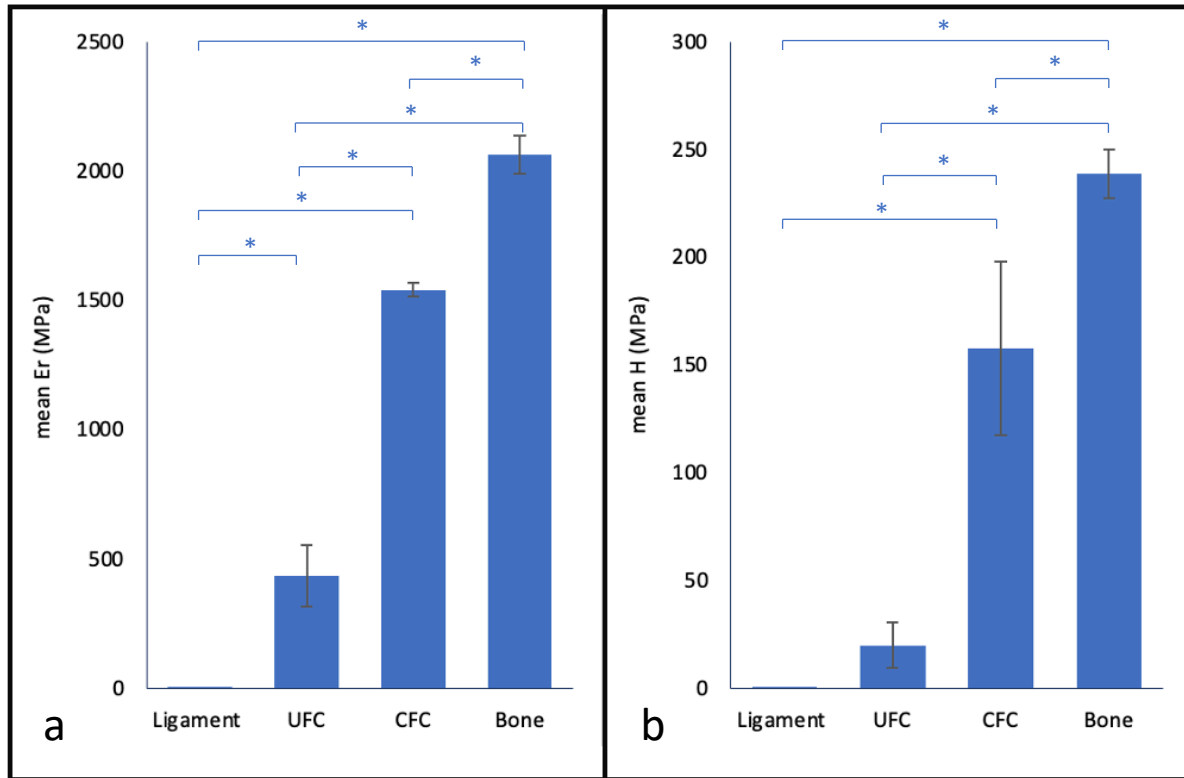


Figure 3.20: Bar charts showing the mean value and standard deviation of reduced modulus (Er, a) and hardness (H, b) of wet enthesis. (N=5, *p<0.05).

	Wet Mean Er (MPa)	SD	Wet Mean H (MPa)	SD	Dry Mean Er (MPa)	SD	Dry Mean H (MPa)	SD
Ligament	2.69	0.4062	0.13	0.04	2230	430	72.79	10.76
UFC	433.31	118.91	20	10.58	6570	1560	199.74	12.65
CFC	1540	26.46	157.71	40.41	7490	330	252.96	56.78
Bone	2063.33	73.71	238.66	11.25	10560	230	291.24	23.56

Table 3.4: Mean values and standard deviations of reduced modulus (Er) and hardness (H) obtained for nanoindented dry and wet enthesis.

For a perfect Berkovich indenter, the contact stiffness (S) is a function of contact depth (h_c) and Elastic modulus (E_r). So for a uniform material with a constant elastic modulus value of E_r is a constant, therefore S is linearly proportional to h_c . However, for a non-uniform material, E_r changes with indentation depth. In this case, the linear relationship between S and h_c does not exist. This phenomenon is illustrated in Figure 3.21. Typical curves of the Young's modulus as a function of tip penetration, obtained from both dry and wet samples, are shown in Figure 3.22.

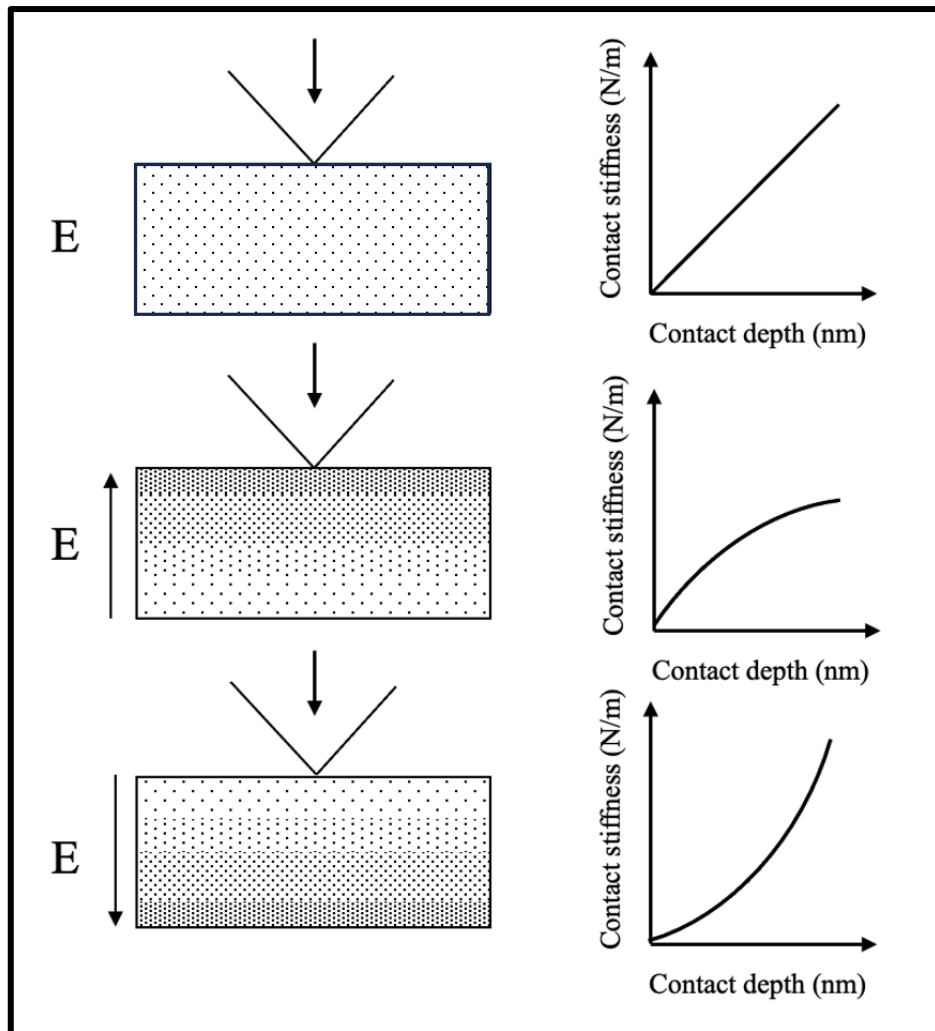


Figure 3.21: Representation of uniform and non-uniform materials sample stiffness with relation to probe tip depth.

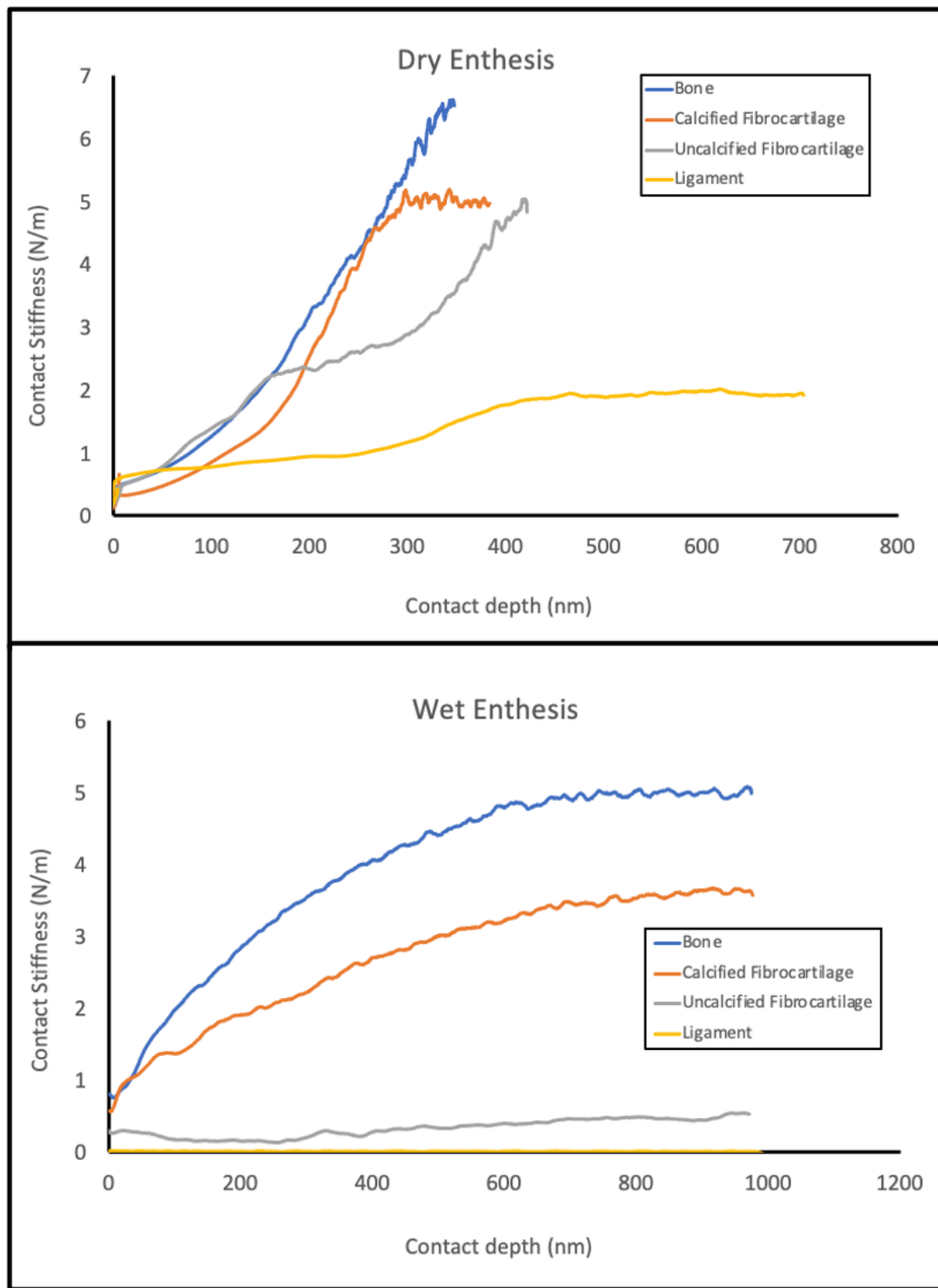


Figure 3.22: Graphs of contact stiffness against contact depth for dry (top) and wet (bottom) samples.

3.5. Discussion

3.5.1. Histology

From histology images, it is seen that collagen fibres assemble to form ligaments. Analysis of these images using ImageJ allowed calculations of fibre diameters, which were found to range between 15-30µm. Among extracellular matrix structures, collagen fibres play a key role in transmitting tensional forces, as supported by many reports that the *in vivo* mechanical properties of tendons and ligaments are strictly related to the fibre collagen arrangement, diameter and length [14]–[18]. The collagen fibres present in ligaments need to be able to oppose high tensional forces. For this reason, collagen fibres run straight and are mainly oriented along the stress direction in ligaments, although fibres in some regions show irregular undulations described as crimps. The crimped nature of the fibres serves to guide joint motion. When a ligament is loaded in tension, these crimps disappear or appear more flattened during the initial elongation of the ligament and act as a mechanical buffer system protecting collagen fibres during ligament elongation. Although there are numerous studies that give insight into the mechanical response of ligaments and tendons to longitudinal loading, information on how crimp is formed is lacking [19], [20], [21].

In cartilaginous entheses, type I collagen disappears and is replaced by type II collagen, before shifting back to type I. Such a region appears like a gap between two collagen I-containing tissues (ligament and bone) and therefore the appearance has been called the ‘gap phenomenon’ [22], [23]. The intensity of safranin O staining is directly proportional to the proteoglycan content within the tissue [24]. As shown in Figure 3.10, the change from blue counterstain to reddish-orange, safranin O staining confirms that the change in material composition is gradual. Furthermore, the CFC region has the greatest intensity of safranin O staining indicating that this region has a higher proteoglycan content. This is also reflected in the Alcian

blue staining. In Figure 3.11, the intensity of blue staining increases as the tissue transitions from UFC to CFC which further confirms the increase of proteoglycan.

Unlike the microstructure of the enthesis in which the transition from ligament to bone is smooth and indistinguishable, the mineral content between these two tissues is very distinct. The basophilic line which separates the uncalcified and calcified fibrocartilage, also known as the “tidemark”, is a visual representation of the mechanical boundary between soft and hard tissue [25]. The tidemark is relatively straight which indicates the production of a flat surface during the mineralisation process which is important clinically as this surface reduces the risk of damage to soft tissues during joint movement [25]. However, this region is certainly a region of mechanical weakness at which horizontal clefts can develop [26], [27], [28]. Although a tidemark separates calcified from uncalcified fibrocartilage, collagen fibres between the two regions are continuous [29].

Sectioning calcified tissue without decalcifying first is very challenging and therefore it was difficult to obtain sections thinner than those stained. Thus, the black staining in calcium-containing sections is fully saturated and hence does not give information on variations in calcium content through CFC to bone.

As the name suggests, fibrocartilaginous tissue is where chondrogenesis has occurred, thus the expression of cartilage-specific collagen II and GAG chondroitin sulphate is expected. Furthermore, as in articular cartilage, its ability to resist compression is linked to the presence of aggrecan and type II collagen in the extracellular matrix [30]. The presence of these chondrogenesis markers was confirmed using safranin O staining in which the region between ligament and bone was stained a reddish-orange, Figure 3.11.

3.5.2. Scanning Electron Microscopy/ Energy Dispersive X-Ray

Spectroscopy

EDX line scans have shown that the content of calcium rises from 0 in the uncalcified fibrocartilage to a maximum in the mineralised fibrocartilage region. The definitive increase of mineral content aligns with the “tidemark” identified through histology von Kossa staining in section 3.3.1.4. Furthermore, utilising point EDX analysis and assuming that the CFC region is 100µm wide and begins at the tide mark, the mineral content through the centre of the calcified fibrocartilage region was identified as having a mean calcium and phosphorus content half of that of bone. These findings can be used when creating scaffolds that closely mimic the ECM of natural tissue.

Because of its compatible structure with natural hard tissues, hydroxyapatite (HAp) has been known as a Ca-P bio ceramic and is most utilised in calcified tissue regeneration endeavours. Using the mineralisation information gained from EDX scans will aid in the development of biometric scaffolds. The content of minerals in human bone is typically 70% with a ratio of calcium to phosphorus of 1.67:1 [31], [32]. Therefore, HAp can be used in combination with collagen I to mimic the ECM of bone whilst calcified fibrocartilage can be mimicked by using half the HAp content of the bone structure.

3.5.3. Mechanical Testing

The values of the strength of native tissues tested *in vitro* will be lower than that of the tissues *in vivo* as only a portion of the tissue was tested. Furthermore, even though great care was taken in avoiding damaging the tissue during dissection and sample preparation, it is possible that samples could have had points of weakness prior to testing.

3.5.3.1. Tensile

Ligaments are an anisotropic material whose mechanical properties depend on loading direction. Thus, ligament samples were stretched longitudinally with collagen fibres running parallel to the direction of elongation.

The values for the modulus obtained were from the linear region, the point at which the stiffness of the ligament increases to restrict any excessive motion in the joint. At this point, the crimps in the collagen fibres have completely flattened out and elongation is the result of stretching imposed in the already aligned fibres. At the end of this linear region, some fibres start to fail thus the ligament stiffness begins to drop. Beyond this point, further fibres fail gradually until ultimate failure occurs. As the fibres which make up the ligaments do not fail all at once, ligaments can extend 2-3 times further once ultimate tensile strength has been reached.

Although these regions are apparent in all samples tested, the shapes of the curves obtained differ. These differences are caused by inter-specimen dimensional differences. Even though the length and the specimens tested were kept the same, there were variations in specimen diameter, as presented in Table 3.2.

SEM images of the fractured ligament samples show that the fracture surface of the ligaments is brush-like. This is because fracture has occurred due to dispersed failure of fibres until the ligament sections can no longer support the load applied [33]. Dispersed failure is connected to “ductile-like” behaviour and a nonlinear stress-strain response [33]. Ligaments have a hierarchical structure (Section 1.4). It can be seen that failure has occurred due to the fracture of ligament fibres ($\sim 10\text{-}100\mu\text{m}$ diameter [34]), which correspond to the more significant drops

in force. This mode of failure is in line with different grades of ligament injuries seen from mild tearing to complete rupture.

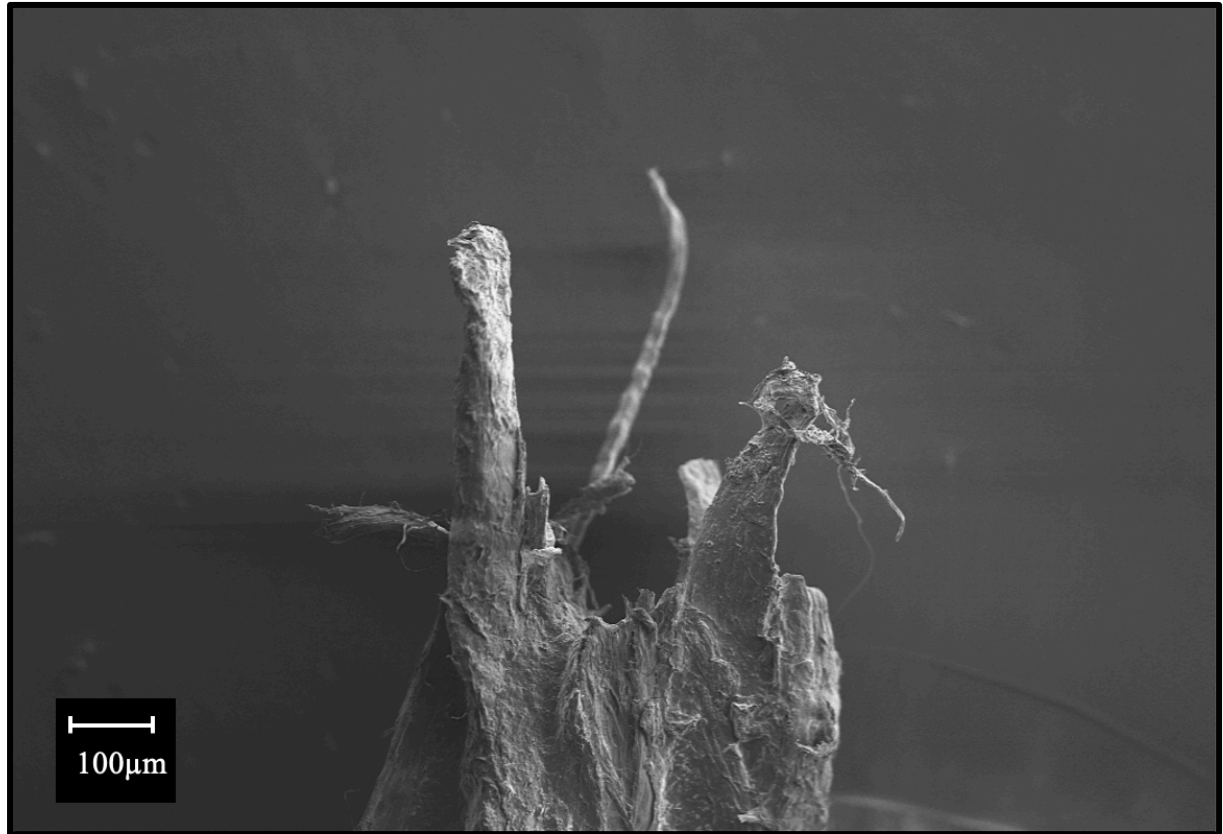


Figure 3.23: SEM image of the fractured ligament.

3.5.3.2. Compression

Bone is an anisotropic material whose mechanical properties depend on loading direction. Thus, bone samples were compressed in the longitudinal direction, whereas enthesis samples were orientated so that ligament fibres ran transversely into the top surface of the specimen.

Although the linear portion of the load-displacement curve is taken by many researchers as the elastic modulus or Young's modulus, bone is a complex, anisotropic, and heterogeneous material that does not behave as a purely elastic material, therefore, strictly speaking, the slope of the linear portion of the curve is not the Young's modulus of the bone specimen because

this deformation is not purely elastic. Bones have complex hierarchical structures and contain many defects, including pores. Some degree of plastic deformation is present, even at a relatively low compressive stress or strain. This likely explains why Young's modulus values are reported with a wide range of variance. An appropriate term used to describe the slope of the initial linear part is the apparent modulus, which is a useful stiffness parameter for comparison purposes. The apparent modulus of bone samples are 2-fold greater than the enthesis samples. As bone is an anisotropic material, bone samples being tested longitudinally whilst enthesis samples being tested at an angle could be the cause for the lower modulus values. Furthermore, variations in modulus are mainly driven by mineral content and porosity. As the enthesis samples include the fibrocartilage regions, the overall mineral content in these samples is far lower.

As the samples were tested in a hydrated state, the samples did not fail in a brittle, sudden manner but rather in a gradual ductile manner. The samples barrelled which can be seen in Figure 3.24 with the sides of the samples splaying outwards.

A large range in apparent modulus is reported in literature 90-209MPa [35], [36], [37], which may be caused by inter-specimen variability, in bone microstructure at different locations in the same bone, between species and age groups, and diversity in experimental testing conditions.

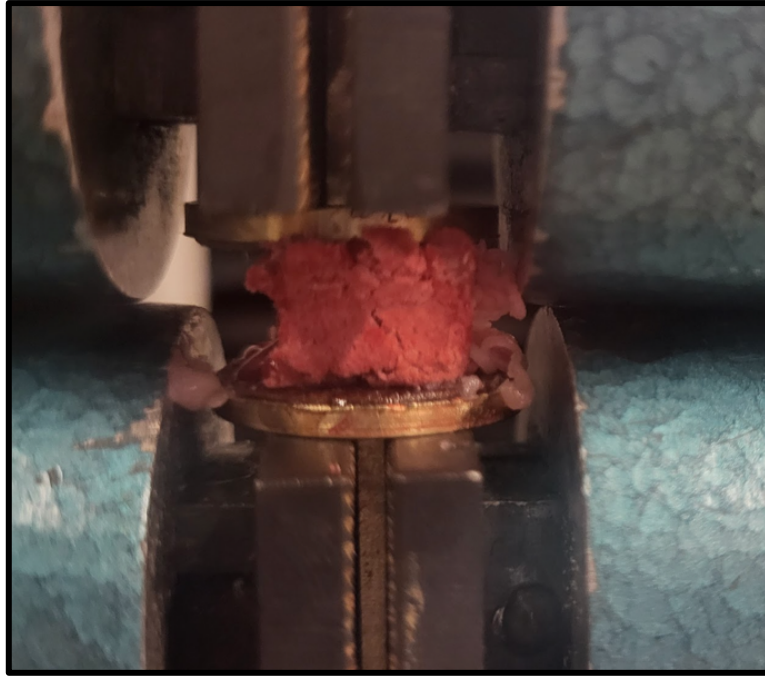


Figure 3.24: Compression testing bone longitudinally. Barrelling deformation with cracks arising.

3.5.4. Nanoindentation

The nanoindentation technique presented here shows the ability of such a method to determine properties at the nano- to micro-scale, leading to a knowledge of the local behaviour of the ligament-bone attachment sites. In this study, the correlation of the individual indentation locations to each transition zone was estimated based on the length of each region determined through EDX scans and histology staining.

Initially, trapezoidal load profiles were used for all dry sample tests. However, when testing the wet samples, the profile showed nonlinear loading and unloading paths, indicating a time-dependent deformation that could not be compensated by the load transducer in a timely fashion. Therefore, to maintain a constant loading and unloading rate, depth control was used. The application of a pre-stress minimised deflection during indentation, reducing the variation in mechanical property measurements. During the hold phase, for a non-compliant material,

neither the load nor displacement would be expected to change with time. However, for the dry samples to maintain a constant load for 3 seconds, the probe tip needed to continue to displace. Furthermore, when the probe tip was held at a constant displacement in the wet samples, the load was reduced. Stress is defined as the indentation load divided by the projected contact area of the indentation (which is driven by the indentation depth). Therefore, a change in either of these measures during a hold period indicates that the mechanical stresses are not consistent. Thus, nanoindentation results highlight the time-dependent behaviour of both dry and wet samples. As expected, the least amount of creep occurred in the bone and the most creep occurred in the ligament.

Overall, a monotonic increase of elastic modulus and hardness across the zones was observed. As the indentations move from the ligament to the bone region, the elastic modulus and hardness increased substantially. Deviations among individual measurements within each zone owe to diversities in tissue architecture and structural heterogeneity. The largest deviations from the mean results were observed with the fibrocartilage zones. As these zones are transitional, a greater amount of structural heterogeneity is expected. These results show that the insertional zones between ligament and bone attachments serve as a transition from the compliant ligament to the much stiffer bone. Furthermore, the fact that results obtained for both reduced stiffness and hardness for both fibrocartilage regions were found to not be significantly different further emphasises this point.

The modulus and hardness results for the dry samples were vastly different from the wet samples. Dehydrating samples before testing is beneficial in eliminating the influence of changing environmental conditions. However, the values obtained were far from the absolute mechanical properties of samples *in vivo*. Testing the dehydrated samples allowed for the

observation of stiffness and hardness trends and highlighted the importance of hydration to the mechanical properties of biological materials.

The projected area of the contact for a Berkovich tip with face angle of 65.3° is given by:

$$A_{\text{proj}} = 24.49 h_c^2$$

(Equation 3.4)

At a maximum indentation depth of 1000nm the length of each side of the base triangle is $\sim 7.5\mu\text{m}$. When considering the hierarchical structure of ligaments and bones discussed in Sections 1.3 and 1.4, this suggests that the stiffness being calculated in the initial unloading is that of collagen fibres.

The elastic modulus and hardness results obtained from the initial unloading of the bone samples were in line with studies carried out by others both in dry and wet conditions [38]–[48]. Variations were expected due to testing on different species, locations and even testing procedures. Unlike bone, there is not a vast number of previous studies which can be utilised to compare results obtained for ligament or fibrocartilage. If we use other non-calcified soft tissue as a suggestion, the results obtained in this study are in line with others' works [49]–[53].

When observing the curves for sample stiffness with relation to probe tip depth (Figure 3.22), each of the tissue regions deformed non-uniformly. Furthermore, the most significant changes in stiffness occurred for indentation depths during the initial loading (less than 300nm). These depths are attributed to the indentation of collagen fibrils. Once the probe penetrated (displaced) deeper into the specimen and began to probe the collagen fibres instead, these changes plateaued.

If we focus on the initial loading, we can see that whilst the dry samples were getting stiffer with depth, the wet samples appeared to be getting less stiff. An explanation for the increase in stiffness of dried samples is that the collagen fibrils were compressing and becoming more condensed as the tip probed deeper into the samples. On the other hand, the wet samples were seemingly getting less stiff which is a behaviour reflective of the effects of hydration. Although measures were taken to try and prevent this by keeping samples in PBS until ready for testing, any excess water on the surface of the sample would prevent the probe from being able to identify the sample surface therefore there was a limit to how wet the sample surfaces could be. Therefore, the surfaces of tested samples were the least hydrated and hydration increased progressing deeper into the sample. This is in line with a study by Andriotis *et al.* [54], which illustrated the influence of hydration on the mechanics of collagen fibrils, which they described as “nature’s highly tuneable nonlinear springs”. At high levels of hydration, the intermolecular distances are larger in collagen fibrils compared to the ones with lower levels of hydration. A decrease of intermolecular distance leads to an increase in packing density and in turn, to an increase of the density of non-covalent interactions.

Again, if we focus on the initial 300nm probe tip depths, it is very easy to see the effects of calcification on the stiffness of samples. At this depth, collagen fibrils were being indented. For uncalcified tissues, this was just collagen fibrils, however, for calcified tissue, this was mineralised collagen fibrils (Figure 3.25). EDX results showed that the amount of mineralisation increased from calcified fibrocartilage to bone therefore, it can be said that the amount of mineralisation within the collagen fibrils are having significant effects on the material properties down to the nanoscale. These results are in agreement with other studies that show that the stiffness of bone increases with increasing mineralisation [55], [56].

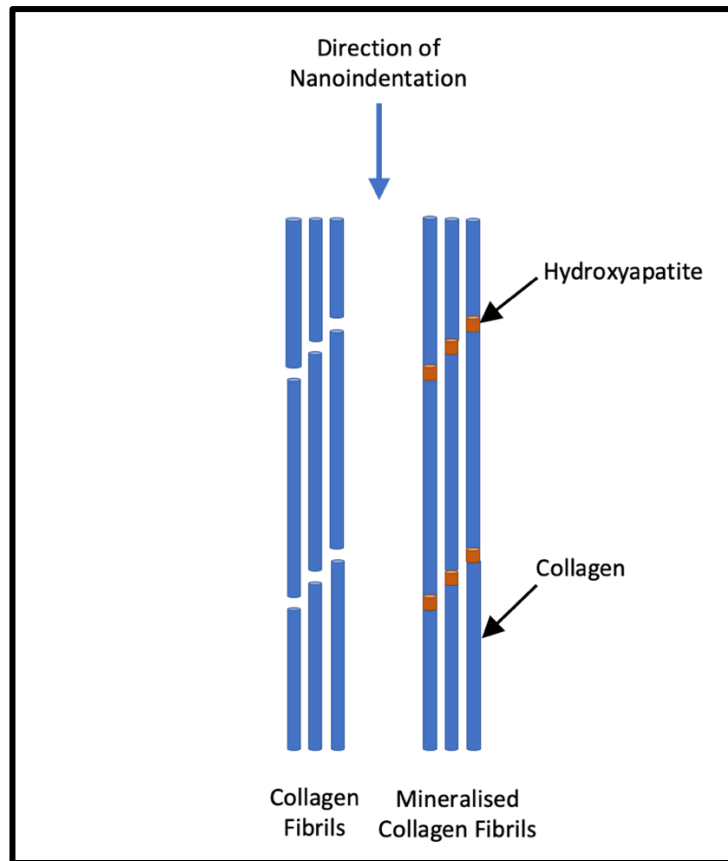


Figure 3.25: Collagen fibrils in uncalcified and calcified tissue.

Although nanoindentation has emerged as a promising tool for mechanical characterisation there are still several significant challenges. In particular, there is a scarcity of a consensus on the appropriate testing method and data analysis for the mechanical characterisation of soft biomaterials. This leads to large disparities in reported results between different studies, greatly linked to variations in the protocols rather than specimens [57]. The nanoindentation method is very sensitive to sample preparation technique and testing conditions. For example, according to Thurner *et al*, the elastic modulus of the human femur middle portion varied between 17 and 27 GPa, depending on sample hydration conditions, maximum applied force, tip type, and anatomical direction [58]. Furthermore, as soft tissue specimens cannot be polished to provide a flat surface for testing the high surface roughness, this is also a cause for discrepancies.

3.6. Summary

This chapter presented the histological characterisation of the ligament-bone enthesis and the mechanical properties of these regions from the macro- to nano-scale. The main findings were as follows:

- ⇒ SEM micrographs and stained images showed that the bounds between the different layers were not distinct. There appeared to be microstructural gradations going from one region to the next. This is a clear difference from made composites where interfaces are generally sharp.
- ⇒ Most of the enthesis tissue was found to be collagen, and the fibre orientation changed from parallelly aligned fibres in ligaments to a random orientation in bone. Differences in cell types were also observed between the zones.
- ⇒ Differences in biochemical composition occurred across the four zones of the enthesis. Collagen type I was expressed in the ligament and both calcified regions, whereas collagen type II and aggrecan were expressed mainly within the uncalcified fibrocartilage tissue.
- ⇒ Simple uniaxial longitudinally oriented tension and compression tests are useful for studying the effects of various modifications to the tissue. However, they are insufficient for characterising the anisotropy and heterogeneity, and viscoelasticity. However, nanoindentation was utilised to gain a greater insight into these characteristics.
- ⇒ Nanoindentation testing confirmed that the stiffness of soft tissue is highly related to calcification from macro- down to the nano-scale.

3.7. References

- [1] M. Benjamin and J. R. Ralphs, ‘Fibrocartilage in tendons and ligaments--an adaptation to compressive load’, *J. Anat.*, vol. 193 (Pt 4), pp. 481–494, Nov. 1998, doi: 10.1046/j.1469-7580.1998.19340481.x.
- [2] W. Murphy, J. Black, and G. Hastings, *Handbook of biomaterial properties, second edition*. 2016, p. 676. doi: 10.1007/978-1-4939-3305-1.
- [3] Y. C. Fung, *Biomechanics: Mechanical Properties of Living Tissues*. Springer Science & Business Media, 2013.
- [4] I. G. Mackie, M. Green, H. Clarke, and D. H. Isaac, ‘Human bone microstructure studied by collagenase etching’, *J Bone Joint Surg Br*, vol. 71, no. 3, pp. 509–513, May 1989, doi: 10.1302/0301-620X.71B3.2542341.
- [5] T. Kawamoto and K. Kawamoto, ‘Preparation of thin frozen sections from nonfixed and undecalcified hard tissues using Kawamot’s film method (2012)’, *Methods Mol Biol*, vol. 1130, pp. 149–164, 2014, doi: 10.1007/978-1-62703-989-5_11.
- [6] Z.-J. Zhu, Y. Zhu, J.-F. Liu, Y.-P. Wang, G. Chen, and X.-Y. Xu, ‘Posterolateral ankle ligament injuries affect ankle stability: a finite element study’, *BMC Musculoskeletal Disorders*, vol. 17, no. 1, p. 96, Feb. 2016, doi: 10.1186/s12891-016-0954-6.
- [7] F. Rupin *et al.*, ‘Experimental determination of Young modulus and Poisson ratio in cortical bone tissue using high resolution scanning acoustic microscopy and nanoindentation.’, *The Journal of the Acoustical Society of America*, vol. 123, no. 5, p. 3785, 2008, doi: DOI:10.1121/1.2935440.
- [8] K. Hu, P. Radhakrishnan, R. V. Patel, and J. J. Mao, ‘Regional structural and viscoelastic properties of fibrocartilage upon dynamic nanoindentation of the articular condyle’, *J Struct Biol*, vol. 136, no. 1, pp. 46–52, Oct. 2001, doi: 10.1006/jsbi.2001.4417.

- [9] B. J. Briscoe, L. Fiori, and E. Pelillo, ‘Nano-indentation of polymeric surfaces’, *J. Phys. D: Appl. Phys.*, vol. 31, no. 19, pp. 2395–2405, Oct. 1998, doi: 10.1088/0022-3727/31/19/006.
- [10] S. Font Tellado, E. R. Balmayor, and M. Van Griensven, ‘Strategies to engineer tendon/ligament-to-bone interface: Biomaterials, cells and growth factors’, *Adv. Drug Deliv. Rev.*, vol. 94, pp. 126–140, Nov. 2015, doi: 10.1016/j.addr.2015.03.004.
- [11] A. Deymier, J. Pasteris, G. Genin, and S. Thomopoulos, ‘Allometry of the Tendon Enthesis: Mechanisms of Load Transfer Between Tendon and Bone’, *Journal of biomechanical engineering*, vol. 137, Sep. 2015, doi: 10.1115/1.4031571.
- [12] M. Benjamin, H. Toumi, J. Ralphs, G. Bydder, T. Best, and S. Milz, ‘Where tendons and ligaments meet bone: Attachment sites (‘entheses’) in relation to exercise and/or mechanical load’, *Journal of anatomy*, vol. 208, pp. 471–90, May 2006, doi: 10.1111/j.1469-7580.2006.00540.x.
- [13] A. Tits and D. Ruffoni, ‘Joining soft tissues to bone: Insights from modeling and simulations’, *Bone Rep*, vol. 14, p. 100742, Dec. 2020, doi: 10.1016/j.bonr.2020.100742.
- [14] S. L. Woo, R. E. Debski, J. D. Withrow, and M. A. Janaushek, ‘Biomechanics of knee ligaments’, *Am J Sports Med*, vol. 27, no. 4, pp. 533–543, 1999, doi: 10.1177/03635465990270042301.
- [15] Y. Lanir, ‘Structure-strength relations in mammalian tendon’, *Biophys J*, vol. 24, no. 2, pp. 541–554, Nov. 1978, doi: 10.1016/S0006-3495(78)85400-9.
- [16] D. A. Parry, G. R. Barnes, and A. S. Craig, ‘A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties’, *Proc R Soc Lond B Biol Sci*, vol. 203, no. 1152, pp. 305–321, Dec. 1978, doi: 10.1098/rspb.1978.0107.

- [17] C. J. Doillon, M. G. Dunn, E. Bender, and F. H. Silver, 'Collagen fiber formation in repair tissue: development of strength and toughness', *Coll Relat Res*, vol. 5, no. 6, pp. 481–492, Dec. 1985, doi: 10.1016/s0174-173x(85)80002-9.
- [18] R. A. Hart, S. L. Woo, and P. O. Newton, 'Ultrastructural morphometry of anterior cruciate and medial collateral ligaments: an experimental study in rabbits', *J Orthop Res*, vol. 10, no. 1, pp. 96–103, Jan. 1992, doi: 10.1002/jor.1100100112.
- [19] A. D. Freed and T. C. Doehring, 'Elastic Model for Crimped Collagen Fibrils', *Journal of Biomechanical Engineering*, vol. 127, no. 4, pp. 587–593, 2005, doi: 10.1115/1.1934145.
- [20] S. P. Reese, S. A. Maas, and J. A. Weiss, 'Micromechanical models of helical superstructures in ligament and tendon fibers predict large Poisson's ratios', *J Biomech*, vol. 43, no. 7, pp. 1394–1400, May 2010, doi: 10.1016/j.jbiomech.2010.01.004.
- [21] M. Lavagnino, S. P. Arnoczky, E. Kepich, O. Caballero, and R. C. Haut, 'A finite element model predicts the mechanotransduction response of tendon cells to cyclic tensile loading', *Biomech Model Mechanobiol*, vol. 7, no. 5, pp. 405–416, Oct. 2008, doi: 10.1007/s10237-007-0104-z.
- [22] S. Milz *et al.*, 'Molecular composition and pathology of entheses on the medial and lateral epicondyles of the humerus: a structural basis for epicondylitis', *Ann Rheum Dis*, vol. 63, no. 9, pp. 1015–1021, Sep. 2004, doi: 10.1136/ard.2003.016378.
- [23] A. A. Boszczyk, B. M. Boszczyk, R. Putz, M. Benjamin, and S. Milz, 'Expression of a wide range of fibrocartilage molecules at the entheses of the alar ligaments - possible antigenic targets for rheumatoid arthritis?', *J. Rheumatol.*, vol. 30, no. 7, pp. 1420–1425, Jul. 2003.

- [24] K. L. Camplejohn and S. A. Allard, 'Limitations of safranin "O" staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies', *Histochemistry*, vol. 89, no. 2, pp. 185–188, 1988, doi: 10.1007/BF00489922.
- [25] M. E. Angeline and S. A. Rodeo, 'Biologics in the management of rotator cuff surgery', *Clin Sports Med*, vol. 31, no. 4, pp. 645–663, Oct. 2012, doi: 10.1016/j.csm.2012.07.003.
- [26] M. Benjamin, H. Toumi, D. Suzuki, S. Redman, P. Emery, and D. McGonagle, 'Microdamage and altered vascularity at the enthesis-bone interface provides an anatomic explanation for bone involvement in the HLA-B27-associated spondylarthritides and allied disorders', *Arthritis Rheum*, vol. 56, no. 1, pp. 224–233, Jan. 2007, doi: 10.1002/art.22290.
- [27] I. Redler, V. C. Mow, M. L. Zimny, and J. Mansell, 'The ultrastructure and biomechanical significance of the tidemark of articular cartilage', *Clin Orthop Relat Res*, no. 112, pp. 357–362, Oct. 1975.
- [28] S. Havelka, V. Horn, D. Spohrová, and P. Valouch, 'The calcified-noncalcified cartilage interface: the tidemark', *Acta Biol Hung*, vol. 35, no. 2–4, pp. 271–279, 1984.
- [29] K. L. Moffat *et al.*, 'Characterization of the structure-function relationship at the ligament-to-bone interface', *Proc. Natl. Acad. Sci. U.S.A.*, vol. 105, no. 23, pp. 7947–7952, Jun. 2008, doi: 10.1073/pnas.0712150105.
- [30] M. Benjamin *et al.*, 'Anatomy and Biochemistry of entheses', *Annals of the Rheumatic Diseases*, vol. 59, no. 12, pp. 995–999, 2000, doi: 10.1136/ard.59.12.995.
- [31] A. C. Deymier *et al.*, 'Micro-mechanical properties of the tendon-to-bone attachment', *Acta Biomater*, vol. 56, pp. 25–35, Jul. 2017, doi: 10.1016/j.actbio.2017.01.037.
- [32] Y. Xu *et al.*, 'Biomimetic Trachea Engineering via a Modular Ring Strategy Based on Bone-Marrow Stem Cells and Atelocollagen for Use in Extensive Tracheal

- Reconstruction’, *Adv Mater*, vol. 34, no. 6, p. e2106755, Feb. 2022, doi: 10.1002/adma.202106755.
- [33] S. Phoenix and I. BEYERLEIN, ‘Statistical Strength Theory for Fibrous Composite Materials’, in *Comprehensive Composite Materials*, vol. 1, 2000, pp. 559–639. doi: 10.1016/B0-08-042993-9/00056-5.
- [34] C. Laurent, ‘Micromechanics of Ligaments and Tendons’, in *Multiscale Biomechanics*, 2018, pp. 489–509. doi: 10.1016/B978-1-78548-208-3.50013-7.
- [35] A. H. Burstein, D. T. Reilly, and M. Martens, ‘Aging of bone tissue: mechanical properties’, *J Bone Joint Surg Am*, vol. 58, no. 1, pp. 82–86, Jan. 1976.
- [36] M. P. Ginebra, ‘10 - Cements as bone repair materials’, in *Bone Repair Biomaterials*, J. A. Planell, S. M. Best, D. Lacroix, and A. Merolli, Eds., in Woodhead Publishing Series in Biomaterials. , Woodhead Publishing, 2009, pp. 271–308. doi: 10.1533/9781845696610.2.271.
- [37] E. F. Morgan, G. U. Unnikrisnan, and A. I. Hussein, ‘Bone Mechanical Properties in Healthy and Diseased States’, *Annu Rev Biomed Eng*, vol. 20, pp. 119–143, Jun. 2018, doi: 10.1146/annurev-bioeng-062117-121139.
- [38] H. H. Bayraktar, E. F. Morgan, G. L. Niebur, G. E. Morris, E. K. Wong, and T. M. Keaveny, ‘Comparison of the elastic and yield properties of human femoral trabecular and cortical bone tissue’, *J Biomech*, vol. 37, no. 1, pp. 27–35, Jan. 2004, doi: 10.1016/s0021-9290(03)00257-4.
- [39] M. P. Akhter, Z. Fan, and J. Y. Rho, ‘Bone intrinsic material properties in three inbred mouse strains’, *Calcif Tissue Int*, vol. 75, no. 5, pp. 416–420, Nov. 2004, doi: 10.1007/s00223-004-0241-7.
- [40] S. Pathak, J. G. Swadener, S. R. Kalidindi, H.-W. Courtland, K. J. Jepsen, and H. M. Goldman, ‘Measuring the dynamic mechanical response of hydrated mouse bone by

- nanoindentation', *J Mech Behav Biomed Mater*, vol. 4, no. 1, pp. 34–43, Jan. 2011, doi: 10.1016/j.jmbbm.2010.09.002.
- [41] N. Rodriguez-Florez, M. L. Oyen, and S. J. Shefelbine, 'Insight into differences in nanoindentation properties of bone', *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 18, pp. 90–99, Feb. 2013, doi: 10.1016/j.jmbbm.2012.11.005.
- [42] M. J. Silva, M. D. Brodt, Z. Fan, and J.-Y. Rho, 'Nanoindentation and whole-bone bending estimates of material properties in bones from the senescence accelerated mouse SAMP6', *J Biomech*, vol. 37, no. 11, pp. 1639–1646, Nov. 2004, doi: 10.1016/j.jbiomech.2004.02.018.
- [43] P. K. Zysset, X. Edward Guo, C. Edward Hoffler, K. E. Moore, and S. A. Goldstein, 'Elastic modulus and hardness of cortical and trabecular bone lamellae measured by nanoindentation in the human femur', *Journal of Biomechanics*, vol. 32, no. 10, pp. 1005–1012, Oct. 1999, doi: 10.1016/S0021-9290(99)00111-6.
- [44] T. Hoc, L. Henry, M. Verdier, D. Aubry, L. Sedel, and A. Meunier, 'Effect of microstructure on the mechanical properties of Haversian cortical bone', *Bone*, vol. 38, no. 4, pp. 466–474, Apr. 2006, doi: 10.1016/j.bone.2005.09.017.
- [45] A. J. Bushby, V. L. Ferguson, and A. Boyde, 'Nanoindentation of bone: Comparison of specimens tested in liquid and embedded in polymethylmethacrylate', *Journal of Materials Research*, vol. 19, no. 1, pp. 249–259, Jan. 2004, doi: 10.1557/jmr.2004.19.1.249.
- [46] C. E. Hoffler, X. E. Guo, P. K. Zysset, and S. A. Goldstein, 'An Application of Nanoindentation Technique to Measure Bone Tissue Lamellae Properties', *Journal of Biomechanical Engineering*, vol. 127, no. 7, pp. 1046–1053, Aug. 2005, doi: 10.1115/1.2073671.

- [47] J.-Y. Rho, T. Y. Tsui, and G. M. Pharr, ‘Elastic properties of human cortical and trabecular lamellar bone measured by nanoindentation’, *Biomaterials*, vol. 18, no. 20, pp. 1325–1330, Oct. 1997, doi: 10.1016/S0142-9612(97)00073-2.
- [48] C. H. Turner, J. Rho, Y. Takano, T. Y. Tsui, and G. M. Pharr, ‘The elastic properties of trabecular and cortical bone tissues are similar: results from two microscopic measurement techniques’, *J Biomech*, vol. 32, no. 4, pp. 437–441, Apr. 1999, doi: 10.1016/s0021-9290(98)00177-8.
- [49] D. M. Ebenstein and L. A. Pruitt, ‘Nanoindentation of soft hydrated materials for application to vascular tissues’, *J Biomed Mater Res A*, vol. 69, no. 2, pp. 222–232, May 2004, doi: 10.1002/jbm.a.20096.
- [50] D. M. Ebenstein, A. Kuo, J. J. Rodrigo, A. H. Reddi, M. Ries, and L. Pruitt, ‘A nanoindentation technique for functional evaluation of cartilage repair tissue’, *Journal of Materials Research*, vol. 19, no. 1, p. 27, Jan. 2004, doi: 10.1557/jmr.2004.19.1.273.
- [51] K. N. Hauch, M. L. Oyen, G. M. Odegard, and T. L. Haut Donahue, ‘Nanoindentation of the insertional zones of human meniscal attachments into underlying bone’, *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 339–347, Aug. 2009, doi: 10.1016/j.jmbbm.2008.10.005.
- [52] O. Franke, M. Göken, and A. M. Hodge, ‘The nanoindentation of soft tissue: Current and developing approaches’, *JOM*, vol. 60, no. 6, pp. 49–53, Jun. 2008, doi: 10.1007/s11837-008-0071-6.
- [53] D. M. Ebenstein, ‘Nanoindentation of Soft Tissues and Other Biological Materials’, in *Handbook of Nanoindentation*, Jenny Stanford Publishing, 2011.
- [54] O. G. Andriotis, S. Desissaire, and P. J. Thurner, ‘Collagen Fibrils: Nature’s Highly Tunable Nonlinear Springs’, *ACS Nano*, vol. 12, no. 4, pp. 3671–3680, Apr. 2018, doi: 10.1021/acsnano.8b00837.

- [55] N. M. B. K. Willems, L. Mulder, J. M. J. den Toonder, A. Zentner, and G. E. J. Langenbach, 'The correlation between mineralization degree and bone tissue stiffness in the porcine mandibular condyle', *J Bone Miner Metab*, vol. 32, no. 1, pp. 29–37, Jan. 2014, doi: 10.1007/s00774-013-0464-7.
- [56] C. H. Turner, 'Biomechanics of bone: determinants of skeletal fragility and bone quality', *Osteoporos Int*, vol. 13, no. 2, pp. 97–104, 2002, doi: 10.1007/s001980200000.
- [57] S. Chatelin, A. Constantinesco, and R. Willinger, 'Fifty years of brain tissue mechanical testing: from in vitro to in vivo investigations', *Biorheology*, vol. 47, no. 5–6, pp. 255–276, 2010, doi: 10.3233/BIR-2010-0576.
- [58] P. J. Thurner, 'Atomic force microscopy and indentation force measurement of bone', *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, vol. 1, no. 6, pp. 624–649, 2009, doi: 10.1002/wnan.56.

Chapter 4 - Collagen Fibres

4.1. Introduction

The ideal material for the repair of a ligament should have the biomechanical characteristics of native tissue and should provide a scaffold for the aligned deposition of collagen. As collagen is the major acellular component of ligaments, materials based on reconstituted collagen have been developed for the repair of ligaments or tendons. Although, the high levels of porosity of sponge-like collagen scaffolds typically are essential for providing sufficient surface area for initial cell attachment as well as facilitating nutrient bio-transport, they also lead to low mechanical strength, with typical elastic moduli of order 20–200 kPa (dry) and 1–50 kPa (hydrated) [1]–[5]. Stiffness can be increased through the selective addition of mineral content or cross-linking, however, the stiffness of these scaffolds typically remains too low in comparison to native tissues [5]. Healthy tendons and ligaments have elastic moduli well over 1 GPa [6]–[8]. Although tissue engineering constructs do not need to exactly match the tissues they are implanted into, they do need to withstand daily *in vivo* mechanical forces.

Therefore, collagen which has been formed into thin fibres has been developed for the repair of soft tissue such as ligaments and tendons (section 2.6.1) in endeavours to improve mechanical properties. Kato *et al.* [9] first reported the fabrication of extruded collagen fibres for tissue engineering purposes with mechanical properties similar to that of native tissue (rat tail tendon).

Although the presence of elastin in ligaments is scarce, it plays an important role in the extracellular matrix of ligaments, localised between and along the collagen fibres and fascicles. In a study by Henninger *et al.* [10] It was shown that elastin supported up to 30% of tensile strength along the primary collagen axis in medial collateral ligament despite only contributing to 4% of the dry weight. Therefore, incorporating elastin into structures for ligament

regeneration could provide greater mechanical strength and microenvironment for ligament tissue growth.

Recent studies have shown that reinforcing scaffolds with fibres have the potential to improve the performance of the materials, providing an adequate mechanical environment during the healing state and improve biocompatibility by facilitating cell adhesion, migration, differentiation and proliferation [11]–[16]. Therefore, it has also been investigated whether the extruded collagen fibres could be utilised to reinforce porous collagen scaffolds and enhance their mechanical strength.

4.2. Materials and Methods

Type I atelocollagen monomers from amniotic membranes (AM) were donated by Veterans Affairs Medical Center. Tris-buffered saline (TBS), Phosphate-buffered saline (PBS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich UK.

4.2.1. Monomeric Collagen Suspension

A 1% wt/v monomeric collagen suspension was prepared by dissolving Type I lyophilised monomeric atelocollagen from amnion (0.5g), in acetic acid solution (50 ml, pH=3.2). The suspension was homogenised with a conventional blender to create a uniform dispersion. The homogenised suspension was centrifuged (Thermo, IEC CL10, UK) at room temperature for 5 mins at 3000 rpm to remove entrapped air bubbles. This produced a monomeric collagen suspension which was stored in centrifuge tubes at 4°C.

4.2.2. Collagen Fibre Production

Fibres were produced by extruding the collagen suspension through a silicon tube of inner diameter 1mm, at a controlled rate of 70 ml/hr using a syringe pump (IVAC P6000 syringe pump). The tube was secured to the bottom of a bath containing a fibre formation buffer (FFB, 30mM TBS and 30mM PBS) and cross-linking agents EDC/NHS (15mM EDC, 6mM NHS) at pH 7.5. Setup is illustrated in Figure 4.1. The acrylic bath with wells of 300mm x 20mm x 20mm, was specially designed and built for this study by the author using a laser cutter.

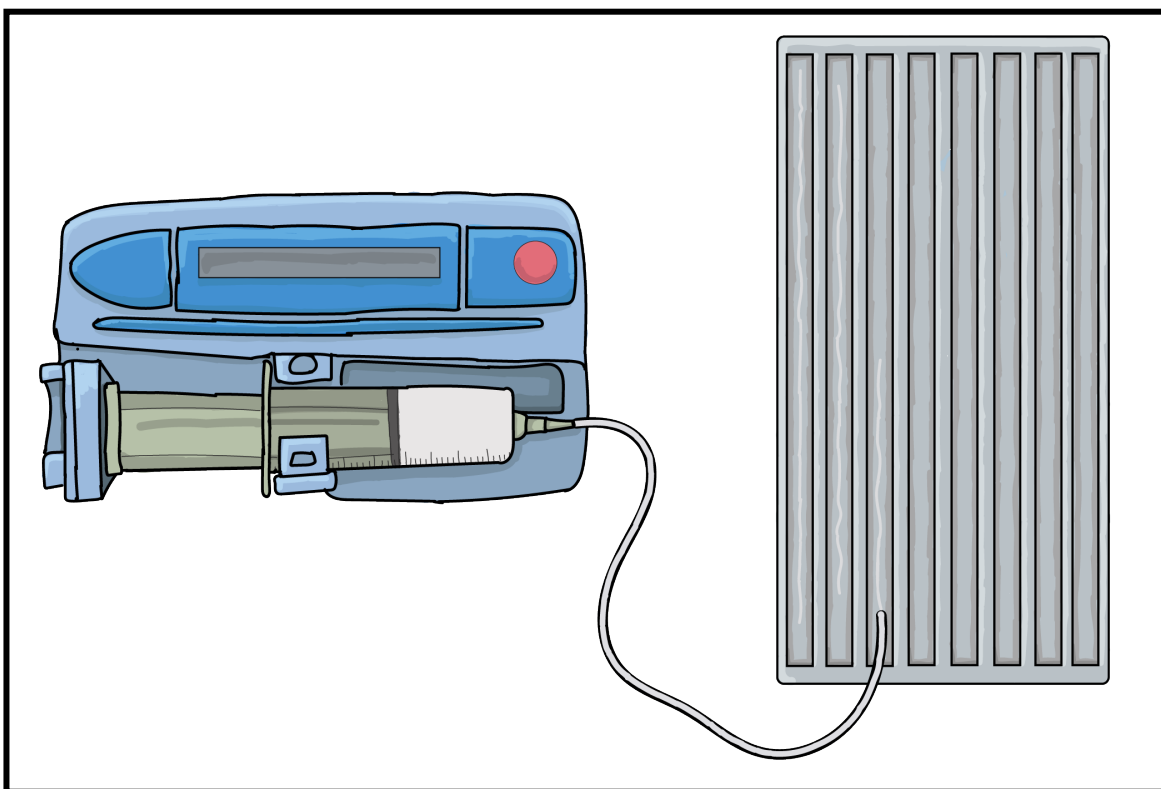


Figure 4.1: Collagen extrusion setup

The extruded fibres were incubated at 37°C for 4 hours to self-assemble, and crosslinked as desired, before being taken out and washed with 0.1 M Disodium Phosphate (Na_2HPO_4) followed by deionised water. After washing the fibres were left to air dry and suspended under their weight for 24hrs.



Figure 4.2: Extruded fibres air drying under their own weight.

4.2.3. Elastin Production

Commercially available insoluble Elastin (Bovine neck ligament, Sigma-Aldrich, UK) was isolated and purified into a workable gel using a bottom-up method pioneered by a previous member of our research group [17]. The method is briefly described.

1:10 w/v of Elastin powder was gently mixed with 0.25M of oxalic acid (purified grade, Sigma Aldrich, UK). The mixture was transferred to a 100ml round-bottomed flask and attached to an air condenser. The mixture was magnetically stirred at a speed of 300rpm for 70 mins whilst heated in a water bath placed on a hot stirring plate (Stuart US152 Hot Stirrer, RS Components, U.K.) at a temperature over 95 °C. The mixture was then rapidly quenched using an ice bath

for 5 mins. After quenching the mixture was transferred to 50ml centrifuge tubes and centrifuged at 5000rpm for 5 mins and the supernatant was discarded. The pellet obtained after centrifugation was mixed with fresh 0.25M oxalic acid and subjected to two further hydrolysis. After three sequential hydrolysis, the elastin pellet was purified and converted into a workable gel-like substance by washing in excess di-water for 1 hour and then a further 4 times for 20 mins each. Excess water was discarded after each washing step by centrifugation. The elastin gel was frozen and then lyophilised and stored in an air-tight bag at 4°.

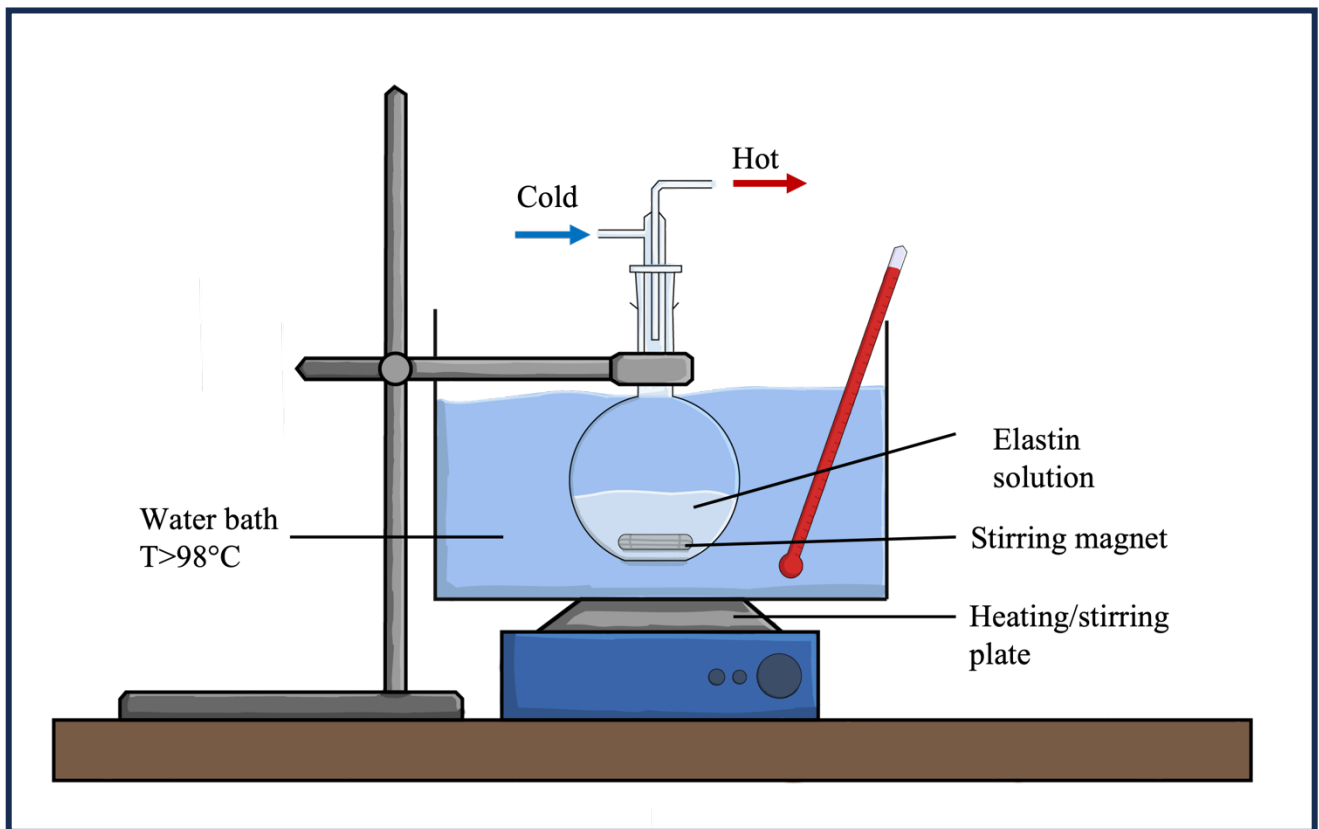


Figure 4.3: Elastin production setup

4.2.4. Cross-Linking

A portion of the partially cross-linked dry fibres was further cross-linked. The fibres were placed in 200mL deionised water with cross-linking agents EDC/NHS (60mM EDC, 24mM NHS), pH 4. The fibres in the solution were gently shaken overnight to fully cross-link. They were then washed with 0.1 M Disodium Phosphate (Na_2HPO_4) followed by deionised water. And left to air dry suspended under their weight for 24hrs again.

4.2.5. Elastin Infiltration

A portion of the partially cross-linked dry fibres was infiltrated with elastin. The lyophilised elastin was redissolved in acidified deionised water at a pH of 2.7 to produce a solution of 1% w/v. Cross-linking agents EDC/NHS (60mM EDC, 24mM NHS) were added to this solution and the pH was adjusted to 3.4. The fibres were added to this solution and were gently shaken overnight to cross-link with the elastin. They were then washed with 0.1 M Disodium Phosphate (Na_2HPO_4) followed by deionised water. And left to air dry suspended under their weight for 24hrs again.

4.2.6. Polymeric Collagen Scaffolds (Commercial)

For reinforced scaffolds, commercially available collagen was used due to the scarcity of monomeric collagen available caused by the global pandemic.

A 1% wt/v collagen suspension was prepared by adding type I collagen powder (0.5g) from bovine Achilles tendon (Sigma Aldrich, UK), in acetic acid solution (50 ml, pH=3.2). The collagen-acid mixture was stirred magnetically for 15 mins before being placed in an ice bath before the suspension was homogenised with a conventional blender to create a uniform dispersion.

The suspension of polymeric collagen was centrifuged (Thermo, IEC CL10, UK) at room temperature for 5 mins at 3000 rpm to remove entrapped air bubbles. The suspension was cast directly into PTFE moulds using a syringe, frozen at -20°C and then lyophilised to obtain the porous scaffolds.

4.2.7. Reinforced Scaffolds

Polymeric collagen scaffolds were reinforced using single and bundles consisting of 10 fibres. The suspension of polymeric collagen was centrifuged (Thermo, IEC CL10, UK) at room temperature for 5 mins at 3000 rpm to remove entrapped air bubbles. The suspension was cast into PTFE moulds using a syringe halfway up the mould. The single or bundles of 10 fibres were then placed longitudinally on the middle of the suspension before the top half of the mould was filled with more of the suspension. The moulds were then frozen at -20°C and then lyophilised to obtain the reinforced scaffolds.

A portion of the polymeric collagen scaffolds were cross-linked. The scaffolds were placed in 200mL deionised water with cross-linking agents EDC/NHS (60mM EDC, 24mM NHS), pH 4. The scaffolds in solution were gently stirred overnight to fully cross-link. They were then washed with 0.1 M Disodium Phosphate (Na_2HPO_4) followed by deionised water. The scaffolds were then frozen at -20°C and lyophilised for a second time.

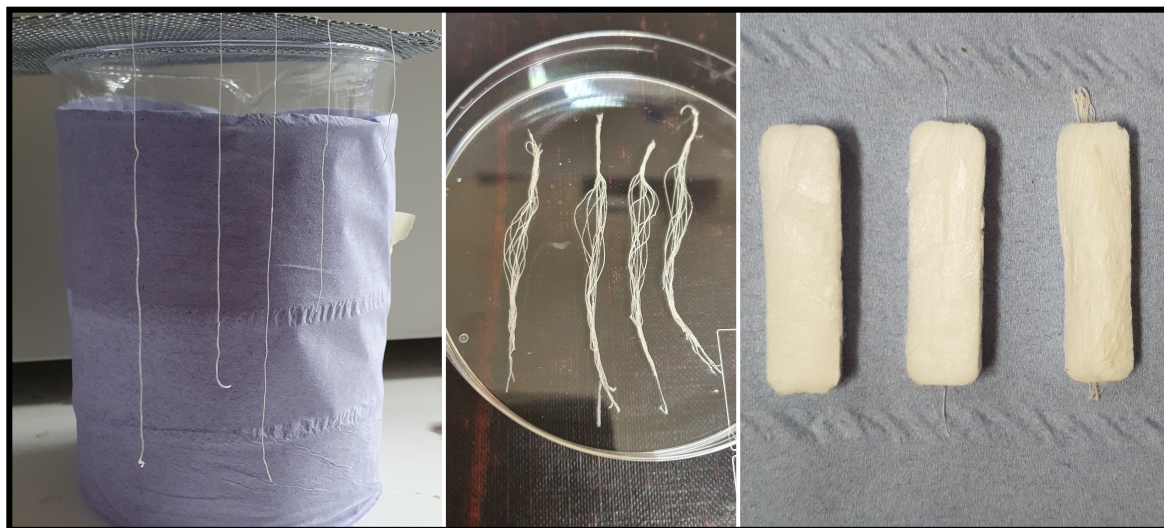


Figure 4.4: Fabrication of fibre reinforced scaffolds. (A) individual collagen fibres air-drying under their own weight, (B) Bundles of 10 fibres, (C) reinforced scaffolds with: no fibre (left), single fibre (centre) and fibre bundle (right).

4.2.8. Scanning Electron Microscopy

The morphology and microstructure of fibres were studied using scanning electron microscopy (SEM), as it has a large depth of focus and the samples are relatively easy to prepare. Specimens were sectioned with a sharp blade, adhered to aluminium stubs using carbon tabs and tweezers, and sputter coated with platinum (thickness 6nm). Samples were imaged using a Zeiss EVO operated at an accelerating voltage of 3 kV and a probe current of 100pA at a working distance of 7-8 mm. The chamber was maintained under a vacuum of 1×10^{-5} - 1×10^{-6} mbar, preventing the molecules or atoms already present in the microscope column from interacting with the electron beam and protecting the electron source from vibrations and noise. Post-imaging analysis was conducted using ImageJ.

4.2.9. Water Uptake

The water uptake ratio (G) was determined for all Type I atelocollagen fibres by comparing the increase in mass to the original mass of the dry samples. The samples were weighed in their dry state and then immersed in 0.1M phosphate-buffered saline (PBS) at room temperature. After 24hrs of hydration, the samples were removed from the PBS and placed on a wire mesh rack for 1 minute to drain any excess PBS. PBS was chosen as a solvent for swelling as it simulates physiological conditions in terms of osmolarity and ion concentration. The hydrated samples were weighed, and the water uptake was expressed as a ratio of the mass of the PBS absorbed by the sample to the dry weight of the sample. Shown in Equation 4.1.

$$G = \frac{M_{wet} - M_{dry}}{M_{dry}}$$

(Equation 4.1)

Where G is the water uptake ratio, M_{dry} is the mass of the dry sample and M_{wet} is the mass of the hydrated sample.

4.2.10. Deflection Testing

Deflection tests were carried out due to the extremely low loads required to characterise the hydrated fibres. Deflection tests for samples were carried out as shown in Figure 4.5, in which the hydrated samples were allowed to deflect over a span of 20mm, with the weight of the hydrated samples as the uniform load, w . For fibres, an additional load was placed across the centre of each fibres, v . Scaffolds were allowed to deflect under their weight only (self-deflect). Pieces of an abrasive material were placed on the edges of each of the gaps to prevent the samples from completely falling through. The samples were left to deflect for 2 mins before an

image of the deflection was captured by a camera. Before deflection testing, the dry samples were incubated overnight in PBS.

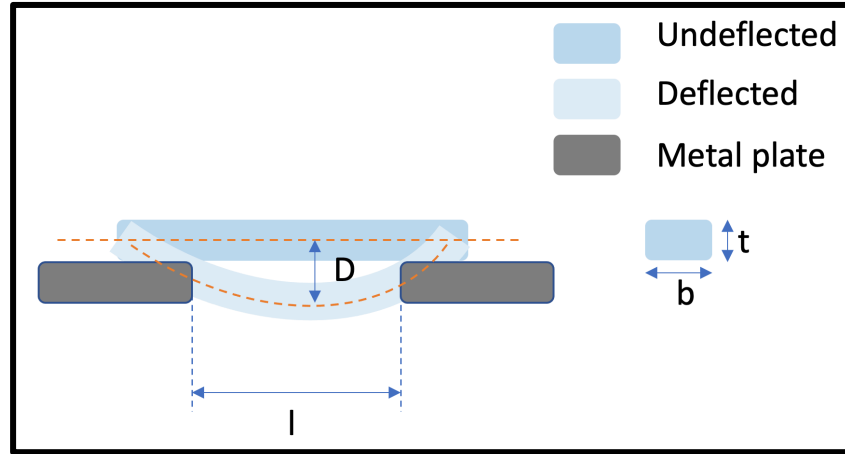


Figure 4.5: Schematic of self-deflection set up.

Using the metal plate of a known thickness (3.02mm) as a scale bar, the thickness and deflection of the samples were measured using ImageJ.

For deflection of fibres, values for Young's modulus were obtained using the following formula:

$$E = \frac{5wl^4}{384\delta I} + \frac{vL}{384\delta I} (8l^3 - 4Ll^2 + L^3)$$

(Equation 4.2)

Where w is the hydrated weight of the fibres, l is the length of the beam, L is the width of the additional load, v is the weight of the additional load (8mg), E is the Young's modulus and I is the second moment of area. Loads acting upon the fibres are illustrated in Figure 4.6.

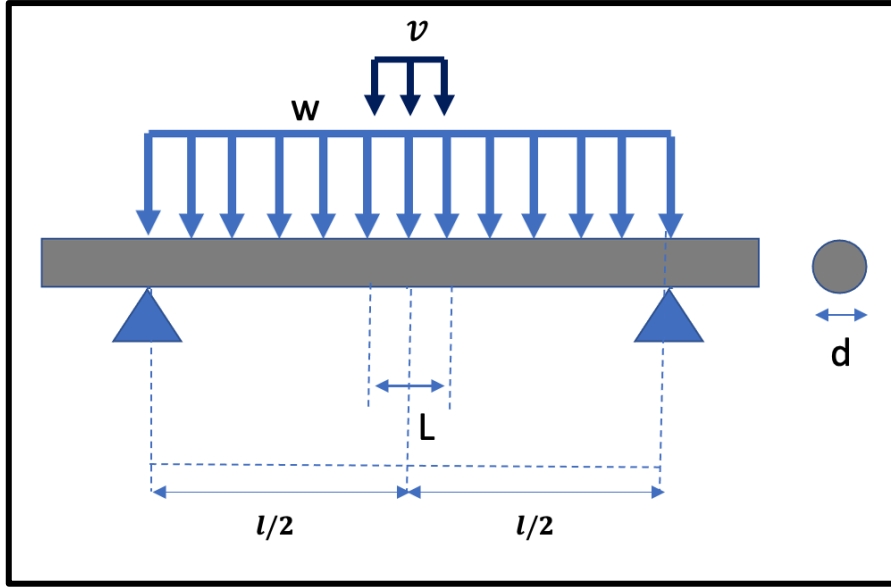


Figure 4.6: Illustration of uniform beam distribution, w , with additional load, v , for fibres deflecting over a span of 20mm.

It was assumed that fibres were circular. Therefore, for a cylindrical beam,

$$I = \frac{\pi d^4}{64}$$

(Equation 4.3)

For reinforced scaffolds, values for Young's modulus were obtained using the following formula:

$$E = \frac{5wl^4}{384\delta I}$$

(Equation 4.4)

Where w is the hydrated weight of the scaffolds, l is the length of the beam, E is the Young's modulus and I is the second moment of area. Loads acting upon the scaffolds are illustrated in Figure 4.7.

It was assumed that scaffolds were rectangular. Therefore, for a rectangular beam,

$$I = \frac{bh^3}{12}$$

(Equation 4.5)

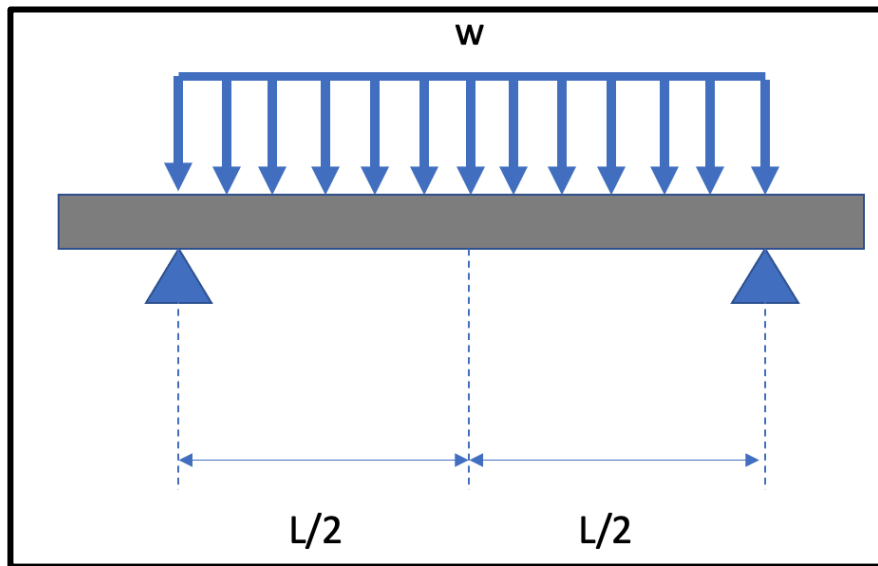


Figure 4.7: Illustration of uniform beam distribution for scaffolds deflecting over a span of 20mm.

4.2.11. Tensile Testing

Composite samples (fibre-reinforced collagen scaffolds) were tested under tension, using an INSTRON 3360 series with BlueHill Universal system. After soaking in PBS solution for one hour, the rectangular cross-section samples (approximately 60mm*10mm*5mm as produced) were tightened within the grips with the fibre direction parallel to the tensile axis. A constant extension rate of 10 mm/minute was applied. Load was recorded using a 500 N load cell and tensile moduli was calculated.

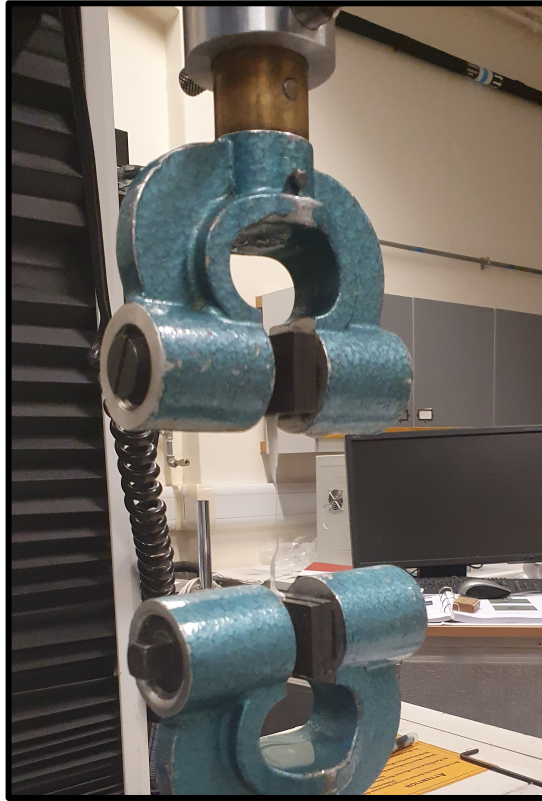


Figure 4.8: Experimental set up for tensile testing on INSTRON

4.2.12. Rule of mixtures

For a fibrous composite that is loaded in the direction of fibre alignment, the total load sustained by the composite F_c is equal to the sum of the loads carried by the matrix phase F_m and the fibre phase F_f , or

$$F_c = F_m + F_f$$

(Equation 4.6)

Assuming that the fibre-matrix interfacial bond is very good such that the deformation of both matrix and fibres is the same (isostrain situation).

From the definition of stress, $\sigma = F/A$, an expression for the composite stress is obtained.

$$\sigma_c = \sigma_m \frac{A_m}{A_c} + \sigma_f \frac{A_f}{A_c}$$

(Equation 4.7)

Where A_m/A_c and A_f/A_c are the area fractions of the matrix and fibre phases, respectively. If the composite, matrix, fibre phase lengths are all equal then A_m/A_c and A_f/A_c are equivalent to the volume fractions. Equation 4.7 now becomes,

$$\sigma_c = \sigma_m V_m + \sigma_f V_f$$

(Equation 4.8)

The previous assumption of an isostrain state means that

$$\epsilon_c = \epsilon_m = \epsilon_f$$

(Equation 4.9)

Furthermore, if composite, matrix and fibre deformations are all elastic then the modulus of a continuous and aligned fibrous composite in the direction of alignment is given as:

$$E_c = E_m V_m + E_f V_f$$

(Equation 4.10)

Which is commonly referred to as the “rule of mixtures”.

4.2.13. Statistical Analysis

Results were expressed as mean $\pm$ standard deviation. The results were compared with one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test. For values of $p < 0.05$, the difference was considered statistically significant.

4.3. Results

4.3.1. Sample Preparation

4.3.1.1. Fabrication and Cross-linking of Fibres

During the fabrication of Type I collagen-based fibres, using the technique mentioned above (section 4.2.2), fibres were suspended under their weight to prevent the formation of a flat edge when drying against a surface, and to prevent the fibres from sticking to the drying plate. The fibres formed this way had uniformly circular cross-sections. As previously mentioned in Section 2.5, there needed to be some cross-linking agents present in the FFB solution for the fibres to successfully overcome the tension of the FFB and not break during extraction.

Label	EDC/NHS concentration in FFB (mM)	EDC/NHS concentration in cross-linking solution (mM)	Presence of Elastin
NXL	15/6	—	N
XL	15/6	60/24	N
EXL	15/6	60/24	Y

Table 4.1: Labels used in describing fibre types.

Diameters of the fibres in their dry state were obtained from SEM images using ImageJ and diameters in their hydrated state from camera photos using ImageJ. The diameters of the fibres produced (Table 4.2), in particular, the hydrated state show a difference between the fibres which were fully cross-linked (XL and EXL) and those which were not (UXL). Although differences in diameter were found between XL and EXL fibres in their dry state, after

hydration no difference was found. Upon analysis of the ratio between hydrated and dry diameters, a value for the swelling ratio can be ascertained.

	Dry diameter (μm)	Wet diameter (μm)	Swelling ratio
UXL	179.09 $\pm$ 0.49	352.29 $\pm$ 8.35	0.97 $\pm$ 0.04
XL	135.71 $\pm$ 1.10	246.25 $\pm$ 3.82	0.81 $\pm$ 0.01
EXL	166.28 $\pm$ 1.06	249.34 $\pm$ 5.79	0.50 $\pm$ 0.03

Table 4.2: Diameters of fibres produced.

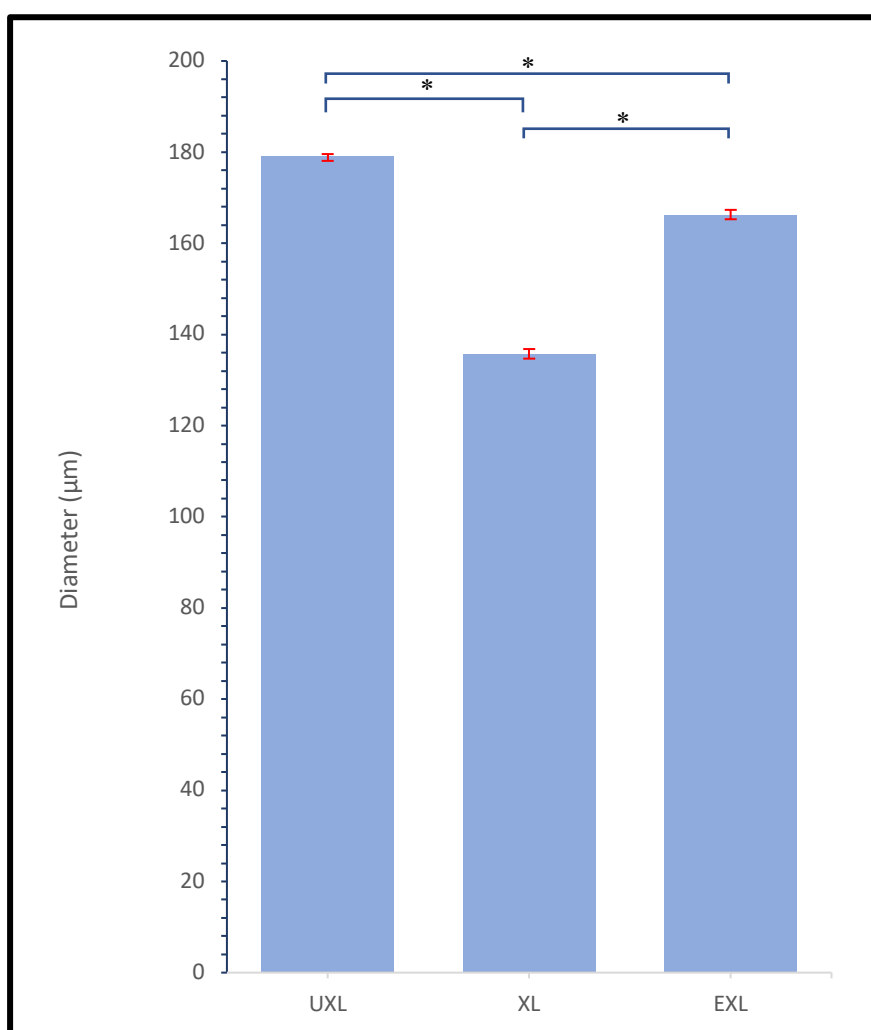


Figure 4.9: Diameter of dry fibres. Data expressed at mean $\pm$ SD

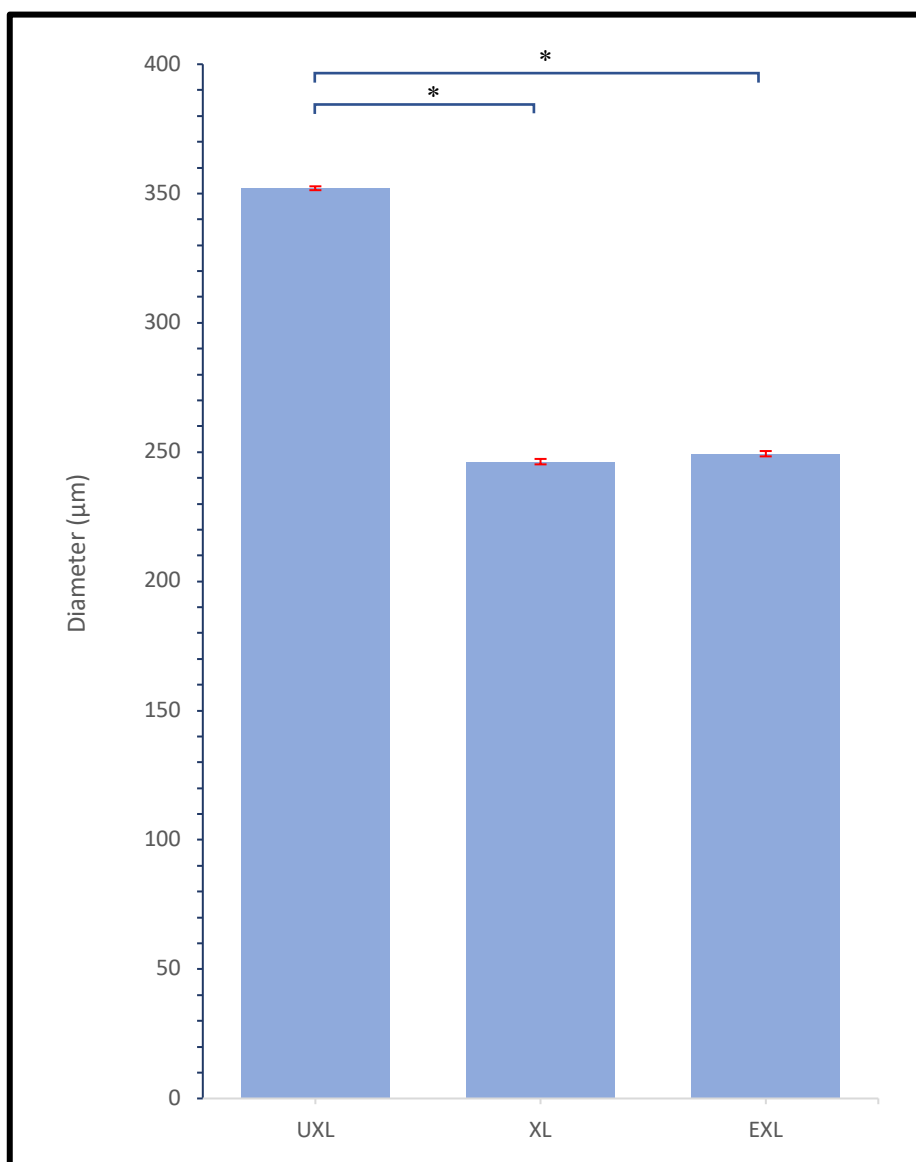


Figure 4.10: Diameter of wet fibres. Data expressed at mean $\pm$ SD

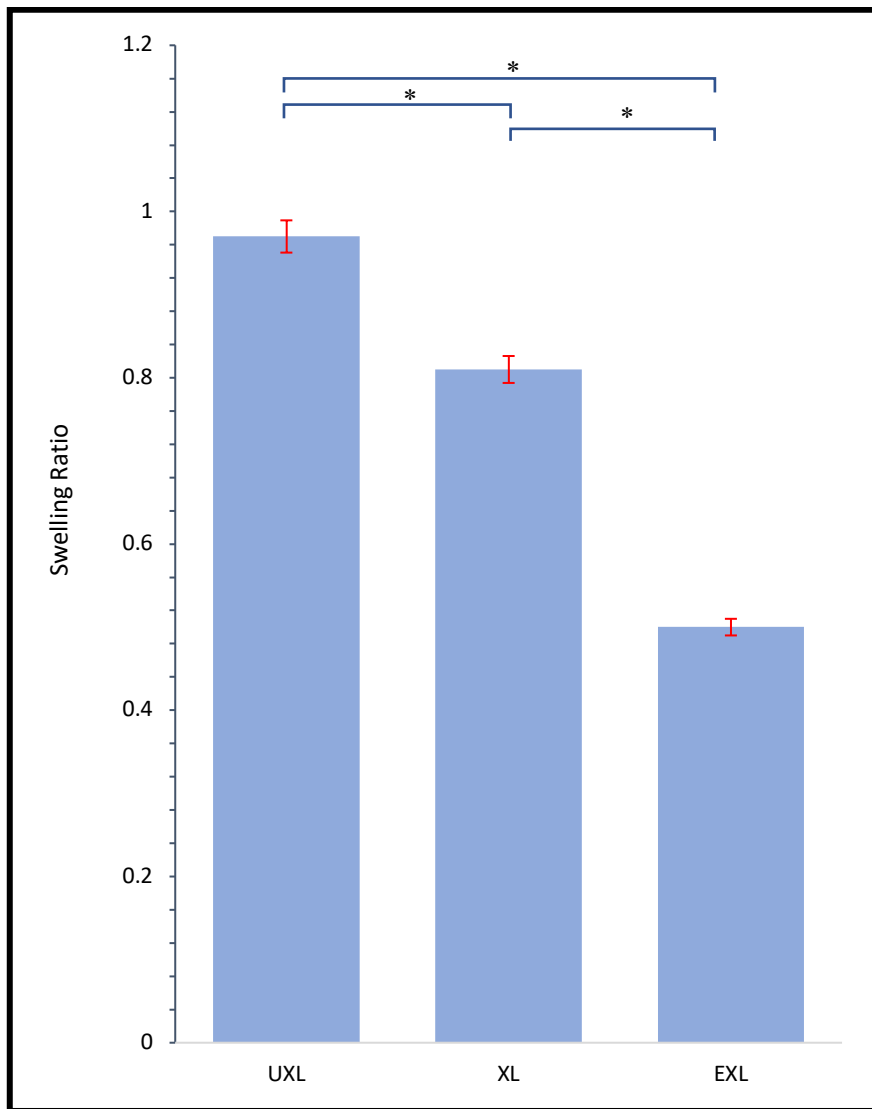


Figure 4.11: Swelling Ratio. Data expressed as mean $\pm$ SD

4.3.1.2. Fabrication of Reinforced Porous Scaffolds

Figure 4.12, Shows photographs of the scaffolds uncrosslinked and crosslinked. After cross-linking and the second round of lyophilisation, scaffolds had noticeably shrunk. This was most prominent with scaffolds containing the bundle of fibres. This was caused by the fibres shrinking by a different amount and causing the outer structure (matrix) to pucker. This does however show that there is a strong fibre-matrix interfacial bond as the dissimilarity in shrinkage between the fibres and matrix is causing the matrix to deform.

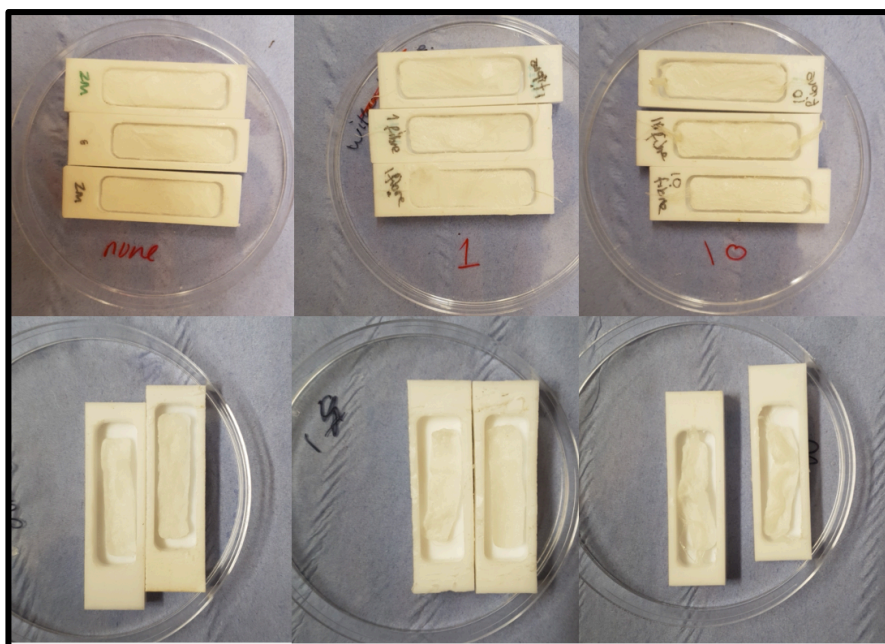


Figure 4.12: Photographs of reinforced collagen scaffolds (A1) no fibres, uncrosslinked (A2) no fibre, crosslinked (B1) single fibre, uncrosslinked (B2) single fibre, crosslinked (C1) bundle of 10 fibres, uncrosslinked (C2) bundle of 10 fibres, crosslinked.

4.3.2. Scanning Electron Microscopy

SEM was used to determine the surface morphology and the dry thickness of the fibres in their dry state. SEM images revealed similarly structured fibres with a consistent surface morphology across all three fibre types. The fibres were presented to be uniformly cylindrical with the thickness of the uncross-linked (UXL), cross-linked (XL) and elastin cross-linked (EXL) fibres within the ranges 178-180 μ m, 134-136 μ m and 166-168 μ m, respectively. Figure 4.13 presents SEM images of the fibres. The uniaxially aligned ridges running along the fibres are very clear to see in figure 4.13a. By observing the fractured end of a fibre, figure 4.14, it can be seen that the aligned arrangement runs throughout the entire fibre, not just at the surface level, thus confirming that the ridges on the surface are from fibril formation rather than caused by the extrusion process.

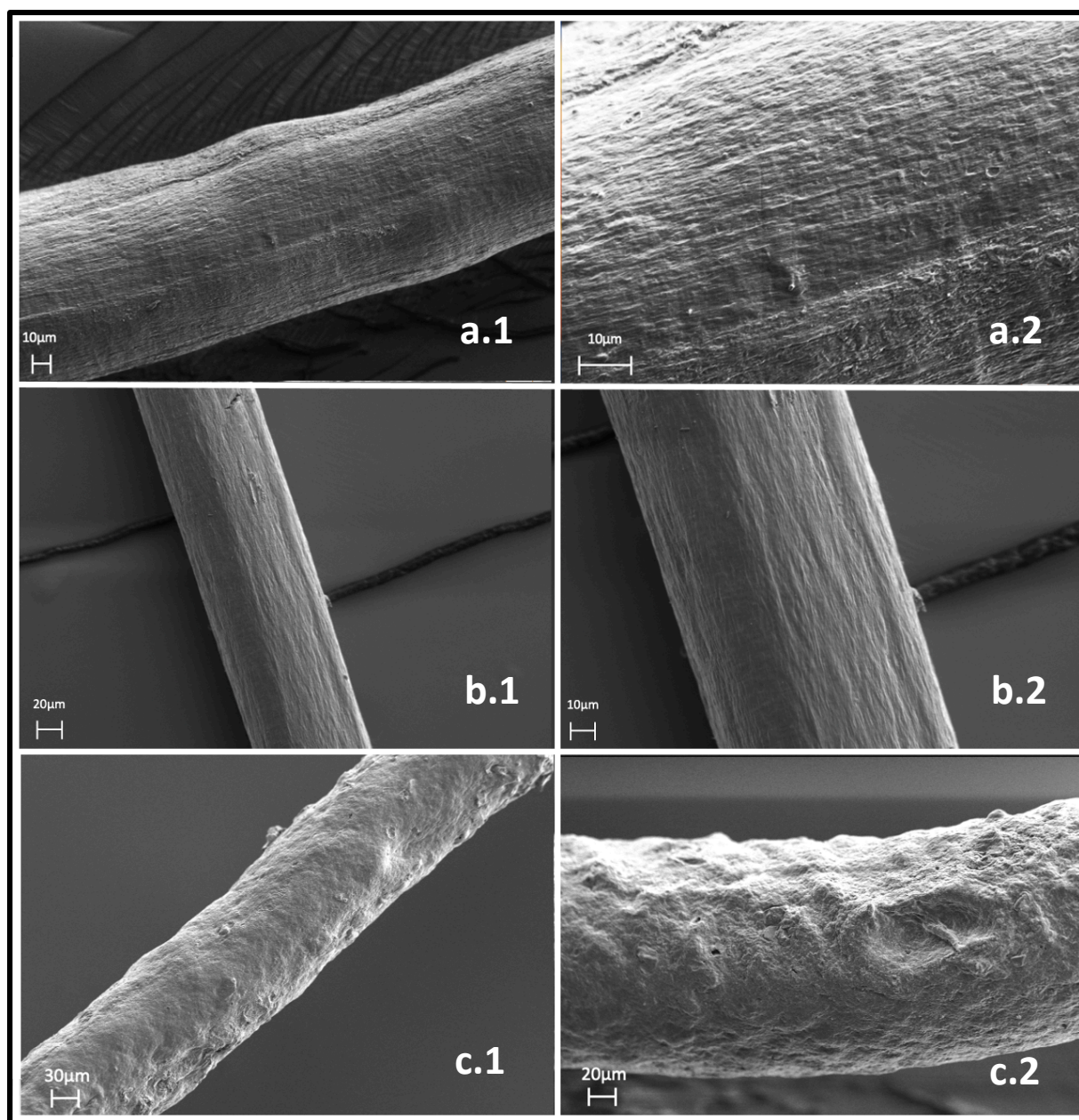


Figure 4.13: Sample SEM images of dry Type I atelocollagen extruded fibres with aligned ridges (a) uncrosslinked (UXL) (b) crosslinked (XL) (c) elastin cross-linked (EXL) .

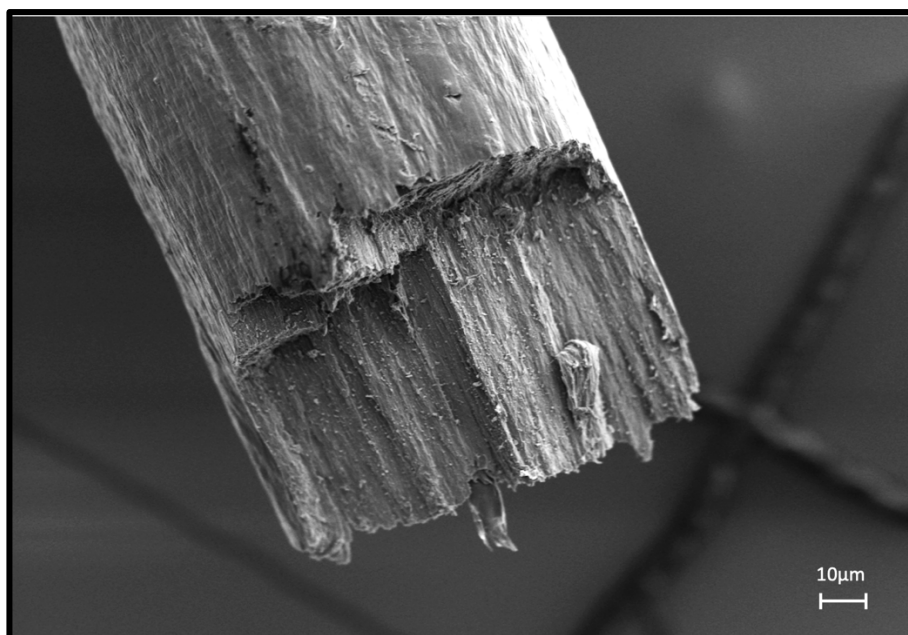


Figure 4.14: fractured end of UXL fibre with aligned ridges running throughout cross-section.

4.3.3. Water Uptake

4.3.3.1. Water Uptake of Fibres

Figure 4.15 shows the results for the ratio of PBS absorbed by each of the fibres after 24 hrs of swelling. It was shown that the additional round of cross-linking resulted in a decrease in the water uptake ratio as the fibres that were not further cross-linked (UXL) presented water uptake ratios which were over 5-fold greater than the samples which were fully cross-linked (XL) and those which were cross-linked with elastin. There was no statistical difference in water uptake ratios for XL fibres and EXL fibres ($p < 0.05$).

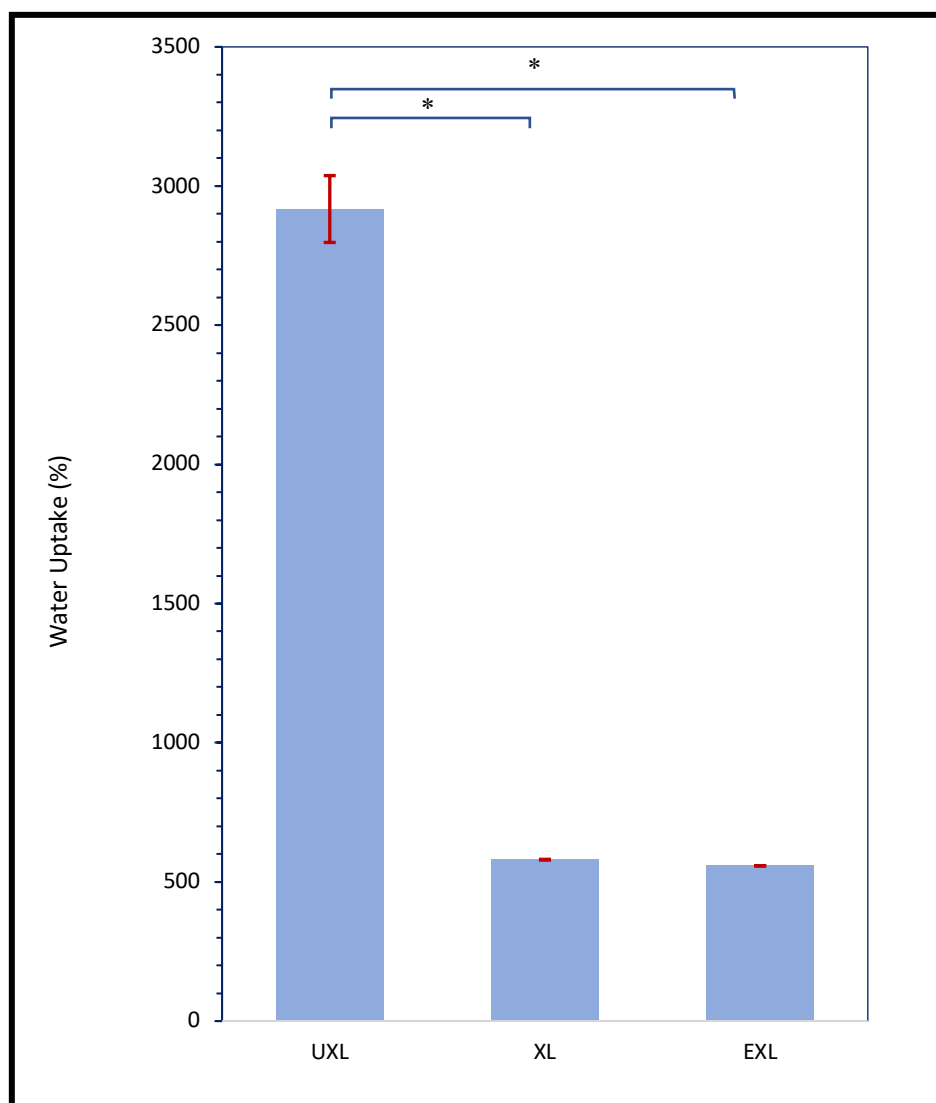


Figure 4.15: Graph of water uptake ratio of extruded fibres. Data expressed as mean $\pm$ SD, $n=5$.

4.3.4. Deflection Testing

4.3.4.1. Deflection of Fibres

The stiffness of each of the fibres was compared by a deflection test. It was found that further crosslinking of fibres significantly increased the stiffness of the fibres. Although there was no significant difference between the cross-linked and elastin cross-linked samples, the fibres which were not further cross-linked were significantly more compliant in bending with XI and EXL samples being 4.5-fold and 5-fold stiffer, respectively.

	Average wet thickness (mm)	E bending (MPa)
NXL	0.35	15.0
XL	0.24	35.9
EXL	0.24	40.2

Table 4.3: Average wet thickness of fibres and average bending modulus.

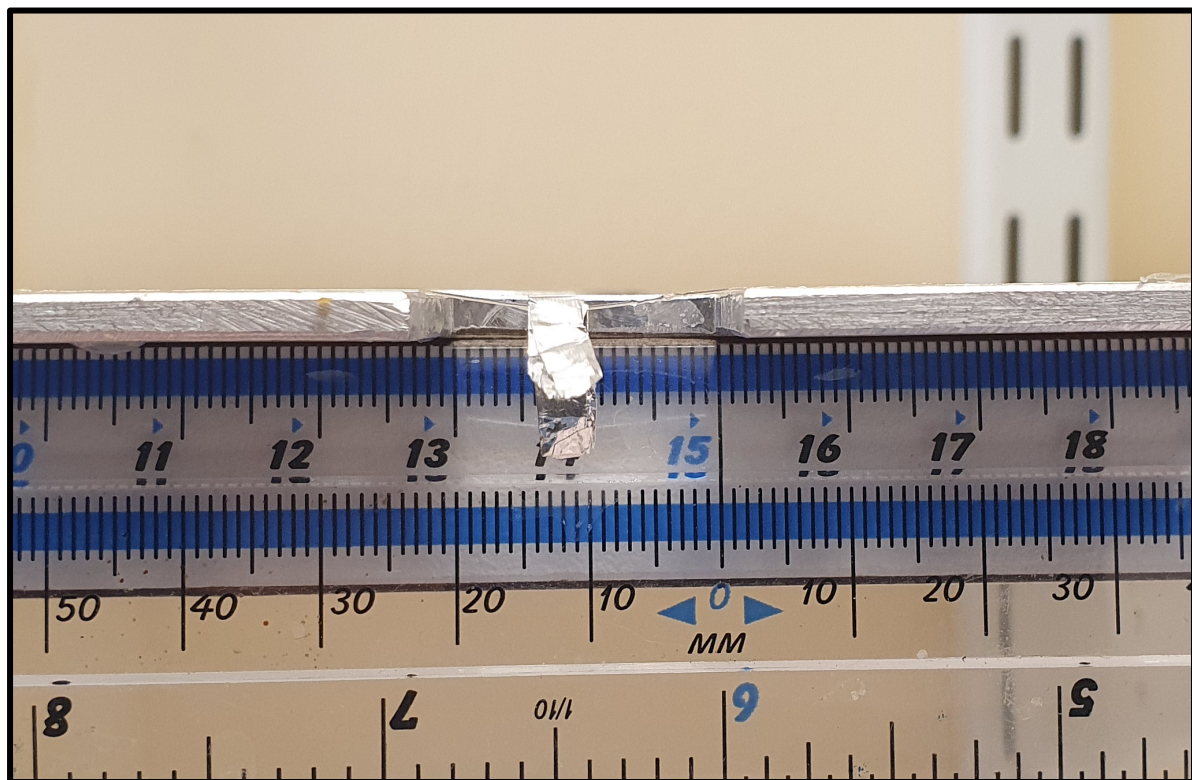


Figure 4.16: Fibre self-deflection testing setup

4.3.4.2. Deflection Testing of Scaffolds

The bending modulus of the scaffolds was calculated by measuring the self-deflection of the hydrated scaffolds. E bending values of the reinforced scaffolds are visualised in Figure 4.17. The E bending values for uncrosslinked scaffolds with no fibre, a single fibre and a bundle of 10 fibres were 0.005 MPa, 0.011 MPa and 0.009 MPa, respectively. Whilst E bending results for the cross-linked scaffolds were 2-fold higher than their uncross-linked counterparts at, 0.010 MPa, 0.022 MPa and 0.021 MPa, respectively. There was no significant difference between scaffolds with a single fibre or a bundle of fibres in E bending modulus.

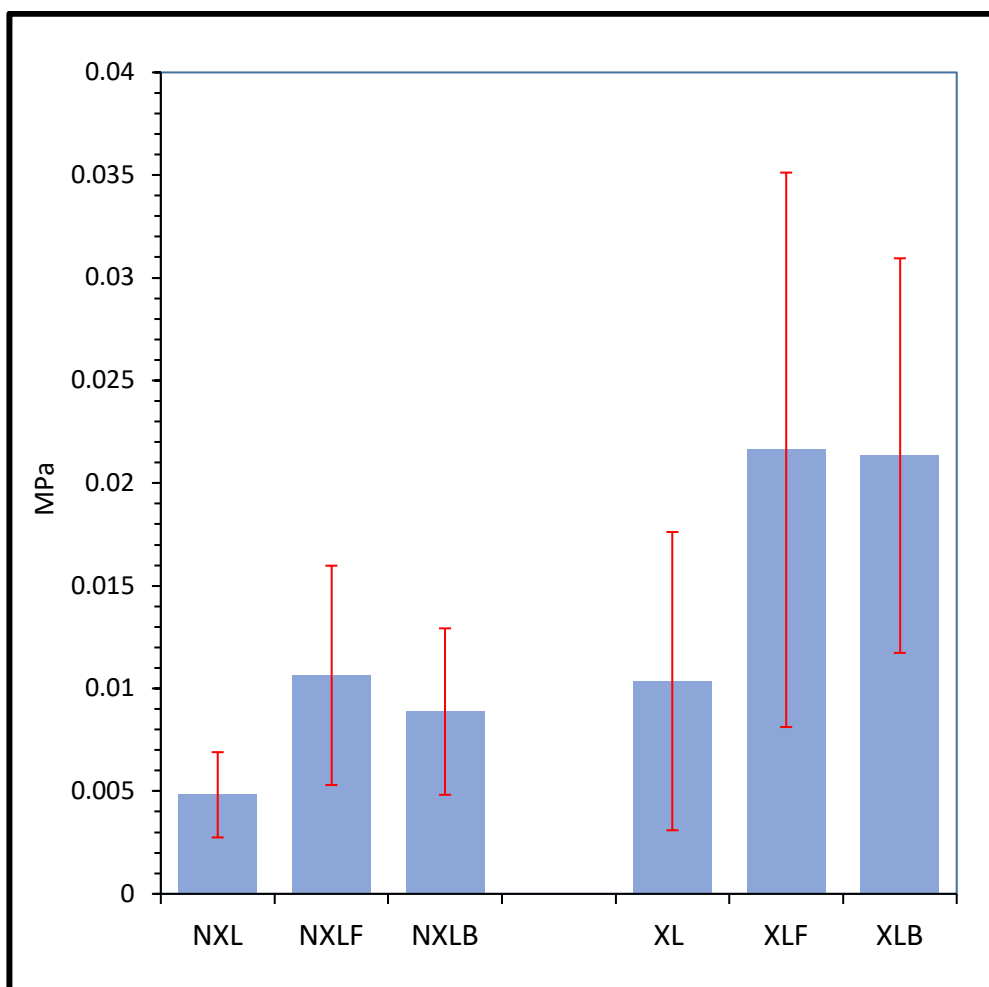


Figure 4.17: Bending modulus (MPa) of reinforced polymeric collagen-based scaffolds, left to deflect under their own hydrated weight. (A1) no fibres, uncrosslinked (A2) no fibre, crosslinked (B1) single fibre, uncrosslinked (B2) single fibre, crosslinked (C1) bundle of 10 fibres, uncrosslinked (C2) bundle of 10 fibres, crosslinked. $n=5$.

4.3.5. Tensile Testing

Tensile strength experiments were performed to determine the mechanical properties of the polymeric collagen scaffolds with and without fibres. By observing the displacement-force behaviour of the polymeric collagen scaffolds being stretched longitudinally (Figure 4.18), it can be seen that cross-linking increased the strength of the scaffolds, however, the addition of fibres did not significantly impact the ultimate strength. However, the inclusion of fibres did

affect the stiffness of the scaffolds. Theoretical values for the stiffness of reinforced scaffolds can be calculated using the rule of mixtures (Equation 4.10). These results are presented alongside the mean experimentally obtained values in Table 4.4. It was not possible to perform tensile testing on the fibres alone due to the small load cell required, therefore values used for theoretical calculations were obtained from a previous study performed in our lab [18].

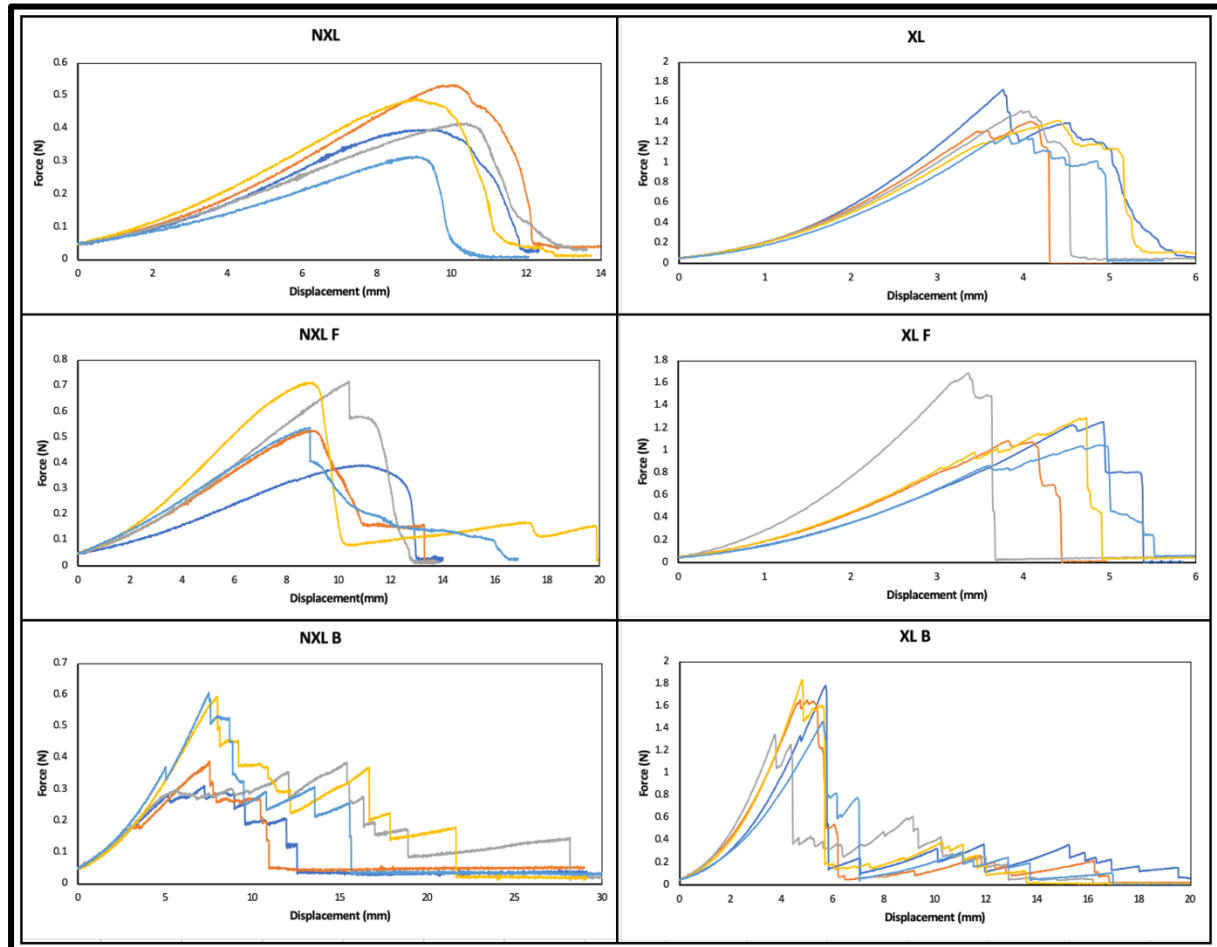


Figure 4.18: Tensile force-displacement plot of polymeric collagen scaffolds, (A1) no fibres, uncrosslinked (A2) no fibre, crosslinked (B1) single fibre, uncrosslinked (B2) single fibre, crosslinked (C1) bundle of 10 fibres, uncrosslinked (C2) bundle of 10 fibres, crosslinked. $n=5$.

	V_m	V_f	E Rule of mixtures (MPa)	E Experimental (MPa)
Fibre (NXL)	0	1	29	29 ± 7 [18]
NXL	1	0	0.07	0.07
NXL F	0.9948	0.0052	0.2181	0.098
NXL B	0.9511	0.0489	1.4849	0.102
Fibre (XL)	0	1	34	34 ± 18 [18]
XL	1	0	0.336	0.336
XL F	0.9985	0.0015	0.3881	0.503
XL B	0.9847	0.0153	0.8498	0.738

Table 4.4: Values for experimentally obtained modulus and theoretical calculated using rule of mixtures with volume fractions of matrix (V_m) and fibre (V_f).

4.4. Discussion

4.4.1. Cross-linking of fibres

Elastin is a highly insoluble protein that is rich in amino acids and contains few polar groups [19], [20]. Although the exact molecular assembly of elastin is still poorly understood, it is generally agreed that natural cross-links in elastin are derived from lysine in peptide linkage and that the cross-linking of collagen and elastin are related. The greatest evidence for this arises from the study of a toxic condition known as lathyrism, which is a toxic condition in animals which produces a loss of integrity in connective tissue. Lathyrogens inhibit cross-linking by creating a block at the conversion of lysine in peptide linkage to the aldehyde. This was shown as early as 1966 to be the case for collagen[21], [22] [23] and was later shown to be the case for elastin by Miller *et al.*[24] and O'Dell *et al*[25].

Therefore, it is reasonable to assume that the aldehyde is involved in both cross-linking processes and that cross-linking of collagen and elastin are closely linked. From this, the crosslinking mechanism of Collagen type I and elastin in the presence of EDC/NHS cross-linking agents shown in figure 4.19 is suggested.

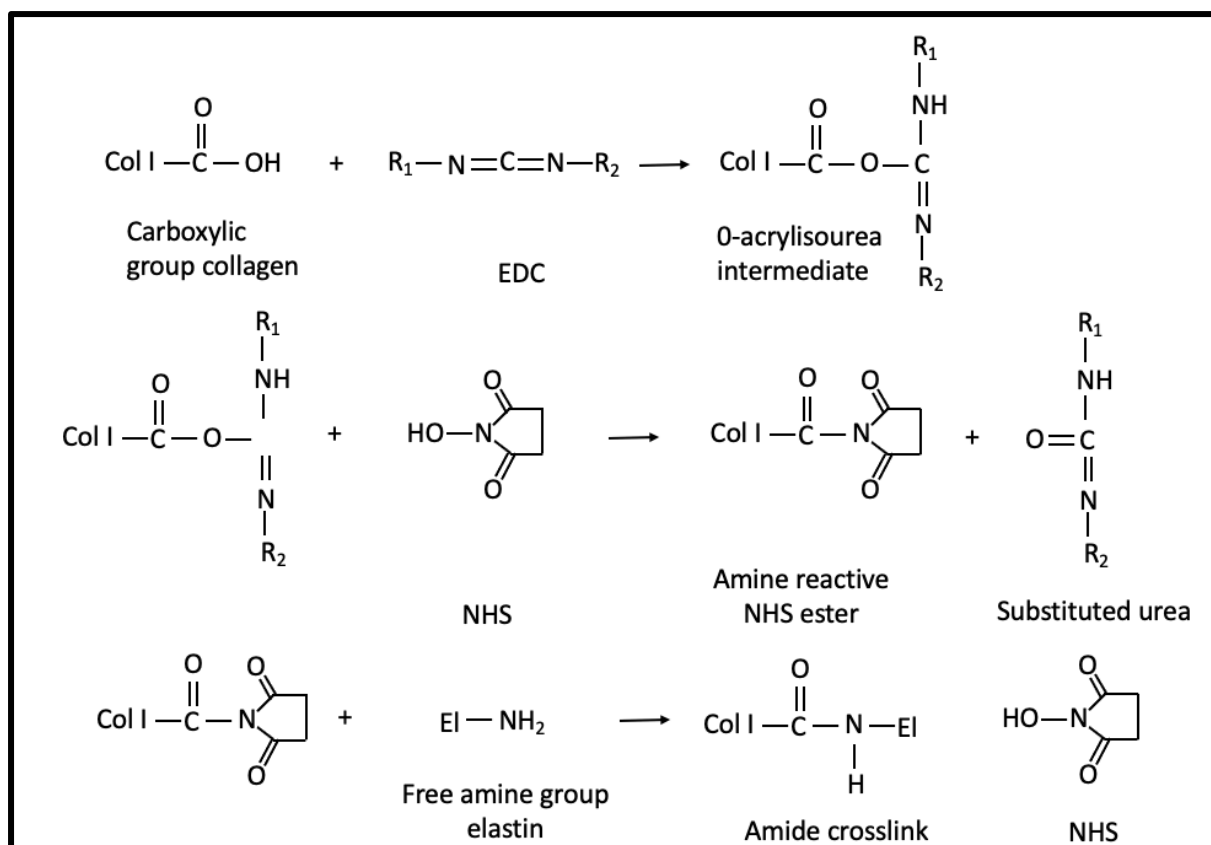


Figure 4.19: Proposed EDC/NHS cross-linking mechanism of elastin to collagen

However, Daamen *et al.* Observed amine group content in scaffold with varying ratios of collagen to elastin and found that 100% collagen scaffolds had 281 ± 7 nmol/mg of amine group content whilst scaffolds of 1:1 ratio collagen to elastin had only 147 ± 12 nmol/mg of amine group content[26]. This is almost half the amount of amine groups for half the amount of collagen suggesting that elastin has very few amine groups available to be forming cross-links with collagen in the presence of EDC/NHS through the mechanism suggested above.

4.4.2. Scanning Electron Microscopy

The SEM examination of the fibre surfaces showed rough surfaces with uniaxially aligned ridges (fibrils). In figure 4.13a,, a closer view of a fibre, it can be seen that the fibrils within the fibres are crimped, which allow the fibres to expand (swell) when hydrated. These results are similar to other studies of collagen fibres [27]–[31]. The interior view of the fibrillar

alignment of the fibres provided by the fractured sample in Figure 4.14 demonstrates how the fibres were made *in vitro* from an assembly of fibrils. Analysis of this image using ImageJ, and the fibrils found within, allowed calculations of fibril diameter, which was found to range around 230nm-400nm. This is not too far from literature. Beisbayeva *et al.* reported the collagen fibril diameter of healthy bovine ACL to range between 20-320nm (weighted mean 147.6nm) and healthy bovine PCL to range between 80-1000nm (weighted mean 617nm), and another study which found the fibril diameter of the suspensory ligament in horses at various ages to range from 100-300nm[32], [33].

4.4.3. Swelling behaviour of Type I collagen fibres

The water absorption (water uptake ratio) decreased with cross-linking. The water absorption of glassy polymers is generally explained by the dual mode sorption theory, including the concept of microvoids [34]–[36]. According to this theory, the sorption isotherm for glassy polymer can be split into two parts: ordinary sorption, which is represented by Henry's law; and the collection of water in pre-existing holes, which is represented by the Langmuir isotherm.

When the fibres are added to the PBS, the first water molecules which diffuse into the network will hydrate the most polar, hydrophilic groups “*primary bound water*”. As the polar groups are hydrated, the network swells to expose the hydrophobic groups which interact with the water leading to “*secondary bound water*”. After the polar and hydrophobic sites have interacted with and bound water molecules, the network will imbibe additional water “*bulk water*”. This additional swelling of collagen is governed by the osmotic pressure differences arising between the protein phase and the external solution as a result of the formation of protein salts and by the cohesion of the protein, i.e. the intermolecular forces which opposes

the osmotic driving force of the network chain towards infinite dilution. This additional water absorption continues until equilibrium is reached between these opposing forces[37], [38].

The decrease in swelling observed after cross-linking of collagen fibres suggest that hydrophilic sites such as lysine were consumed by the cross-linking process, thus reducing the “*primary bound water*”. Additionally, the decrease of water uptake of cross-linked samples XL and EXL is likely also associated with the increased stiffness as intermolecular forces are greater, therefore, less water is needed to reach equilibrium of osmotic swelling. Furthermore, cross-linking forms a close polymer network structure which is not easily swollen by water absorption. Less water can bind to the cross-linked fibres than the uncross-linked fibres, therefore, they do not swell as much.

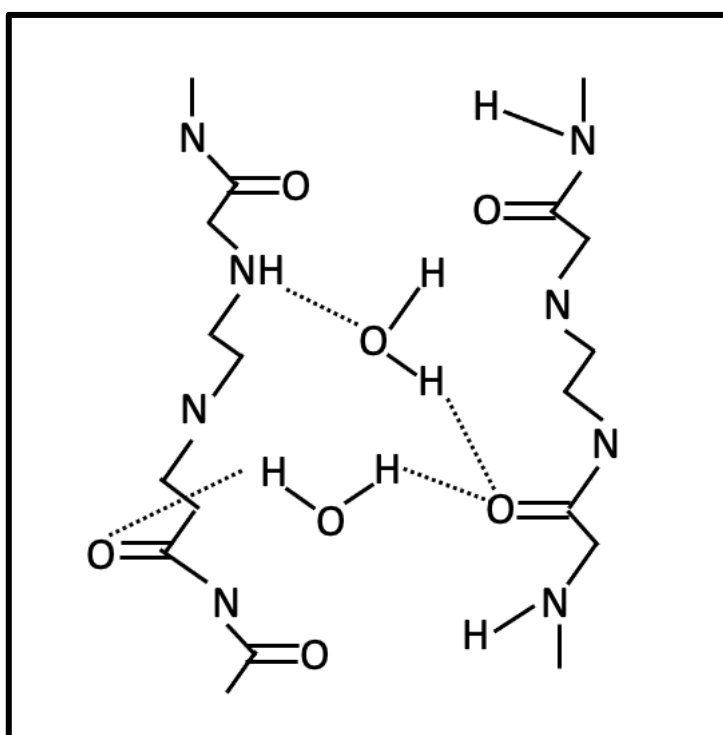


Figure 4.20: Water molecules binding in the collagen.

Elastin cross-linked fibres were of larger diameters in their dry form compared to their cross-linked counterparts. However, once the fibres were rehydrated there was no significant

difference in diameters. While, collagen is relatively homogenous as three single peptide chains form a collagen triple helix and is regarded as a largely hydrophobic molecule, elastin is structurally heterogeneous with hydrophilic segments containing some α -helix and the hydrophobic segments being mostly of a random coil design [39]–[41]. After cross-linking fibres were left to air dry, however, it is the disordered structure and hydrophilic segments which could be the cause of elastin cross-linked fibres exhibiting a greater diameter in their dry state as is likely that the samples were retaining water after being left to air dry. Therefore, water uptake and swelling ratios were presented as being lower.

4.4.4. Self-deflection

In ideal elastic conditions, the flexural and tensile modulus of a material should be similar since they are both representations of mechanical strain. It is necessary to test these collagen fibres in a hydrated environment as *in vivo* they will be in a hydrated state. The self-deflection study produced bending modulus results ranging from 15-40 MPa which is in line with mechanical property results obtained in other studies for fabricated collagen fibres[30], [42], [43]. Furthermore, Graham et al. [77] stretched *in vitro*-assembled type I collagen fibrils from humans with a diameter of ~ 30 nm the Young's modulus was determined to be 32 MPa [44]. Whilst, Sasaki and Odajima studied the stress-strain behaviour of collagen fibrils in bovine Achilles tendon and found that the elastic modulus of collagen fibrils in the hydrated state was estimated to be around 43MPa [45]. Therefore, obtained modulus results are also in line with results obtained in other studies for native soft fibrous tissue.

Results presented Table 4.3, show that cross-linked fibres were two-folds stiffer than uncross-linked fibres, and agrees with previous discussion (section 2.5.3). Cross-linking forms intramolecular links within collagen fibrils (figure 2.10) and is what gives fibres their increased

mechanical properties. Although elastin cross-linked fibres had a greater modulus, this was found to not be significantly different from that of collagen only cross-linked fibres and cannot confirm that elastin cross-linking affects mechanical properties. However, a study by Daamen *et al.*[26] suggested that elastin does not bond with collagen intramolecularly, which is what affects mechanical properties. Instead, the ability of elastin cross-linked fibres to retain water after being left to air dry is an indication that elastin could instead be binding to collagen intermolecularly as fibre hydration is driven by intermolecular bonds (Figure 2.9).

4.4.5. Tensile

Figure 4.21 shows how the matrix failed before the fibres and that the fibres would continue to stretch far beyond the limit of the matrix. Even after the matrix fails the fibres undergo densification, this is most evident in the samples with the bundles which can withstand more stress before another fibre fails. This allows for the samples to withstand stresses for longer before ultimate failure. However, this is not of great interest for tissue engineering purposes as the scaffolds should ideally not experience forces that push them beyond their elastic region, therefore stiffness and strength are of greater importance.



Figure 4.21: Photograph of fibre-reinforced collagen scaffold being stretched. Matrix has failed, fibre is still being loaded.

Further, during the loading of the scaffolds with the bundle of fibres, there are a few samples with a kink in the curve, and a sudden drop of the force. Rather than being caused by the failure of a fibre it is likely caused by the fibres slipping past each other, a sudden release of energy. This makes the loading of these samples unsmooth and unpredictable which are not ideal characteristics for tissue scaffolds.

The embedded length for which the fibre fails is known as critical length (l_c) i.e. for lengths below l_c the complete interfacial debonding occurs while at higher lengths, fibre failure occurs without debonding of the fibre/matrix interface. Scaffolds containing fibres puckered after cross-linking due to a mismatch of swelling ratios, however, this confirmed that the length of the fibres embedded were greater than the critical length and that there was interfacial bonding between the matrix and fibres. The interfacial shear strength directly influences the mechanical behaviour of the composite [46], therefore it is expected that the fibres will effectively transfer

their mechanical properties to the matrix. This adhesion is likely attributed to the textured surface of the fibres (ridges).

The theoretical values for scaffolds reinforced by bundles of fibres were significantly lower than the calculated values. This is likely caused by poor bonding of fibres to the matrix because of reduced surface area. The fibres in the middle of the bundles had less surface area exposed to bind with the matrix and therefore were unable to effectively transfer their mechanical strength. Embedding the scaffolds one by one and ensuring that they do not come into contact with one another could be a way to improve the interfacial bonding and improve the performance of the bundle-reinforced scaffolds.

4.5. Summary and Future Work

This chapter focused on the fabrication of collagen fibres and their use in creating reinforced composite scaffolds. The main findings were as follows:

- ⇒ The successful fabrication of uniformly cylindrical collagen fibres and composite scaffolds with strong fibre-matrix interfacial bonding.
- ⇒ SEM used to examine the microstructure of these formed fibres, revealed that fibres were formed from an assembly of uniaxially aligned fibrils.
- ⇒ Whilst cross-linking reduced solvent uptake, it significantly increased elastic modulus.
- ⇒ Single fibres embedded into porous scaffolds were effective in transferring their mechanical properties to the matrix. However, bundles of fibres were unable to reach values similar to theoretically obtained values due to poor bonding of fibres in the centre of the bundle to the matrix.

As mentioned above (Section 4.1), although the presence of elastin in ligaments is scarce, when stretched along the primary collagen axis, elastin supports a relatively high percentage of loads [10]. Although this conclusion couldn't be drawn from the work presented in this chapter, there are signs that elastin was contributing to the behaviour of the fibres which was apparent in their interaction with water. The process of elastin production does not have a very high yield and due to restraints in the availability of consumables caused by the pandemic and the United Kingdom's departure from the single market, it was not possible to try reinforcing scaffolds with elastin-infiltrated fibres. As elastin has been shown to have a positive impact on the cellular response of scaffolds [47] [48], and with results of interest presented in this chapter this would be the next steps to take this work forward.

4.6. References

- [1] D. W. Weisgerber, D. O. Kelkhoff, S. R. Caliarì, and B. a. C. Harley, ‘The impact of discrete compartments of a multi-compartment collagen-GAG scaffold on overall construct biophysical properties’, *J Mech Behav Biomed Mater*, vol. 28, pp. 26–36, Dec. 2013, doi: 10.1016/j.jmbbm.2013.07.016.
- [2] F. J. O’Brien, B. A. Harley, I. V. Yannas, and L. J. Gibson, ‘The effect of pore size on cell adhesion in collagen-GAG scaffolds’, *Biomaterials*, vol. 26, no. 4, pp. 433–441, Feb. 2005, doi: 10.1016/j.biomaterials.2004.02.052.
- [3] C. M. Murphy, M. G. Haugh, and F. J. O’Brien, ‘The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering’, *Biomaterials*, vol. 31, no. 3, pp. 461–466, Jan. 2010, doi: 10.1016/j.biomaterials.2009.09.063.
- [4] S. R. Caliarì, D. W. Weisgerber, W. K. Grier, Z. Mahmassani, M. D. Boppart, and B. A. C. Harley, ‘Collagen Scaffolds Incorporating Coincident Gradations of Instructive Structural and Biochemical Cues for Osteotendinous Junction Engineering’, *Adv Healthc Mater*, vol. 4, no. 6, pp. 831–837, Apr. 2015, doi: 10.1002/adhm.201400809.
- [5] B. A. Harley, J. H. Leung, E. C. C. M. Silva, and L. J. Gibson, ‘Mechanical characterization of collagen-glycosaminoglycan scaffolds’, *Acta Biomater*, vol. 3, no. 4, pp. 463–474, Jul. 2007, doi: 10.1016/j.actbio.2006.12.009.
- [6] C. N. Maganaris and J. P. Paul, ‘In vivo human tendon mechanical properties’, *J Physiol*, vol. 521, no. Pt 1, pp. 307–313, Nov. 1999, doi: 10.1111/j.1469-7793.1999.00307.x.
- [7] C. T. Thorpe, C. P. Udeze, H. L. Birch, P. D. Clegg, and H. R. C. Screen, ‘Specialization of tendon mechanical properties results from interfascicular differences’, *J R Soc Interface*, vol. 9, no. 76, pp. 3108–3117, Nov. 2012, doi: 10.1098/rsif.2012.0362.

- [8] C. T. Thorpe, R. J. F. Stark, A. E. Goodship, and H. L. Birch, 'Mechanical properties of the equine superficial digital flexor tendon relate to specific collagen cross-link levels', *Equine Vet J Suppl*, no. 38, pp. 538–543, Nov. 2010, doi: 10.1111/j.2042-3306.2010.00175.x.
- [9] Y. P. Kato, D. L. Christiansen, R. A. Hahn, S.-J. Shieh, J. D. Goldstein, and F. H. Silver, 'Mechanical properties of collagen fibres: a comparison of reconstituted and rat tail tendon fibres', *Biomaterials*, vol. 10, no. 1, pp. 38–42, Jan. 1989, doi: 10.1016/0142-9612(89)90007-0.
- [10] H. B. Henninger, W. R. Valdez, S. A. Scott, and J. A. Weiss, 'Elastin Governs the Mechanical Response of Medial Collateral Ligament Under Shear and Transverse Tensile Loading', *Acta Biomater*, vol. 25, pp. 304–312, Oct. 2015, doi: 10.1016/j.actbio.2015.07.011.
- [11] X. Li, Q. Feng, Y. Jiao, and F. Cui, 'Collagen-based scaffolds reinforced by chitosan fibres for bone tissue engineering', *Polymer International*, vol. 54, no. 7, pp. 1034–1040, 2005, doi: 10.1002/pi.1804.
- [12] S. Chand, 'Review Carbon fibers for composites', *Journal of Materials Science*, vol. 35, no. 6, pp. 1303–1313, Mar. 2000, doi: 10.1023/A:1004780301489.
- [13] P. Wambua, J. Ivens, and I. Verpoest, 'Natural fibres: can they replace glass in fibre reinforced plastics?', *Composites Science and Technology*, vol. 63, no. 9, pp. 1259–1264, Jul. 2003, doi: 10.1016/S0266-3538(03)00096-4.
- [14] T. Sathishkumar, S. Satheeshkumar, and J. Naveen, 'Glass fiber-reinforced polymer composites – a review', *Journal of Reinforced Plastics and Composites*, vol. 33, no. 13, pp. 1258–1275, Jul. 2014, doi: 10.1177/0731684414530790.
- [15] X. Li and Q. Feng, 'Porous poly-L-lactic acid scaffold reinforced by chitin fibers', *Polym. Bull.*, vol. 54, no. 1, pp. 47–55, May 2005, doi: 10.1007/s00289-005-0364-7.

- [16] X. Wang, Y. Yan, M. J. Yost, S. A. Fann, S. Dong, and X. Li, ‘Nanomechanical characterization of micro/nanofiber reinforced type I collagens’, *J Biomed Mater Res A*, vol. 83, no. 1, pp. 130–135, Oct. 2007, doi: 10.1002/jbm.a.31207.
- [17] Sonia Iftekhhar, ‘Isolation of Fibrillar Elastin Gel (FEG) and its Application in Heart Valve Tissue Engineering’, University of Oxford, 2020.
- [18] Samuel Halliday, ‘Tissue Engineering with Collagen Fibres’, University of Oxford, 2011.
- [19] J. Thomas, D. F. Elsdén, and S. M. Partridge, ‘Degradation Products from Elastin: Partial Structure of Two Major Degradation Products from the Cross-linkages in Elastin’, *Nature*, vol. 200, no. 4907, Art. no. 4907, Nov. 1963, doi: 10.1038/200651a0.
- [20] S. M. Partridge, D. F. Elsdén, J. Thomas, A. Dorfman, A. Telser, and P. L. Ho, ‘Biosynthesis of the desmosine and isodesmosine cross-bridges in elastin’, *Biochem J*, vol. 93, no. 3, pp. 30C-33C, Dec. 1964.
- [21] K. A. Piez, G. R. Martin, A. H. Kang, and P. Bornstein, ‘Heterogeneity of the α Chains of Rat Skin Collagen and Its Relation to the Biosynthesis of Cross-Links*’, *Biochemistry*, vol. 5, no. 12, pp. 3813–3820, Dec. 1966, doi: 10.1021/bi00876a011.
- [22] P. Bornstein, A. H. Kang, and K. A. Piez, ‘The nature and location of intramolecular cross-links in collagen.’, *Proceedings of the National Academy of Sciences*, vol. 55, no. 2, pp. 417–424, Feb. 1966, doi: 10.1073/pnas.55.2.417.
- [23] M. Rojkind and H. Juárez, ‘The nature of the collagen defect in lathyrism’, *Biochemical and Biophysical Research Communications*, vol. 25, no. 4, pp. 481–486, Nov. 1966, doi: 10.1016/0006-291X(66)90231-2.
- [24] E. J. Miller, G. R. Martin, C. Mecca, and K. Piez, ‘The Biosynthesis of Elastin Cross-links’, *Journal of Biological Chemistry*, vol. 240, pp. 3623–3627, Sep. 1965, doi: 10.1016/S0021-9258(18)97189-9.

- [25] B. L. O'Dell, D. F. Elsdon, J. Thomas, S. M. Partridge, R. H. Smith, and R. Palmer, 'Inhibition of the biosynthesis of the cross-links in elastin by a lathyrogen', *Nature*, vol. 209, no. 5021, pp. 401–402, Jan. 1966, doi: 10.1038/209401a0.
- [26] W. F. Daamen *et al.*, 'Preparation and evaluation of molecularly-defined collagen–elastin–glycosaminoglycan scaffolds for tissue engineering', *Biomaterials*, vol. 24, no. 22, pp. 4001–4009, Oct. 2003, doi: 10.1016/S0142-9612(03)00273-4.
- [27] G. D. Pins, D. L. Christiansen, R. Patel, and F. H. Silver, 'Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties', *Biophys J*, vol. 73, no. 4, pp. 2164–2172, Oct. 1997, doi: 10.1016/S0006-3495(97)78247-X.
- [28] D. I. Zeugolis, G. R. Paul, and G. Attenburrow, 'Cross-linking of extruded collagen fibers--a biomimetic three-dimensional scaffold for tissue engineering applications', *J Biomed Mater Res A*, vol. 89, no. 4, pp. 895–908, Jun. 2009, doi: 10.1002/jbm.a.32031.
- [29] G. D. Pins, E. K. Huang, D. L. Christiansen, and F. H. Silver, 'Effects of static axial strain on the tensile properties and failure mechanisms of self-assembled collagen fibers', *Journal of Applied Polymer Science*, vol. 63, no. 11, pp. 1429–1440, 1997, doi: 10.1002/(SICI)1097-4628(19970314)63:11<1429::AID-APP5>3.0.CO;2-O.
- [30] D. Enea *et al.*, 'Extruded collagen fibres for tissue engineering applications: effect of crosslinking method on mechanical and biological properties', *J Mater Sci Mater Med*, vol. 22, no. 6, pp. 1569–1578, Jun. 2011, doi: 10.1007/s10856-011-4336-1.
- [31] D. I. Zeugolis, R. G. Paul, and G. Attenburrow, 'Engineering extruded collagen fibers for biomedical applications', *Journal of Applied Polymer Science*, vol. 108, no. 5, pp. 2886–2894, 2008, doi: 10.1002/app.27208.
- [32] D. A. D. Parry, A. S. Craig, and G. R. G. Barnes, 'Tendon and Ligament from the Horse: An Ultrastructural Study of Collagen Fibrils and Elastic Fibres as a Function of Age',

- Proceedings of the Royal Society of London. Series B, Biological Sciences*, vol. 203, no. 1152, pp. 293–303, 1978.
- [33] Z. Beisbayeva, A. Zhanbassynova, G. Kulzhanova, F. Mukasheva, and C. Eriskien, ‘Change in Collagen Fibril Diameter Distribution of Bovine Anterior Cruciate Ligament upon Injury Can Be Mimicked in a Nanostructured Scaffold’, *Molecules*, vol. 26, no. 5, Art. no. 5, Jan. 2021, doi: 10.3390/molecules26051204.
- [34] M. S. Majerus, D. S. Soong, and J. M. Prausnitz, ‘Experimental measurements and monte-carlo simulation of water diffusion into epoxy matrices’, *Journal of Applied Polymer Science*, vol. 29, no. 8, pp. 2453–2466, 1984, doi: 10.1002/app.1984.070290803.
- [35] W. R. Vieth, J. M. Howell, and J. H. Hsieh, ‘Dual sorption theory’, *Journal of Membrane Science*, vol. 1, pp. 177–220, Jan. 1976, doi: 10.1016/S0376-7388(00)82267-X.
- [36] R. J. Pace and A. Datyner, ‘Model of sorption of simple molecules in polymers’, *Journal of Polymer Science: Polymer Physics Edition*, vol. 18, no. 5, pp. 1103–1124, 1980, doi: 10.1002/pol.1980.180180515.
- [37] M. Rizwan *et al.*, ‘pH Sensitive Hydrogels in Drug Delivery: Brief History, Properties, Swelling, and Release Mechanism, Material Selection and Applications’, *Polymers (Basel)*, vol. 9, no. 4, p. 137, Apr. 2017, doi: 10.3390/polym9040137.
- [38] A. S. Hoffman, ‘Hydrogels for biomedical applications’, *Advanced Drug Delivery Reviews*, vol. 54, no. 1, pp. 3–12, Jan. 2002, doi: 10.1016/S0169-409X(01)00239-3.
- [39] I. Streeter and N. H. de Leeuw, ‘A molecular dynamics study of the interprotein interactions in collagen fibrils’, *Soft Matter*, vol. 7, no. 7, pp. 3373–3382, Apr. 2011, doi: 10.1039/C0SM01192D.
- [40] S. M. Mithieux and A. S. Weiss, ‘Elastin’, *Adv. Protein Chem.*, vol. 70, pp. 437–461, 2005, doi: 10.1016/S0065-3233(05)70013-9.

- [41] G. C. Na, L. J. Phillips, and E. I. Freire, ‘In vitro collagen fibril assembly: thermodynamic studies’, *Biochemistry*, vol. 28, no. 18, pp. 7153–7161, Sep. 1989, doi: 10.1021/bi00444a004.
- [42] D. I. Zeugolis, R. G. Paul, and G. Attenburrow, ‘The influence of a natural cross-linking agent (*Myrica rubra*) on the properties of extruded collagen fibres for tissue engineering applications’, *Materials Science and Engineering: C*, vol. 30, no. 1, pp. 190–195, Jan. 2010, doi: 10.1016/j.msec.2009.09.017.
- [43] D. I. Zeugolis, R. G. Paul, and G. Attenburrow, ‘Post-self-assembly experimentation on extruded collagen fibres for tissue engineering applications’, *Acta Biomater*, vol. 4, no. 6, pp. 1646–1656, Nov. 2008, doi: 10.1016/j.actbio.2008.05.015.
- [44] J. S. Graham, A. N. Vomund, C. L. Phillips, and M. Grandbois, ‘Structural changes in human type I collagen fibrils investigated by force spectroscopy’, *Exp Cell Res*, vol. 299, no. 2, pp. 335–342, Oct. 2004, doi: 10.1016/j.yexcr.2004.05.022.
- [45] N. Sasaki and S. Odajima, ‘Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy’, *Journal of Biomechanics*, vol. 29, no. 9, pp. 1131–1136, Sep. 1996, doi: 10.1016/0021-9290(96)00024-3.
- [46] S. N. Monteiro *et al.*, ‘Interfacial Shear Strength in Lignocellulosic Fibers Incorporated Polymeric Composites’, in *Cellulose Fibers: Bio- and Nano-Polymer Composites: Green Chemistry and Technology*, S. Kalia, B. S. Kaith, and I. Kaur, Eds., Berlin, Heidelberg: Springer, 2011, pp. 241–262. doi: 10.1007/978-3-642-17370-7_9.
- [47] D. V. Bax, H. E. Smalley, R. W. Farndale, S. M. Best, and R. E. Cameron, ‘Cellular response to collagen-elastin composite materials’, *Acta Biomaterialia*, vol. 86, pp. 158–170, Mar. 2019, doi: 10.1016/j.actbio.2018.12.033.

- [48] J. J. Perdomo *et al.*, ‘Interaction between cells and elastin fibers: an ultrastructural and immunocytochemical study’, *J Cell Physiol*, vol. 158, no. 3, pp. 451–458, Mar. 1994, doi: 10.1002/jcp.1041580309.

Chapter 5 - Porous Scaffolds

5.1. Introduction

As shown in Chapter 3, the ligament-bone enthesis consists of 4 regions with different compositions and mechanical functions. Therefore, each of these regions needs to be tackled individually before the development of the more complicated multi-phase scaffolds. The development and use of highly porous, freeze-dried scaffolds have shown great promise in both *in vitro* and *in vivo* studies [1], [2], [3], [4], [5], [6], [7]. The success of these scaffolds depends on the ability of the implanted cells to attach to the surrounding environment and to stimulate angiogenesis (new tissue formation) [8]. Therefore, the rationale behind mimicking the compositional and structural organisation of the main organic and inorganic components of tissue in a single scaffold is to create an environment that more closely resembles the natural Extracellular Matrix (ECM), to guide cell response and stimulate tissue formation [9]. Therefore, this chapter will cover the fabrication of pure collagen I, collagen II + chondroitin sulphate (CS), and collagen I + hydroxyapatite (HAp) porous scaffolds to mimic the ECM of the ligament, noncalcified fibrocartilage and the two calcified tissues, respectively. Furthermore, this chapter will discuss the microstructure, swelling, degradation characteristics and mechanical properties of the developed scaffolds to understand their feasibility for enthesis regeneration.

5.2. Manufacture

5.2.1. Preparation of Collagen-Based Suspensions from Monomeric

Atelocollagen

Type I and Type II atelocollagen monomers from bovine skin and Type I atelocollagen monomers from amnion were donated by Prof. David Brand at the Veterans Affairs Medical Center. Tris-buffered saline (TBS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich UK. Monomeric collagen-based scaffolds were prepared based on previous studies from members of our research group [2], [7], [8], [10].

A 1% wt/v monomeric collagen dispersion was prepared by dissolving Type I or Type II lyophilised monomeric atelocollagen from bovine skin (0.5g), in acetic acid solution (50 ml, pH=3.2). The suspensions were homogenised with a conventional blender to create a uniform dispersion.

Collagen monomers were moved to a natural salt solution condition for fibrillogenesis. 50mL TBS solution (50 mM tris-(hydroxymethyl)-aminomethane, 150 mM sodium chloride, pH=7.5) was added to the collagen dispersion. The pH of the combined dispersion was adjusted from 7.2 to 7.5. The dispersion was then put into a 37°C incubator for 1 hour.

Fibrillar Collagen was crosslinked in 200 mL deionised water with 60 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 24 mM N-hydroxysuccinimide (NHS). After reacting overnight, the collagens were washed twice with 0.1 M Disodium Phosphate (Na_2HPO_4) and twice in deionised water. The washed collagen solution was stored at 4°C.

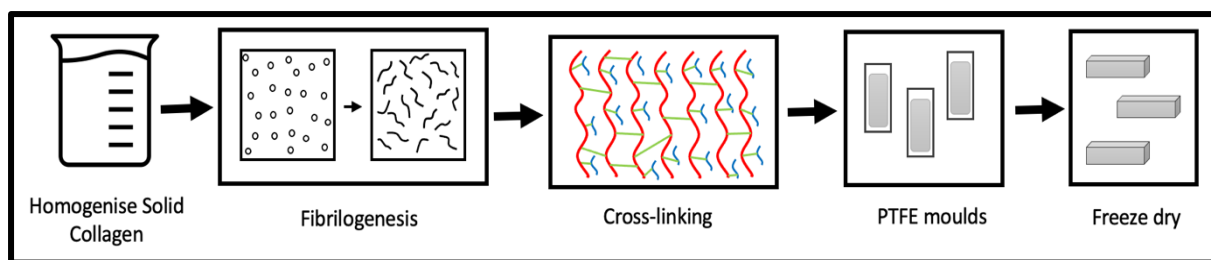


Figure 5.1. Schematic of collagen-based porous scaffold fabrication.

5.2.1.1. Bone and Fibrocartilage Layers

Hydroxyapatite (2 g, HAP: Collagen = 4:1 for bone, and, 1.17g, HAP: Collagen =7:3 for Calcified fibrocartilage) was mixed with washed type I collagen suspension.

5.2.1.2. Uncalcified Fibrocartilage Layer

Chondroitin sulphate (0.5g, CS: Collagen =1:1) was added to type II collagen suspension during the cross-linking process.

5.2.2. Monolayered Scaffold Fabrication

For monolayer scaffold fabrication, individual collagen solutions were cast into rectangular PTFE moulds (40mmx10mmx10mm) using a syringe, frozen at -20°C and then lyophilised to obtain the porous scaffolds.

5.2.3. Bi-layered Scaffold Fabrication

For integrated gradient bi-layered scaffolds, the first layer was syringed into the PTFE moulds and frozen at -20°C. The moulds were then removed from the freezer and left at room temperature for 5mins to allow for the surface of the collagen disc to liquefy before a second layer was poured. Moulds were frozen again at -20°C before being lyophilised to obtain bi-layered porous scaffolds.

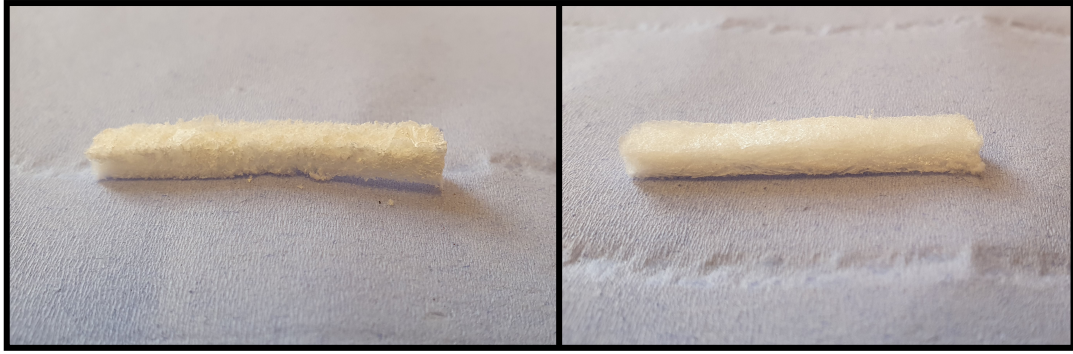


Figure 5.2: Collagen-based bi-layered porous scaffolds, (A) Col I (top) and Col II + CS (bottom), (B) Col II + CS (top) and Col I + HAp (bottom).

5.3. Analysis Methods

5.3.1. Scanning Electron Microscopy

The morphology and microstructure of scaffolds were studied using scanning electron microscopy (SEM). Pore size is an important characteristic of membrane-based scaffolds as it determines the overall surface area available for cell attachment. Specimens were sectioned with a sharp blade, adhered to aluminium stubs using carbon tabs, and sputter coated with platinum (thickness 6nm) and then the cross-sections were examined. Samples were imaged using a Zeiss EVO operated at an accelerating voltage of 10 kV.

5.3.2. Porosity

The total porosity of scaffolds was determined using the dimensions and mass of the scaffold as follows. The dimensions (diameter and height) of all scaffolds of interest were measured using a digital calliper (MitutoyoTM, CD-6" CSX, Japan), and the mass was measured using a balance (Mettler ToledoTM, AB204, UK). The density of each scaffold, ρ_{scaffold} , was determined using

$$\rho_{\text{scaffold}} = \frac{\text{mass (g)}}{\text{volume (cm}^3\text{)}}$$

(Equation 5.1)

Porosity was determined using

$$\text{Porosity} = 1 - \frac{\rho_{\text{scaffold}}}{\rho_{\text{material}}}$$

(Equation 5.2)

Assuming ρ_{material} , is 1.35g/cm³ for collagen [11], 3.16 g/cm³ for hydroxyapatite [12] and 5.5g/cm³ for chondroitin sulphate [13].

Surface porosity was evaluated using SEM images of each scaffold type (n=5) with a minimum of 100 pores in the frame. Using ImageJ software, the images were converted to binary images and a thresholding operation was employed to identify the pixels belonging to the pore space. From this, the area of each pore was recorded and the area fraction of voids to pore walls was determined.

$$\text{Surface Porosity (\%)} = \frac{\text{Sum of pore areas } (\mu\text{m}^2)}{\text{Area of image } (\mu\text{m}^2)} \times 100$$

(Equation 5.3)

5.3.3. Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to investigate the changes in primary and secondary structures of the collagen during crosslinking with chondroitin sulphate and adding hydroxyapatite. The samples were analysed by infrared absorption mode (Varian FTIR spectrometer - UMA600, Palo Alto, CA) over the range of 4000-500 cm⁻¹ with a resolution of 2 cm⁻¹ and were recorded as averages of 64 scans for each sample, with the background scan removed. Numerical analysis was conducted on the spectra with essential FTIR (Operant, LLC. FL).

5.3.4. Water Uptake

Water uptake is an important indicator used to determine the suitability of a biomaterial for tissue engineering as it is a valuable property that will influence the hydrolytic degradation of the scaffolds [1] [2], transport of nutrients [16], whereas cell attachment and proliferation will

be favoured for materials with improved hydrophilicity [17]. For collagen-based scaffolds, water uptake is not only dependent on the amount of collagen in the scaffolds but also on cross-links and the pore characteristics of the scaffolds [18]. The water uptake ratio was determined by following the same protocol as previously outlined in Section 4.2.7. Briefly, scaffolds were weighed in their dry state and then immersed in 0.1M phosphate-buffered saline (PBS) at room temperature for 24 hrs before any excess PBS was drained and the hydrated scaffolds were weighed. The water uptake (G) was expressed using Equation 4.1 as a ratio of the mass of the PBS absorbed to the dry mass.

The volumetric water uptake (G_V) was expressed as a ratio of the mass of the PBS absorbed by the scaffolds to the dry volume of the scaffolds. Shown in Equation 5.4.

$$G_V = \frac{M_{wet} - M_{dry}}{V_{dry}} \times 100$$

(Equation 5.4)

Where V_{dry} is the volume of the dry scaffolds.

5.3.5. Self-Deflection

The mechanical behaviour of the porous scaffolds was characterised by self-deflection testing due to the low loads required. Self-deflection tests were carried out by following the same protocol as previously outlined in Section 4.2.10. Briefly, scaffolds hydrated scaffolds were allowed to deflect over a span of 20mm under their weight. Images captured their deflection and values for bending modulus were determined using Equation 4.4 for a beam bending under a uniform load (weight of the hydrated scaffolds).

5.3.6. Degradation Assays

The degradation of a material is important when considering its use. For many tissue engineering applications, the breakdown of the scaffold and regrowth of host tissue is the ideal case, and if possible, the degradation rate of the material will be tuned to match the regrowth [19].

Degradation assays were performed by cutting down collagen-based porous scaffolds into pieces of roughly 5mm x5mm and were immersed in 2ml of PBS with 0.2 mg/ml sodium azide (Sigma Aldrich, UK) overnight and left in a platform shaker (Jeio Tech BS-31, Medline Scientific, UK) to agitate mildly at 37°C. The following morning, samples were removed from the PBS, and left to drain for 1 minute on a metallic mech. The mass was recorded and taken as the original mass at 0 hrs ($mass_0$).

5.3.6.1. Non-Enzymatic Degradation

The samples were then placed back in 2ml of PBS with sodium azide and returned to the heated platform shaker. Samples were removed each week for 12 weeks, allowed to drain as above and measured for the new mass ($mass$) to determine the mass loss each week using the equation:

$$Mass\ Loss\ (\%) = \frac{mass_0 - mass_x}{mass_0}$$

(Equation 5.5)

5.3.6.2. Enzymatic Degradation

The samples were then either placed in PBS with 0.5mg/mL collagenase, chondroitinase 0.03mg/mL or both 0.5mg/mL collagenase and 0.03 mg/mL chondroitinase. The experiments were as follows:

Scaffold	Enzymes
Collagen I (Bovine Skin)	Collagenase
Collagen I + ½ HAp (Amnion)	Collagenase
Collagen I + ½ HAp (Bovine Skin)	Collagenase
Collagen I + HAp (Amnion)	Collagenase
Collagen I + HAp (Bovine Skin)	Collagenase
Collagen II + CS	Collagenase
Collagen II + CS	Collagenase + Chondroitinase
Collagen II + CS	Chondroitinase
Collagen II	Chondroitinase

Table 5.1. Table of enzymes used with each scaffold type.

The samples were removed at intervals (1hr, 3hrs, 6hrs, 12hrs and 24hrs), allowed to drain as above and measured for the new mass (mass) to determine the mass loss using Equation 5.5.

5.3.7. Statistical Analysis

Results were expressed as mean $\pm$ standard deviation. The results were compared with one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test. For values of $p < 0.05$, the difference was considered statistically significant.

5.4. Results

5.4.1. Microstructure of Scaffolds

5.4.1.1. Scanning Electron Microscopy

Open porous and interconnected networks are essential for cell nutrition, proliferation and migration for tissue vascularisation and the formation of new tissues [20]. The porous network of scaffolds is what supports cell invasion and metabolite biotransport; therefore, mean pore size is an essential aspect of scaffolds for tissue engineering. If pores are too small cells cannot migrate towards the centre of the construct limiting the diffusion of nutrients and removal of waste products. Conversely, if pores are too large there is a decrease in the specific surface area available limiting cell attachment [21], [22], [23], [24].

Scanning Electron Microscopy (SEM) was used to examine the internal structure of the scaffolds. SEM characterisation of the 4 layers is illustrated in Figure 5.3. Sem images were acquired on an EVO SEM system. The results show that each layer of the scaffolds had a high degree of interconnectivity and mixed size of pores, mainly ranging from 50-200µm. Therefore, the vast majority of pore sizes were within a range suitable for the growth of cells, such as fibroblasts, chondrocytes and osteoblasts [25], [26], [27], [28], [29], [30], [31], [32].

Pure collagen I scaffolds exhibited pores with more rounded defined morphology whereas collagen type II + CS scaffolds exhibited pores with discontinuous pore walls and spindly edges. However, this spindle-like morphology is not present for col II + CS layers in either of the multi-phase scaffolds (Figure 5.4). Instead, the col II + CS layers in the multi-phase scaffolds consist of parallel collagen layers connected by single collagen fibres.

Secondary pores which are perforations observed in the struts (pore walls) of the scaffold were observed for all collagen-based scaffolds, this highlights the interconnectivity of pores.

However, as the HAp content increases these secondary pores are less observable. The addition of HAp to the scaffolds provided a microscale surface roughness, which is known to enhance cell attachment [33].

SEM images of the multi-phase scaffolds show that layers are very well integrated. The interface between Col I and Col II + CS is very hard to detect and a soft transition is achieved. The interface between Col II + CS and Col I + HAp scaffolds was more evident however this is because the morphology of these layers is more different, however, it is clear that the layers have integrated. In their dry state, handling of the scaffolds did not cause delamination of the layers.

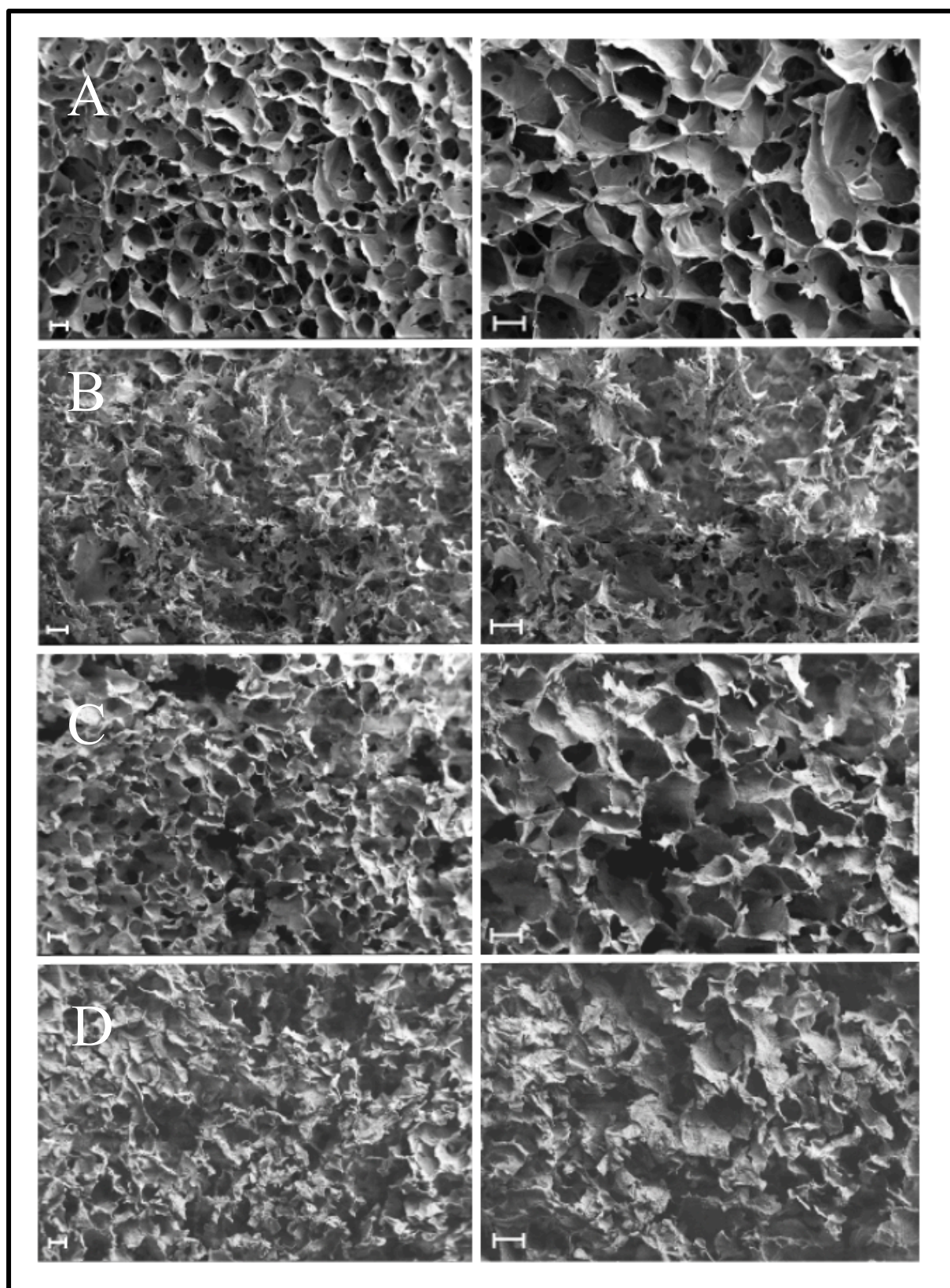


Figure 5.3. Morphology of the 4 collagen-based scaffolds (A) Col I (B) Col II + CS (C) Col I + $\frac{1}{2}$ HAp (D) Col I + HAp. Scale bars 100 μ m.

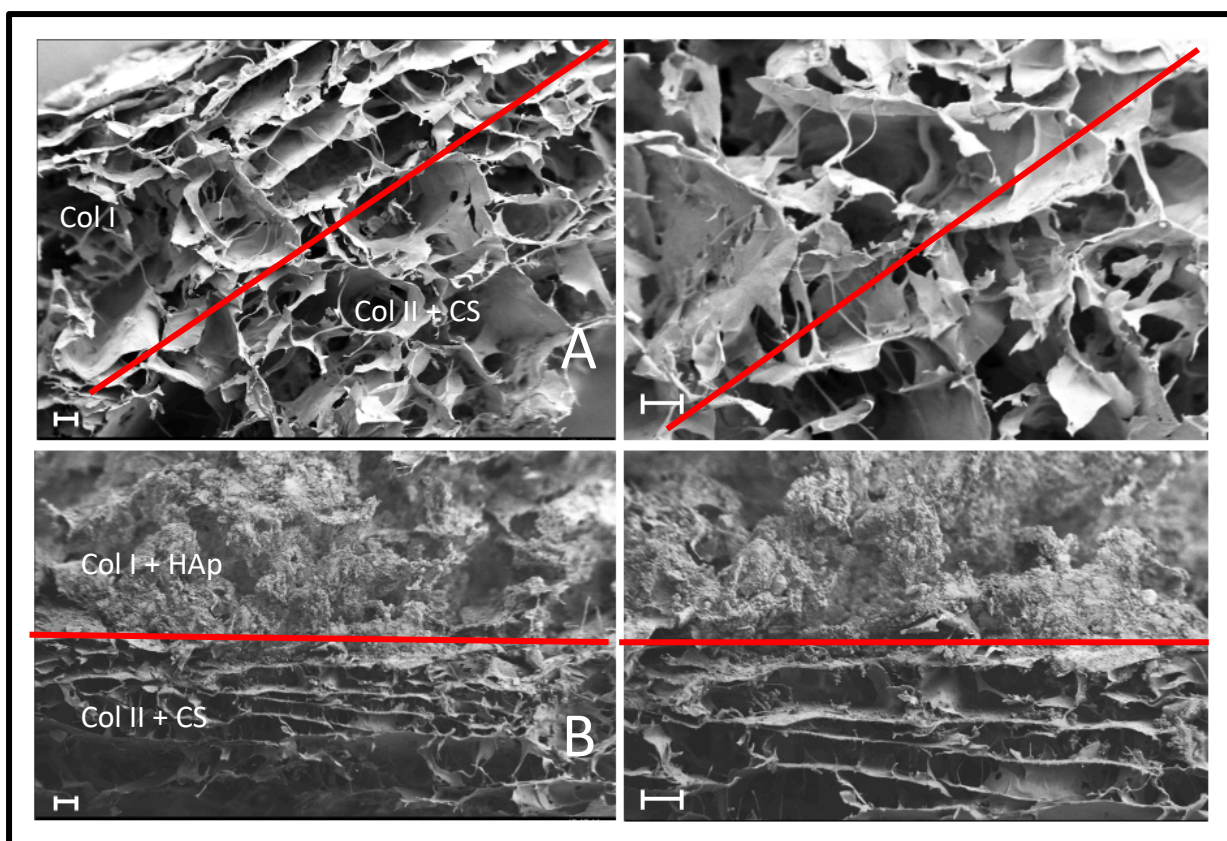


Figure 5.4: Morphology of multi-phase scaffolds (A) Col I and Col II + CS, (B) Col II + CS and Col I + HAp. Scale bars 100 μ m. Red lines indicate layer boundary.

5.4.1.2. Porosity

The total porosity, figure 5.5, of the scaffolds was calculated using the density of the scaffolds. All scaffolds tested were found to have a total porosity greater than 96% with Col II + CS presenting the greatest porosity at 99.1%, followed by Col I scaffolds at 98.9%, Col I + 1/2HAp at 97.2% and finally Col I + HAp at 96.3%. There is no significant difference in porosity between Col I and Col II + CS scaffolds.

The total porosity of multi-phase scaffolds is 98.79% and 98.16% for Col I and Col II + CS, and Col II + CS and Col I + HAp scaffolds, respectively. There is no significant difference in total porosity between these scaffolds.

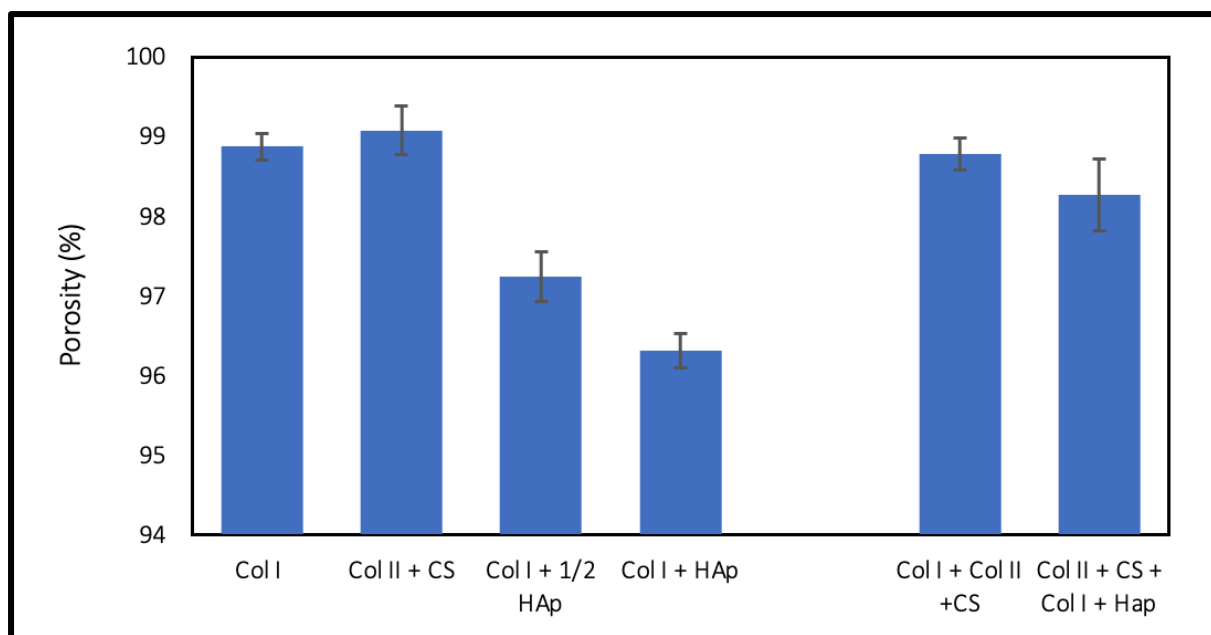


Figure 5.5. Total porosity based on scaffold density.

Surface porosity was evaluated from SEM scans converted to binary images (Figures 5.6 and 5.7) and the area fraction of voids to pore walls was determined. This fraction is reported as the total surface porosity in Figure 5.8.

Only pore areas of order $7000-800000\mu\text{m}^2$ were considered feasible for cell activity (see Section 5.3.1), therefore the percentage of the surface porosity that satisfied this parameter was determined. Surface porosity values were between 60-65% for all scaffolds made from collagen type I. Collagen type II scaffolds exhibited a significantly lower percentage of surface porosity at around 50%. 80-90 percent of all the identified voids satisfied the feasibility parameter. Two-phase scaffold presented surface porosity results which were in between those of its constituents.

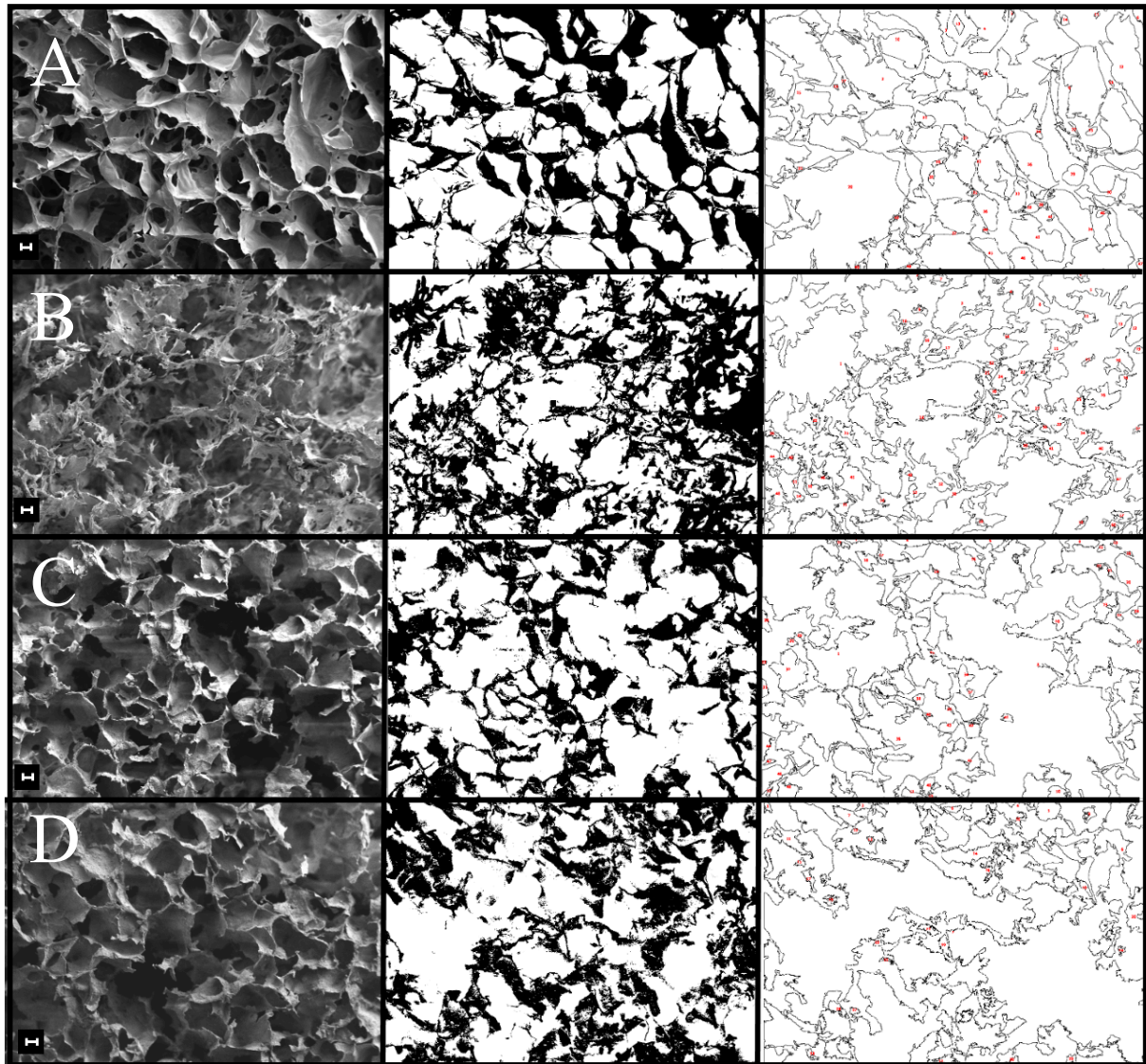


Figure 5.6. Conversion of SEM images into binary and line images for surface porosity analysis (A) Col I (B) Col II + CS (C) Col I + $\frac{1}{2}$ HAp (D) Col I + HAp. Scale bars 100 μ m.

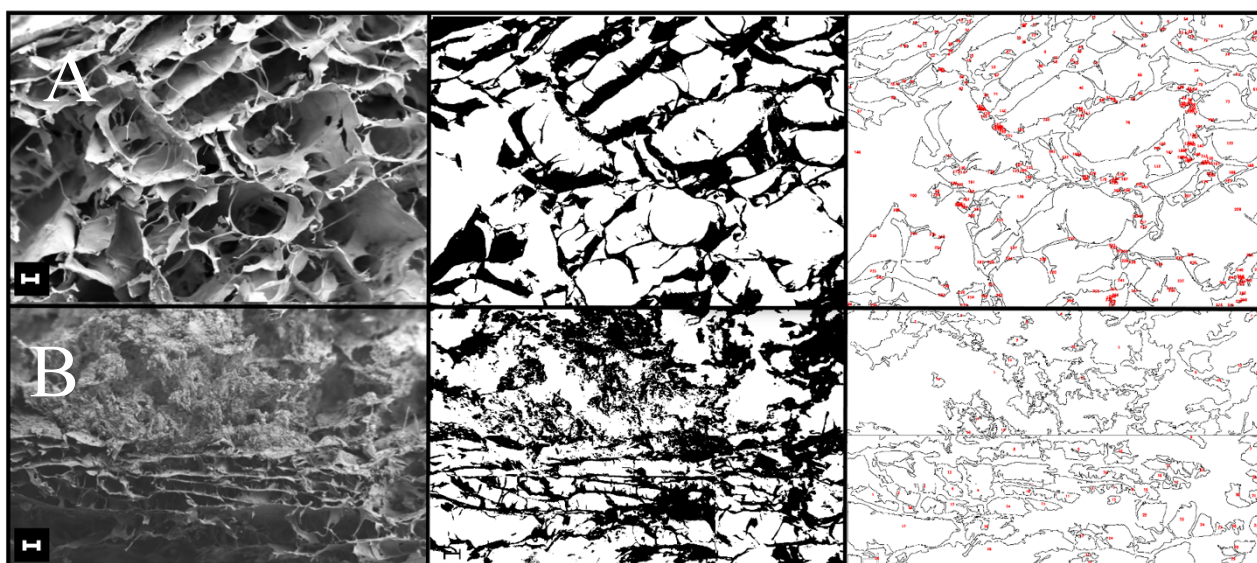


Figure 5.7. Conversion of SEM images into binary and line images for surface porosity analysis of two-phase collagen-based scaffolds (A) Col I and Col II + CS (B) Col II + CS and Col I + HAp. Scale bars 100 μ m.

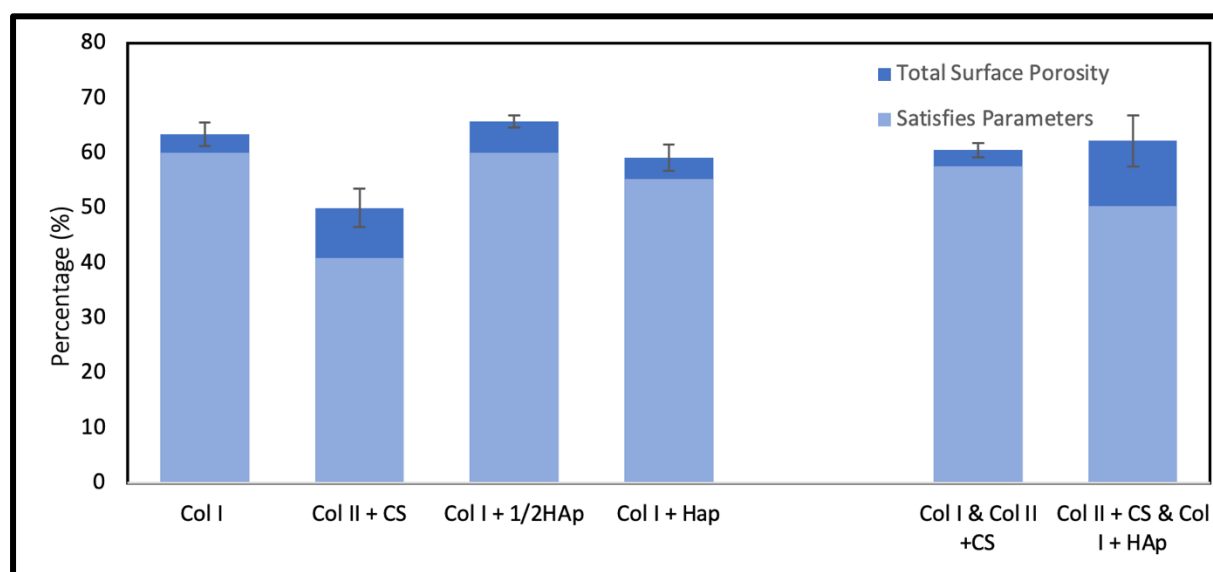


Figure 5.8. Surface porosity from each collagen-based scaffold with the percentage of those which satisfy pore area parameters.

5.4.2. Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy was used to research the structure of collagens and the configuration of the polypeptide chains within. The collagen structure is formed of a right-handed triple super-helical rod consisting of three almost identical polypeptide chains. Each polypeptide chain forms a left-handed helix and consists of repeating triplets (Gly-Xaa-Yaa). In that collagen structure, three polypeptide strands were held together in a helical configuration by a single interstrand N-H(Gly)...O=C(Xaa) hydrogen bond per triplet. (see Section 2.2). Therefore, the characteristic peaks of amide A, B, I, II and III bands arise from the vibration of the peptide groups and provide information about the secondary structure of polypeptides.

Cross-linking agents, EDC and NHS, have been widely accepted as coupling agents that target a molecule's carboxylic group and react with an amine group of another molecule to form an amide bond [34]. The formation of these bonds changes the peaks of any related band group in FTIR analysis. Therefore, this method was chosen to understand the protein structure of the materials used and to give an insight into their cross-linking mechanisms.

All spectra have been normalised against absorption at 2925 cm^{-1} , assigned to the CH_2 group as this peak remains unchanged during the crosslinking reaction [35]. The FTIR spectra of the 4 different layers of collagen scaffolds are present in Figure 5.9. The FTIR spectra of the 2 multi-phase scaffolds are presented in Figure 5.10.

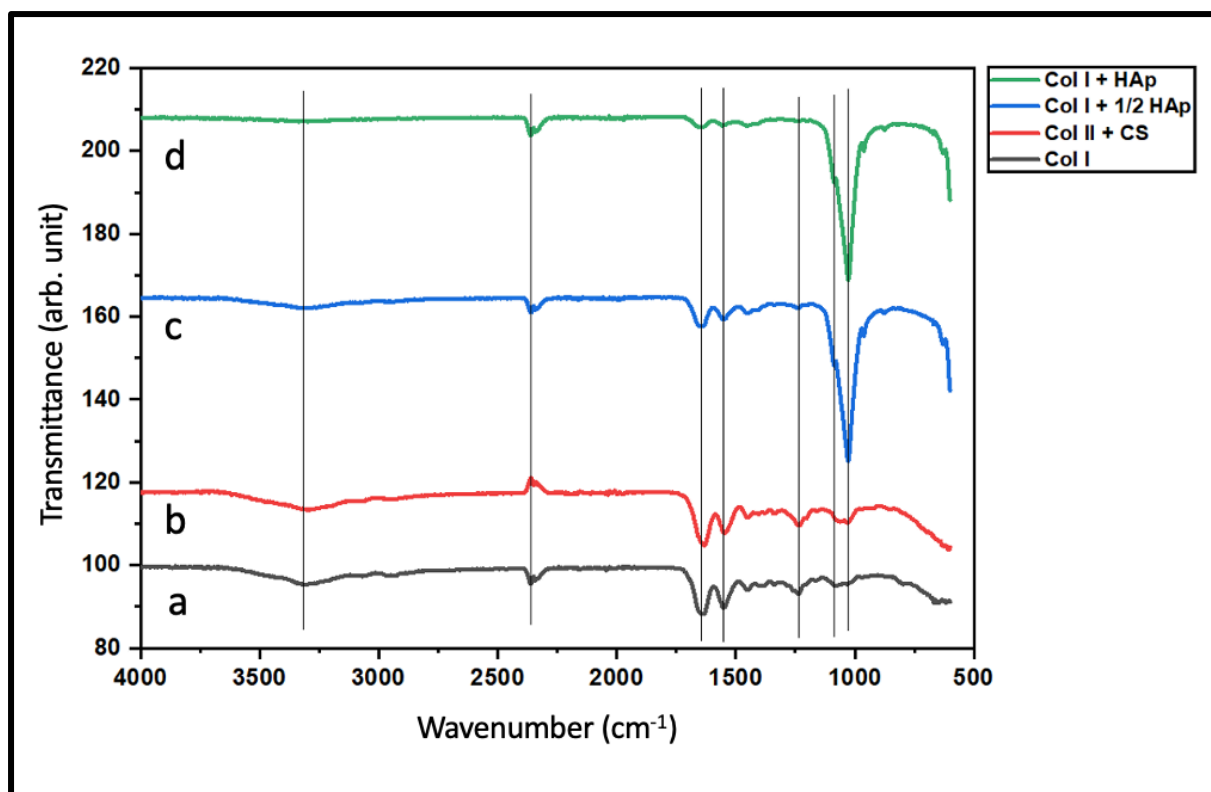


Figure 5.9: FTIR spectra of (a) col I (ligament layer), (b) Col II + CS (uncalcified fibrocartilage layer), (c) Col I + $\frac{1}{2}$ HAp (Calcified fibrocartilage layer) and (d) Col I + HAp (Bone layer).

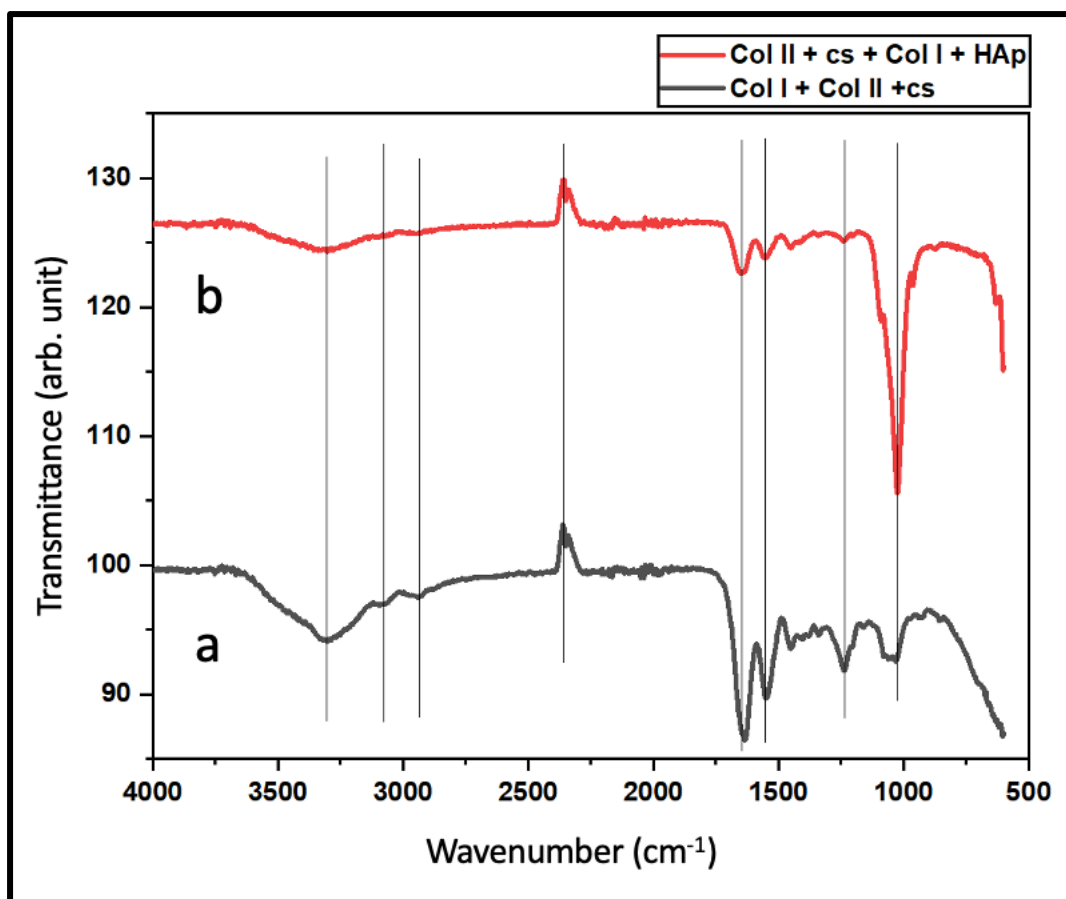


Figure 5.10: FTIR spectra of multi-phase scaffolds (a) Col I and Col II + CS, (b) Col II + CS and Col I + HAp.

Properties	Peak Wavenumber (cm ⁻¹)						Assignment	References
	Single Layer				Multi-layer			
	Col I	Col II + CS	Col I + ½ HAp	Col I + HAp	Col I & Col II +CS	Col II + CS & Col I +HAp		
Amide A	3317	3300	3313	3313	3317	3300	NH stretch coupled with hydrogen bond	[36], [37]
Amide I	1632	1632	1649	1649	1632	1649	C=O stretch/hydrogen bond coupled with COO-	[36], [37], [38]
Amide II	1548	1548	1552	1552	1548	1552	NH bend coupled with CN stretch	[36], [37], [39]
CH ₂ Side chain	1336	1336	-	-	1336	1336	CH ₂ side chain vibrations of collagen	[40], [41]
Amide III	1235	1239	1241	1240	1239	1240	CH bend coupled with CN stretch	[37], [40], [41]
Fibrillar	1082	1082	1082	1082	1082	1082	C-O bond tensile vibrations	[40], [41], [42]5/1/25 3:53:00 PM
PO ₄ ³⁻	-	-	1027	1027	-	1027	PO ₄ ³⁻ asymmetric stretching	[43]

Table 5.2. FTIR analysis values for functional group characterisation.

The band of amide A is bound up with the N-H stretching frequency. The wavenumber of a free N-H stretching vibration is located within the range 3400-3440 cm⁻¹ [44]. The amide A bands of Col I, Col II + CS, Col I + 1/2HAp and Col I + HAp were located at wavenumbers 3313, 3330, 3317 and 3317cm⁻¹, respectively. The amide A bands for Col I + Col II + CS multi-phase and Col II + CS and Col I + HAp multi-phase were at 3317 and 3300cm⁻¹, respectively.

Amide I, amide II and amide III bands are bound up with the triple helical structure of collagen resulting from C=O stretching, N-H bending and C-H stretching, respectively. The amide I bands of col I and Col II + CS and multi-phase scaffolds col I and Col II + CS were located at a wavenumber of 1632cm⁻¹, Col I + 1/2HAp and Col I + HAp and multi-phase scaffolds Col II + CS and Col I + HAp were located at a wavenumber of 1649cm⁻¹. The amide II bands of Col

I and Col II + CS and multi-phase scaffolds Col I and Col II + CS were located at a wavenumber of 1548cm^{-1} , Col I + 1/2HAp and Col I + HAp and multi-phase scaffolds Col II + CS and Col I + HAp were located at a wavenumber of 1552cm^{-1} . The amide III bands of Col I, Col II + CS, Col I + 1/2HAp and Col I + HAp were located at wavenumbers of 1235, 1239, 1241 and 1240cm^{-1} , respectively. Whilst the amide III bands for Col I + Col II + CS multi-phase and Col II + CS and Col I + HAp multi-phase were at 3339 and 3340cm^{-1} , respectively, the intensity of amide III peaks of scaffolds containing HAp were very small to that of Col I + HAp being almost undetectable.

When observing the spectra, we can distinguish a specific feature, the appearance of a peak at 1083 cm^{-1} , which corresponds to tensile vibrations of a bond of CO molecules and is a characteristic of fibrillar collagen [45]. Although the peak at 1082 cm^{-1} is present in all spectra, the transmittance is much smaller in scaffolds cross-linked with HAp.

The major peak at 1027cm^{-1} in sample Col I + $\frac{1}{2}$ HAp and Col I + HAp is associated with the asymmetric stretching of PO_4^{3-} which is a functional group in hydroxyapatite.

5.4.3. Water Uptake

Figure 5.11, Presents the water uptake ratio of the 4 single layer scaffolds per the overall dry weight of the scaffolds and the total volume of the scaffolds. Scaffolds made from type II collagen presented the greatest water uptake ability over all the scaffolds. Pure type I collagen scaffolds had a greater water uptake ability per mass whilst the scaffolds containing HAp had greater water uptake ability per volume.

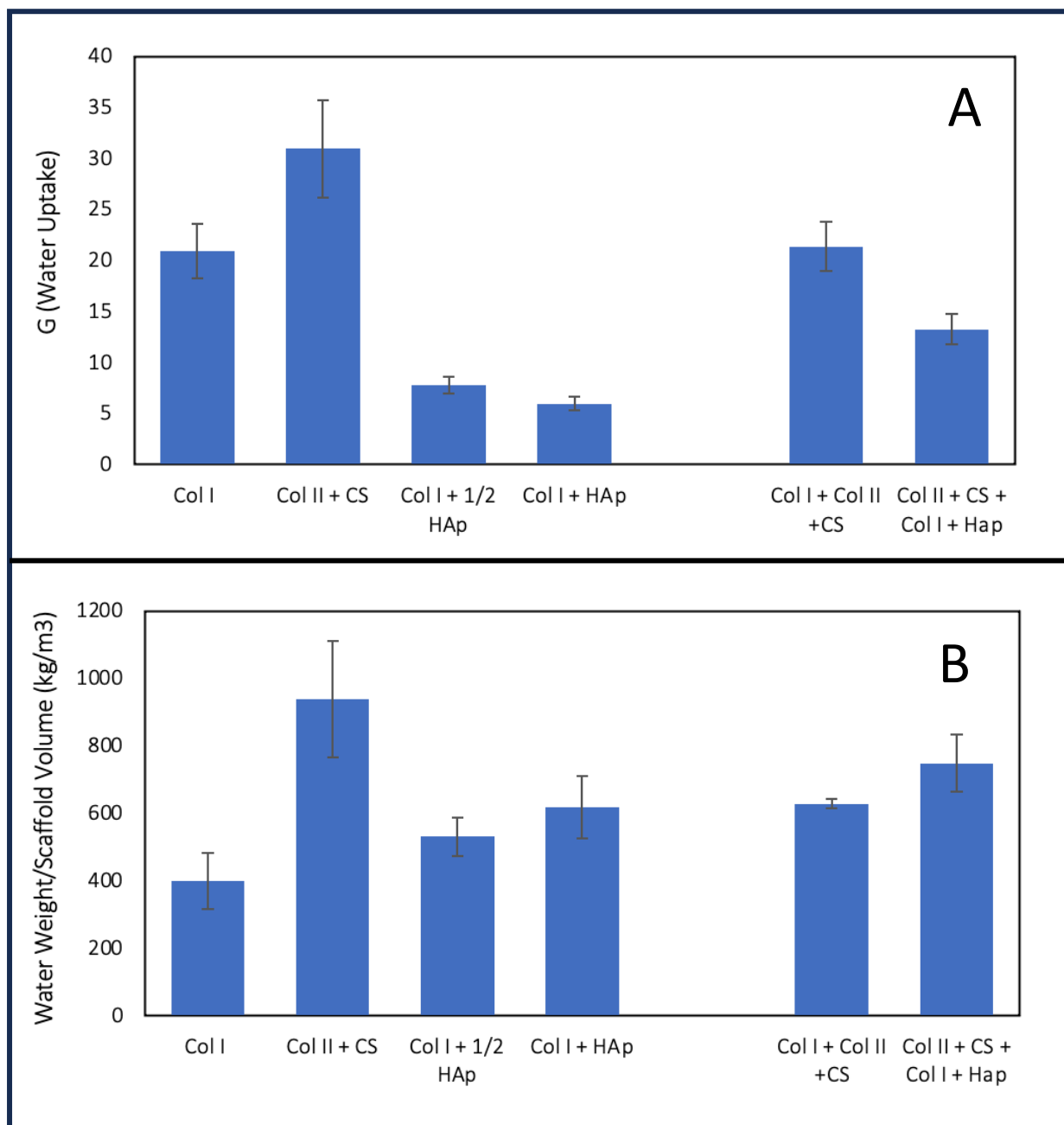


Figure 5.11. Water uptake by scaffolds ($n=5$) after 24hrs of hydration. **A** shows Water uptake per overall mass of the scaffolds, and **B** shows water uptake per total volume of the scaffold.

5.4.4. Self -Deflection

The bending modulus of the scaffolds was measured by measuring the self-deflection of the hydrated scaffolds. E bending values of the single-layer scaffolds are visualised in Figure 5.12. The uncalcified fibrocartilage layer (Col II + CS, 0.0128MPa $\pm$ 0.0006) was found to have the highest bending modulus, more than 2-folds of that of the ligament layer (Col I, 0.005MPa $\pm$ 0.001). The bone layer (col I + HAp, 0.0126MPa $\pm$ 0.003) had higher bending modulus compared to the calcified fibrocartilage layer (Col I + $\frac{1}{2}$ HAp, 0.0088MPa $\pm$ 0.001), and both had greater bending modulus to ligament layer which can be attributed to the higher content of HAp.

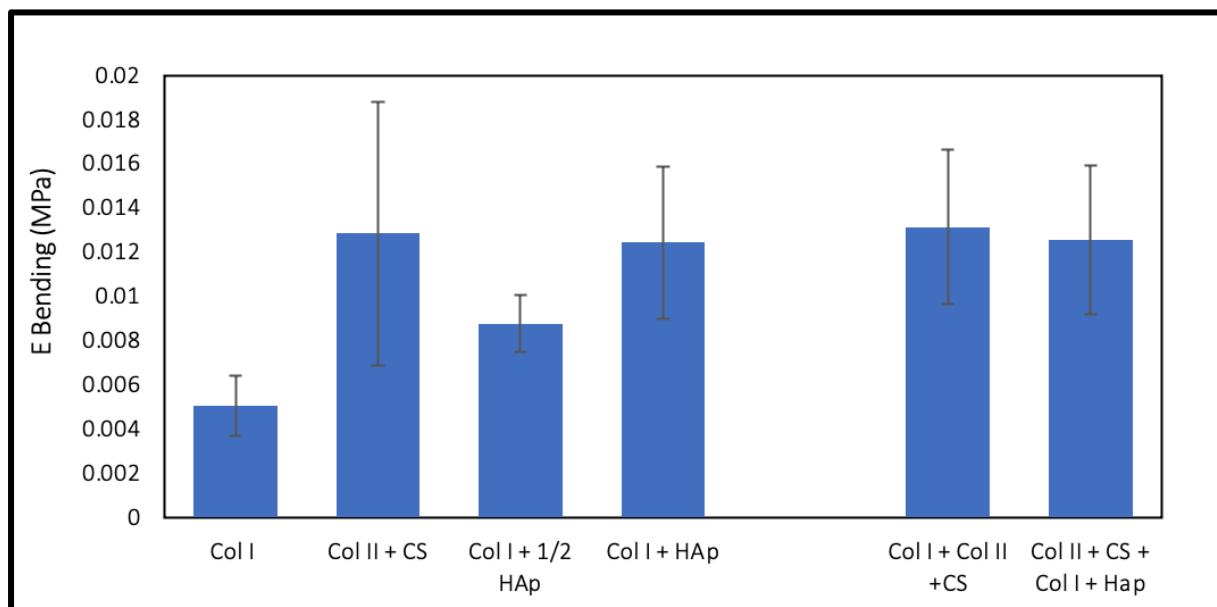


Figure 5.12: Bending modulus (MPa) of collagen-based scaffolds left to deflect under their hydrated weight, $n=5$.

By taking pure type I collagen scaffolds as the baseline. Figure 5.13, Illustrates the rise in bending modulus with HAp content. It can be seen that there is almost a linear increase in bending modulus with HAp content.

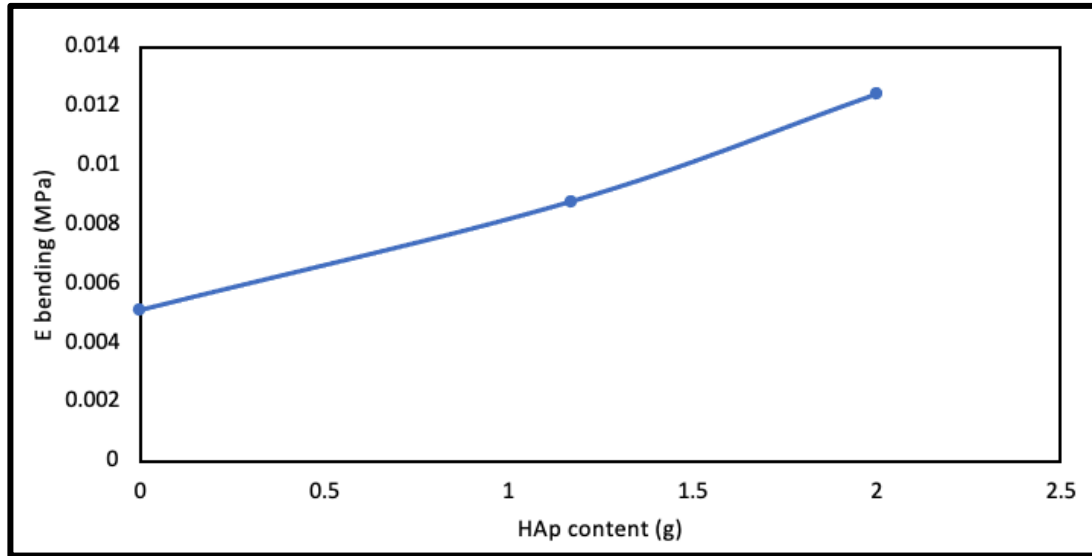


Figure 5.13: Relationship between bending modulus and HAp content in scaffolds.

5.4.5. Degradation

A balance between endogenous neo-tissue formation and host-mediated degradation is essential for achieving successful tissue regeneration. The scaffold must degrade at a similar rate to the replacement of the scaffold. The degradation process is controlled by phagocytic cells which interact with the material upon implantation and mediate the host response. If scaffold degradation is faster than wound healing and native tissue regeneration, the cells will be deprived of the ECM-like structure, and the former tissue can be defective or unfeasible [46]. However, too-slow scaffold degradation may lead to scaffold encapsulation, triggering an immune response and poor integration or rejection from host tissue. The process of degradation of a biopolymer is a chain scission process, during which the polymer chains are cleaved to form oligomers and monomers.

The degradation behaviour of collagen-based scaffolds under physiological conditions (PBS, 37°C, 12 weeks) and in the presence of various enzymes (37°C, 24hrs), are presented in the following sections.

5.4.5.1. Non-Enzymatic

Degradation of scaffolds takes place in a biological environment where water plays an important role. When water enters the polymer body, accompanied by swelling, it triggers the process of creating oligomers and monomers. This water-induced hydrolysis occurs in most scaffold degradation processes. When oligomers and monomers leave the scaffold, the weight of the scaffold will decrease [47]. PBS solutions have osmolarity and ion concentrations that match with those of the human body. Therefore, the non-enzymatic degradation results of collagen-based scaffolds over 12 weeks, where the solvent used was PBS, are presented in Figure 5.14 The percentage mass loss each week for each of the scaffolds is presented in Table 5.3.

During degradation, HAp particles did not degrade, but rather the collagen binding them together did, resulting in HAp breaking away and settling to the bottom of the centrifuge tubes. Once the HAp particles broke away from the main sample, they were no longer taken into account in mass measurements as *in vivo* these particles would be carried away by the body once they broke away.

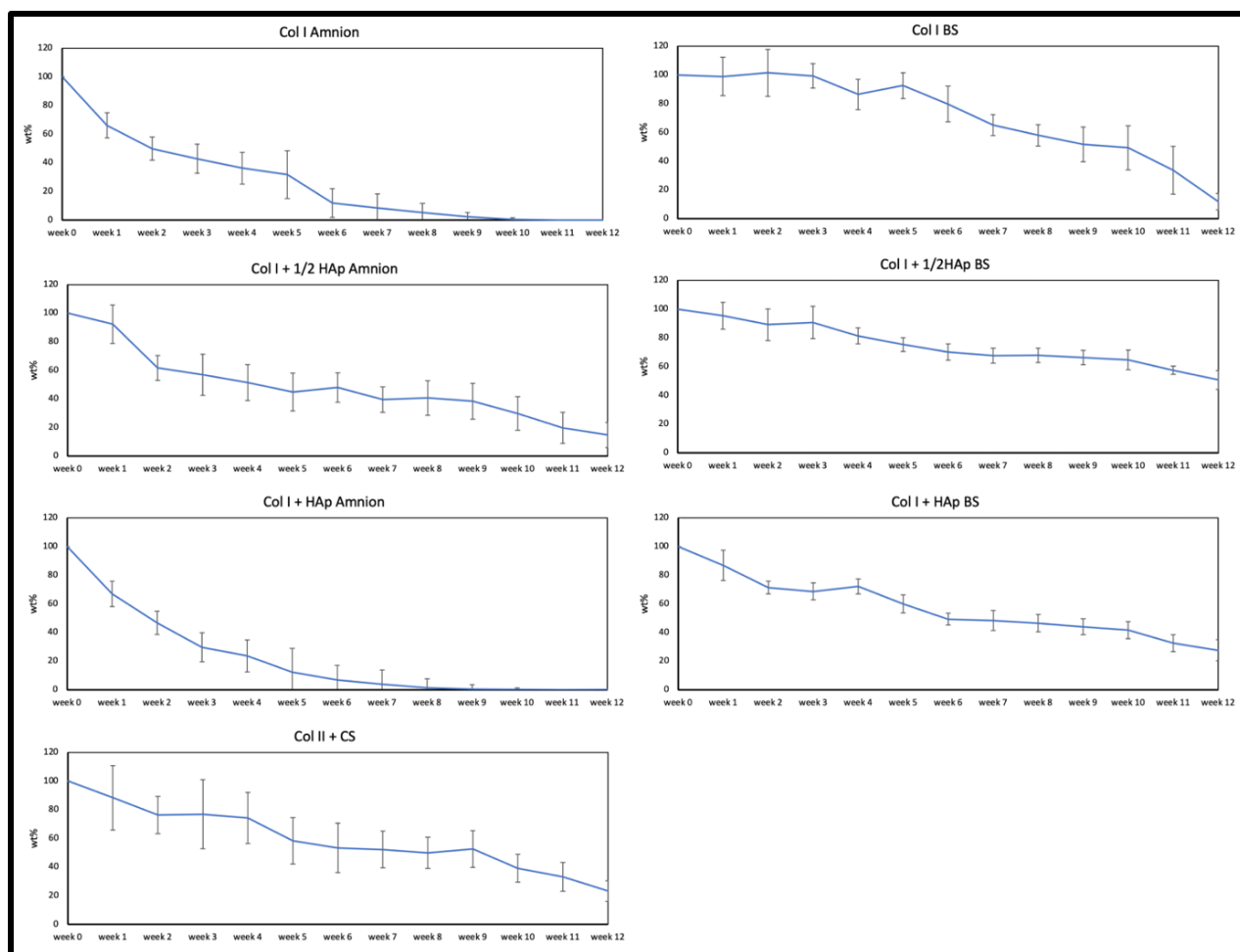


Figure 5.14. Degradation characteristics of collagen-based porous scaffolds in phosphate buffer saline for 12 weeks, $n=5$.

It was found that in the PBS-only degradation assay, there was a significant difference between samples produced from Amnion extracted collagen and those extracted from bovine skin. Particularly, when observing degradation in the 1st 6 weeks, amnion extracted Col I, Col I + $\frac{1}{2}$ HAp and Col I HAp scaffolds on average lost 88.05%, 51.99% and 93.08% of the total mass, respectively, to only 21.69%, 29.78% and 50.63% respectively for bovine skin extracted Col I, Col I + $\frac{1}{2}$ HAp and Col I HAp scaffolds.

Col II scaffolds degraded uniformly over the 12 weeks with the greatest decrease in weight between weeks 4 and 5 with the average mass loss rising from 25.76% to 41.74%, respectively.

Scaffolds	Average Mass Loss Each Week (%)											
	1	2	3	4	5	6	7	8	9	10	11	12
Col I Amnion	33.85	50.12	57.16	63.70	68.21	88.05	91.39	94.61	97.71	99.51	100	100
Col I + ½ HAp Amnion	11.61	23.60	23.04	25.76	41.75	46.58	47.69	49.97	47.41	60.72	66.73	76.68
Col I + HAp	7.70	38.32	43.04	48.48	55.24	52.00	60.45	59.22	61.61	70.18	80.20	85.17
Col I BS	33.03	53.16	70.25	76.37	87.62	93.08	95.97	98.64	99.41	99.66	99.86	99.90
Col I + ½ HAp BS	1.17	3.50	2.72	15.21	9.25	21.69	36.16	43.07	49.22	51.42	67.00	88.36
Col I + HAp BS	4.55	10.76	9.21	18.55	24.53	29.79	32.38	32.12	33.75	35.29	42.53	49.29
Col II + CS	13.14	28.57	31.29	27.77	40.05	50.63	51.66	53.41	55.94	58.30	67.41	72.37

Table 5.3: Summary of the average mass loss per week (%) of collagen-based scaffolds over 12 weeks at 37 °C in PBS gently shaken, n=5.

5.4.5.2. Enzymatic

In addition to the hydrolysis process, enzymes play an important role in biomaterial degradation; enzymolysis or enzymatic hydrolysis. The mechanism behind enzymatic hydrolysis is such that an enzyme is a catalyst during the water-induced hydrolysis process [48]. Previous studies have shown that the measured stability to enzymatic degradation *in vitro* serves to qualitatively identify the degradation behaviour of scaffolds *in vivo*, although the time frames differ greatly. The degradation *in vivo* is observed to be much longer than *in vitro* at ideal conditions [49].

Enzymatic degradation results of collagen-based scaffolds over 24 hours are presented in Figures 5.15, 5.16 and 5.17, where the solvent used was collagenase, collagenase + chondroitinase, and chondroitinase, respectively. The percentage mass loss at each time point: 1hr, 3hrs, 6hrs, 12hrs and 24hrs, for each of the scaffolds is presented in Table 5.4.

Enzymes	Scaffolds	Average Mass Loss at Each Time Point (%)				
		1	3	6	12	24
Collagenase	Col I + 1/2 HAp Amnion	32.28	45.30	76.38	100	100
	Col I + HAp Amnion	22.90	55.28	78.35	100	100
	Col I + 1/2 HAp BS	9.14	26.77	40.43	39.98	53.97
	Col I + HAp BS	10.32	35.67	43.32	54.49	55.74
	Col II + CS	3.78	2.31	13.95	9.82	32.67
Chondroitinase	Col II + CS	1.40	18.64	14.63	27.07	27.76
	Pure CS	8.35	3.77	15.99	24.58	23.58
Chondroitinase + Collagenase	Col II + CS	13.73	11.69	12.41	12.46	45.20

Table 5.4: Summary of the average mass loss (%) at time points 1hr, 3hrs, 6hrs, 12hrs and 24hrs, of collagen-based scaffolds at 37 °C, gently shaken in various enzymes, n=5.

Collagenase

Both Pure Col I Amnion and Col I BS degraded within the hour and therefore there was nothing to measure. However, Pure Collagen from Bovine Skin was able to resist enzymatic degradation slightly more than collagen from Amnion as there we still visible specs after an hour, whereas the Collagen I from Amnion had degraded completely. Similarly to the non-enzymatic degradation assay discussed above, during degradation, HAp particles did not degrade, but rather the collagen binding them did, resulting in HAp breaking away, and once this occurred, they were no longer taken into account in mass measurements.

From Figure 5.15, it can be seen that samples fabricated using collagen I from Bovine skin were better able to resist degradation from collagenase with all the collagen in col I + 1/2HAp and col I + HAP from amnion completely degrading by the 12hrs time point, whereas, col I + 1/2HAp and col I + HAP from bovine skin only losing on average 39.98% and 54.49% of their total mass, respectively.

Col II + CS scaffolds were best at resisting collagenase degradation, losing on average only 9.82% of their total weight by 12 hrs and 32.67% at the end of 24hrs.

Chondroitinase

No change in average mass between hrs 12 and 24 for both col II + CS and pure CS scaffolds.

Collagenase and Chondroitinase

The average mass of the scaffolds is rather stable for the first 12 hrs with a significant jump in the average loss in mass between 12 and 24hrs from 12.46% to 45.19%.

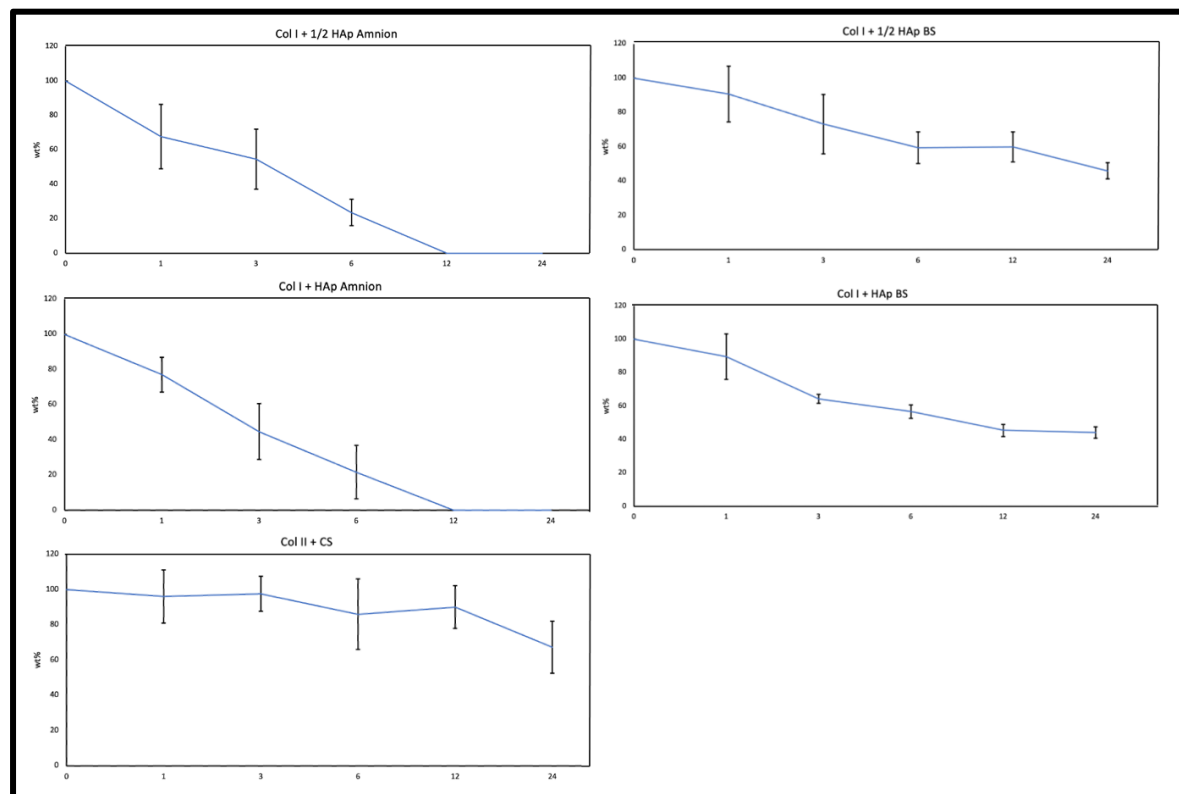


Figure 5.15: Degradation characteristics of collagen-based porous scaffolds with enzyme collagenase.

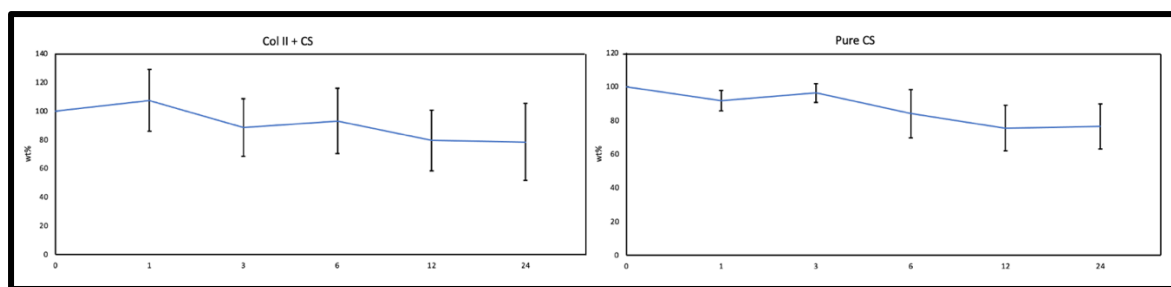


Figure 5.16: Degradation characteristics of collagen-based porous scaffolds with enzyme chondroitinase.

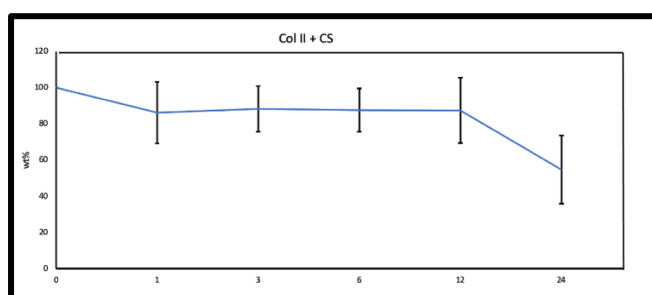


Figure 5.17: Degradation characteristics of collagen-based porous scaffolds with enzymes collagenase and chondroitinase.

5.5. Discussion

This study utilised and expanded upon the existing expertise of our research group in developing collagen-based scaffolds using monomeric collagen for the repair of the ligament-bone interface. Collagen formed the base of each layer, offering the advantage of having natural binding sites and the ability to degrade without releasing harmful bi-products.

5.5.1. Microstructure of Scaffolds

The porous structure of scaffolds provides space for intracellular matrix formation. The size of osteoblasts is in the order of 10-50 μm [50], however, osteoblasts prefer larger pores for regenerating mineralised bone as it promotes vascularisation. Whereas a smaller pore size is associated with the formation of non-mineralised osteoid or fibrous tissue [51].

The relationship between scaffold pore size and cell activity is poorly understood and as a result, there are conflicting reports within the literature on the optimal pore size required for successful tissue engineering. Previous studies reported that optimal scaffold pore sizes for promoting the growth of fibroblasts, chondrocytes, and osteoblasts range from 40-250 [26], [27], 70-400 μm [28], [29], [30] and 200-800 μm [25], [32], [52], respectively. Therefore, pore areas of order 7000-800000 μm^2 were considered feasible for cell activity.

The porous structure of the scaffolds is formed during lyophilisation. When materials are freeze-dried, the frozen water is sublimated directly into the gas phase, which results in pore formation [53]. However, the morphology of pores stems from the ice crystal formation during the freezing process. During freezing, suspensions undergo a heterogeneous nucleation of ice crystals from their exposed ends in the mould [7]. During the growth of ice crystal dendrites, the collagen aggregates are repelled and swept along with the solidification front or remain stationary and become entrapped by the advancing solid-liquid interface; this depends on ice

front velocity facing the particles, which is a function of the freezing temperature and the suspension's viscosity [54]. If a critical velocity is exceeded, the particle becomes encapsulated into the solid, because the lateral flanks of the plate-like ice crystals grow much slower than the ice-crystal tips and the planar ice front, the critical ice-front velocity is not exceeded and the collagen fibre is rejected from the laterally growing ice front.

As the freezing temperatures of all the scaffolds were identical, the spindle-like morphology of col II + CS scaffolds is likely attributed to the viscosity of the suspension. This spindle-like morphology is not present when combined with another collagen-based scaffold (multi-phase), but rather col II + CS layers consist of parallel collagen layers connected by single collagen fibres because col I based scaffolds reduce the ice front velocity and prevent the formation of dendrite.

This parallel collagen structure can be explained by the anisotropy of ice, [55]. Ice crystals have four axes: three a-axes and a c-axis which lies perpendicular to the a-axes and grows 10^2 to 10^3 times slower. The distance between the collagen layers corresponds to the c-axis. Along the a-axes, the collagen aggregates might be incorporated into the solid–liquid interface, as the critical ice-front velocity is exceeded, therefore, in this direction, the collagen fibres are not concentrated between the ice crystals resulting in pores which are not limited by collagen walls but by single collagen fibres [54].

Interconnected pores help to inflow the nutrients and elution of metabolic waste from scaffolds [56]. Although all scaffolds consisted of highly interconnected pores, ideal for cell behaviour (e.g. attachment, proliferation and differentiation), secondary pores observed in the struts of scaffolds reduced with increasing HAp content, likely caused by the nucleation of HAp particles onto the surface of collagen [57].

Not only did all collagen-based scaffolds present incredibly high levels of porosity in agreement with the literature [6], [58], [59], but the size of the pores was also ideal for in vivo conditions, as illustrated by surface porosity results.

5.5.2. Fourier Transform Infrared Spectroscopy

Amide A, amide I, amide II and amide III peaks were observed in all samples. Confirming that the polypeptide structure was maintained after scaffold fabrication.

When water molecules are bound to collagen molecules, with the formation of hydrogen bridges, changes in the collagen spectrum for collagen then show a conspicuous increase in adsorptions for the amide A band. Additionally, the wavenumber would move to a lower frequency if the N-H group participated in the formation of a hydrogen bond. Therefore, both the increased intensity and the shift to lower frequencies, indicate stronger hydrogen bonding of NH groups [60]. This interaction of collagen with water molecules is typically due to the humidity of the environment. The Amide A band in all scaffolds are shifted to lower frequencies suggesting that all scaffolds are interacting with water molecules present within the environment. However, the intensity of the peak reduces with increasing HAp content. This is likely due to collagen cross-linking with HAp molecules and therefore being less readily available to bind with water molecules. The stronger the hydrogen bond, the lower the position of the Amide A band. As the peak of the Amide A band for the Col II + CS is lower, this suggests that this scaffold can form stronger hydrogen bonds.

The amide I and II bands are related to the degree of molecular order involved with the triple helical structure of collagen. The amide I band with strong absorbance in the range of 1600-1700cm⁻¹ is primarily associated with the C=O stretching vibration along the peptide carbonyl group or a hydrogen bond coupled with COO-[61]. The amide II band representing the N-H

bending vibration with a contribution from C-N stretching vibration, generally occurs in the range of 1550-1600 cm^{-1} , which specifies the number of NH groups involved in hydrogen bonding with the adjacent α -chain; therefore, the lower wavenumber of the amide II band is related to the increase of hydrogen bonds by NH groups. Liang et al. reported a shift of amide I and II to higher frequencies which was associated with an increase in the molecular order [44]. The wavenumber of the amide I band of scaffolds with HAp is higher than those without suggesting increased molecular order.

The positive shift of the amide I band associated with carboxylate peaks can be explained according to their mesomeric structure shown in Figure 5.18.

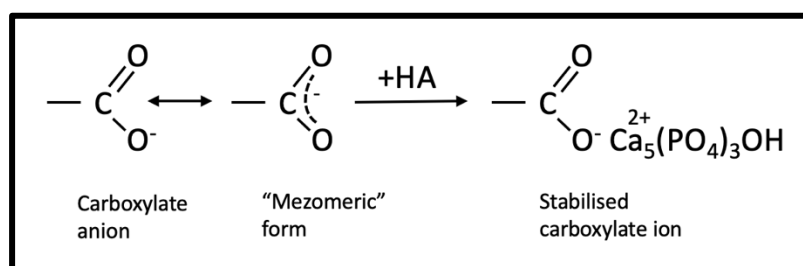


Figure 5.18: Stabilisation of carboxylate group due to mineralisation.

In the absence of HAp, carboxylate groups are predominantly in form I, where the CO bond of interest is quasi-double. When HAp is present, the carboxylic groups are stabilised and this CO bond turns into a double and the CO bond becomes stronger and shifts to a higher corresponding peak [62].

The appearance of a peak at 1082 cm^{-1} allows us to identify fibrillation in collagen as this peak is assigned to C-O, stretching vibrations of the carbohydrate residues present in collagen which is related to the fibril forming process. The intensity of the peak at 1082 cm^{-1} is noticeably less

for HAp-containing scaffolds which means a decrease of bonding energies of C–O bonds and is assumed to be caused by interaction with Ca^{2+} ions on the surfaces of HAp crystals. Studies have shown the nucleation of HAp crystals to the carboxylate group of collagen [62]–[64]. When a calcium ion is bound to the carboxylate group, the positively charged calcium ion makes new chemical bonds with the two oxygen ions of the carboxylate group (figure 5.19), resulting in a decrease of the C-O bond order [64].

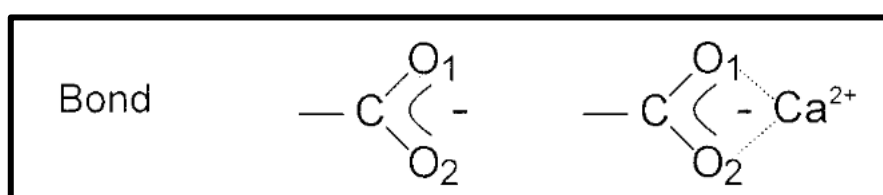


Figure 5.19: Calcium ion forming a bond with oxygen ions of the collagen carboxylic group [64].

Amide III absorption is associated with the triple helix structure of collagen and involves both C-N stretching and N-H in-plane bending from amide linkages, which is typically very weak in FTIR. It also arises from wagging vibrations of CH_2 from the glycine backbone and proline side chains. The results indicate that hydrogen bonds were involved in Col I, Col II + CS and Col I + 1/2HAp but not Col I + HAp.

The peak at around $2300\text{--}2400\text{cm}^{-1}$ is usually due to a different amount of CO_2 in the sample chamber compared to the background scan and indicates that the instrument needs to be thoroughly purged with CO_2 -free gas. However, when observing the peak for each sample, it is interesting that this peak is positive for Col II + cs samples whilst negative for the other 3. This shows that there is less CO_2 present in the sample chamber after the Col II + cs were

placed than when the background scan was taken. This suggests that the Col II + cs samples are interacting with the CO₂ in the chamber and removing it.

Type II collagen contains a greater amount of hydroxylysine, glucosyl and galactosyl residues than type I collagen and these mediate the connection of collagen and proteoglycan components in the matrix [66].

5.5.3. Water Uptake

In general, water absorbed by porous scaffolds is characterised as substrate-bound water and free water. Free water flows within a tissue, fills in pores such that the amount of free water correlates to the porosity of the structure, and is responsible for nutrient transport. Whereas substrate-bound water is substantially more static and contributes to the overall properties of the tissue with which it is associated. Therefore, both forms of ‘hydration’ are important for successful tissue engineering scaffolds.

The amount of bound water is on the order of 0.35 g/g of collagen [67]. Since the amount of water uptake in all our scaffolds is greater than this ratio, most of the water in the scaffolds is free and the scaffolds have suitable water uptake properties for tissue engineering endeavours.

On a per dry weight basis (A), pure collagen has a significantly greater water uptake ability compared to Col I scaffolds with HAp. This is expected as HAp particles notably contribute to the overall mass of the dry scaffolds, as HAp particles have a dry density of 3.16 g/cm⁻³ [12], while the dry density of collagen is only 1.3 g/cm⁻³ [68]. However, on a per volume basis (B), scaffolds containing HAp have a greater water uptake ability than pure col I scaffolds.

The physical combination of HAp and collagen I with crosslinker EDC/NHS are illustrated in Figure 5.20.

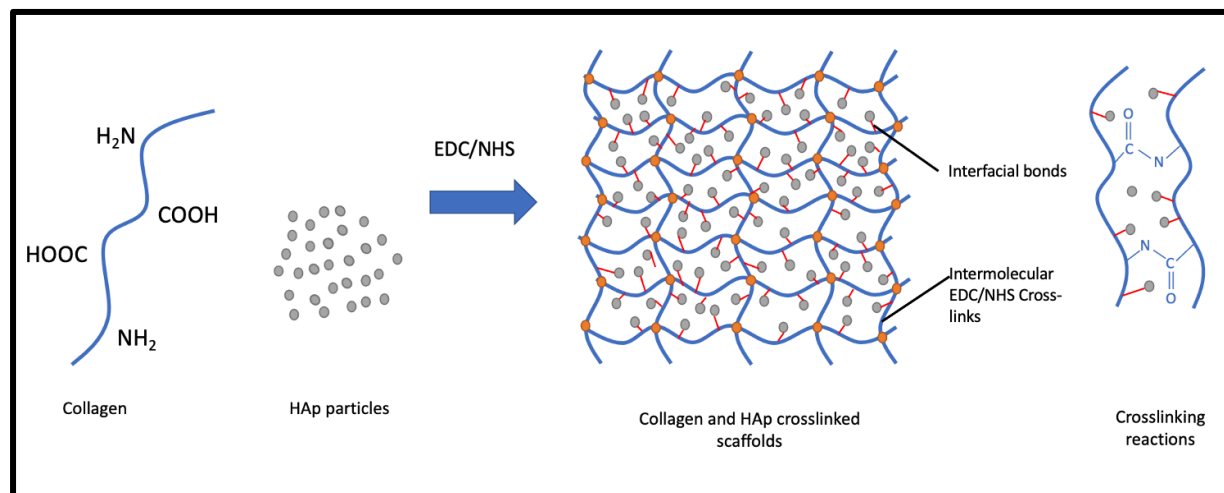


Figure 5.20: Schematic representation of crosslinking reaction of collagen and HAp with EDC/NHS.

Calcium phosphate can't participate in cross-links with organic molecules such as collagen as HAp is mostly ionic whilst collagen is not. Therefore, something more similar to a cross-polymerisation is how HAp particles bind with collagen type I. In a study by Sato *et al.* HAp formation on Langmuir-Blodgett monolayers took place on carboxyl-terminated monolayers but not on amino-terminated monolayer Fields [69]. This along with decreasing bonding energies of C-O bonds identified through FTIR spectra (Section 5.3.2) is a good indication that the driving force for the surface interaction between HAp and collagen is most likely an interfacial interaction between carboxyl groups in collagen and Ca ions in HAp.

During sample preparation, type I collagen was allowed to cross-link before HAp was added to the suspension. Therefore, strong chemical cross-linkage (in the presence of EDC/NHS) between collagen molecules is formed before HAp particles are added. The HAp then nucleates onto the surface of collagen molecules through the carboxylic group [63]. The hydrophilic

nature of HAp lowers the hydrophobic characteristics of collagen scaffolds as the HAp particles nucleate on the surface of the collagen molecules [70]. The greater the amount of HAp particles, the more water per volume the structures were able to uptake.

The ability of scaffolds produced from collagen type II to uptake more water than those using collagen type I agrees with the literature and can be attributed to the lateral spacing of the collagen molecules and the amount of intrafibrillar water. Grynpas *et al.* Reported that fully hydrated tissues containing predominantly type II collagen presented lateral spacing of collagen molecules between 16-17 Å which was significantly higher than the 14-15 Å observed for samples containing predominantly type I [71], whilst no differences were observed before hydration. They also found that polysaccharides and other extrafibrillar constituents were not responsible for the differences in lateral spacing between type I and type II collagen. In a study by Wachtel *et al.* it was found that high ionic strength reduced collagen intermolecular spacing which is consistent with the electrostatic nature of the interactions between the charged groups within the intrafibrillar space [72]. Furthermore, Berthet et al, [73] found that the greater lateral spacing of type II collagen was attributed to the disaccharide residues bound to the collagen field [74], [75]. The hydrophilic nature of disaccharide molecules could decrease the hydrophobicity of collagen molecules and favour more water uptake, by reducing the electrostatic forces acting upon the central triple-helical structure (Section 2.2.1), allowing for greater intermolecular collagen spacing and hence greater intrafibrillar hydration.

5.5.4. Self-Deflection

The modulus of freeze-dried (wet and dry) scaffolds tends to be very low ~1-40kPa, with values usually towards the lower end of the range when hydrated and higher end when dry. Bending modulus results obtained in this study (~ 4 kPa – 20 kPa) are in line with literature [76], [77],

[78], [79], [80], [81], [82]. The main reason for the values being so low is that elastic deformation occurs primarily via the bending of slender structural elements. In the case of porous scaffolds, it is the bending of these thin pore walls which allows for large deflection under low applied loads. However, as these scaffolds are only intended to mimic the ECM, a structural support network for tissue growth, and not the actual native tissue which also includes cells, low values are expected. The mechanical properties of the scaffolds must facilitate cell culture *in vitro* (section 1.8), and meet the demands of *in vivo* implantation and mechanical functionality once implanted [77], [83]. Absorbable collagen sponges used clinically exhibited moduli ranging from 1-20kPa [77], [81]. As the results obtained in this study are similar, it can be assumed that the scaffolds have the necessary mechanical properties to meet *in vivo* demands.

We can utilise values for bending modulus to observe how changes to a scaffold's composition affect its mechanical properties. The results emphasised the contribution of HAp to the increased stiffness. The chemical interactions between Col I and HAp which result in the increased bending modulus are explained in Section 5.5.3. The interfacial interaction between carboxyl groups in collagen and Ca ions in HAp further stabilises the structure and increases its stiffness.

When a scaffold is in bending, the top of the scaffold is in compression whilst the bottom of the scaffold is in tension, therefore, the effective bending modulus obtained from deflection testing will take into account both the compressive and tensile modulus of the scaffolds. Therefore, collagen II + CS scaffolds presenting the highest bending modulus values are very promising. Unlike ligaments which have mechanical properties favourable in tensile and bone which have properties which favour compression, fibrocartilage tissue needs to be able to

effectively withstand forces from both directions to dissipate the stress formation within this region. The high bending modulus of these scaffolds can be explained by results obtained from FTIR spectroscopy which were discussed in section 5.5.3.

5.5.5. Degradation

5.5.5.1. Collagen Type I Scaffolds

Hydrolysis degradation can be classified into two kinds: surface degradation (surface erosion), where degradation occurs primarily in the outermost polymer layer, and bulk degradation, where degradation occurs in the whole polymer volume in a simulation manner [84].

When comparing the degradation of samples from the 2 types of collagen sources, amnion and bovine skin, the scaffolds using col I amnion degraded with large chunks detaching from the main bulk whereas scaffolds fabricated using col I from bovine skin degraded with the overall size of the scaffolds getting smaller each week. This suggests that the collagen I amnion scaffolds were degrading through bulk degradation whilst collagen I scaffolds from bovine skin degraded through surface degradation.

Unlike the pure collagen I sample, when hydroxyapatite was added to the scaffolds, they were able to resist degradation from collagenase far better. This is likely due to the HAp particles acting as a protective barrier and shielding the collagen particles from attack.

5.5.5.2. Collagen II + Chondroitin Sulphate

During non-enzymatic degradation, Col II + CS scaffolds degraded similarly to Col I BS scaffolds with the outermost layers degrading and the size of the scaffolds reducing evenly all over.

Interstitial collagens consist of three α chains of approximately 1000 residues with repeating Gly–X–Y triplets, where X and Y are often proline and hydroxyproline, respectively. Each α chain adopts a left-handed poly-Pro II-like helix, and three left-handed α chains intertwine to form a right-handed superhelix. This triple-helical conformation makes interstitial collagens resistant to most proteinases, however, enzymes which can cleave the triple-helical structure are known as collagenases [85]. For Collagenases to cleave collagen they must first bind and then unwind the rigid triple-helical substrate before they cleave the three α chains of native triple-helical type I and II collagens after Gly in particular sequences (Gly-Ile and Gly-Leu), located approximately three-quarters away from the N-terminus of the collagen molecule [86].

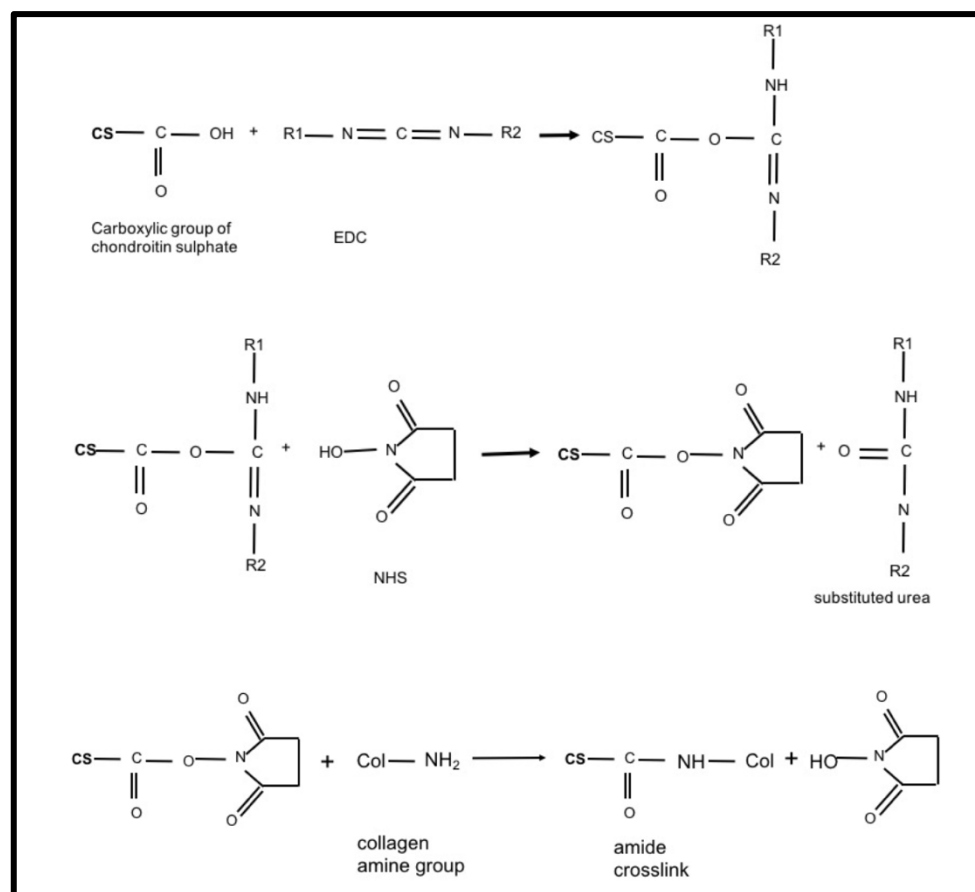


Figure 5.21: Mechanism of covalent attachment of chondroitin sulphate (CS) to collagen (Col) using EDC and NHS [87].

Chondroitin sulphate chemically attaches to the collagen triple helical structure by bonding to the amine group -NH_2 , which is present in Gly. As collagenase cleaves the triple-helical structure of collagen after Gly, the ability of collagen II + CS scaffolds to resist collagenase degradation is likely attributed to the chemically bonded CS to Gly in collagen preventing the attachment of collagenase to the structure. After EDC/NHS treatment collagen Type II and chondroitin sulphate, there are not only col-col cross-links but also col-GAG cross-links formed [88]. Therefore, the incorporation of CS into collagen significantly increases the degradation resistance of collagen. However, the removal of the GAGs by chondroitinase may therefore result in the collagen scaffolds becoming less resistant to attacks from collagenase. As there was no change in the average mass loss for scaffolds treated with chondroitinase only, it is evident that the CS degradation of the scaffolds by that concentration of collagenase has reached its max. This explains the more prominent drop in average mass loss for samples in both collagenase and chondroitinase as enough of the GAGs have degraded leaving the structure open to attacks by the collagenase and speeding up the rate of degradation [89].

5.6. Summary

This chapter focused on fabricating 4 types of collagen-based porous scaffolds to correspond with each of the distinct regions present in the enthesis and their combination to form two-phase scaffolds. Both SEM and FTIR were utilised to examine their microstructure and physiological properties. The swelling and degradation were of interest for structural integrity. The mechanical properties of these scaffolds were also investigated to investigate the biocompatibility of the designed scaffolds to native tissue. The main findings were as follows:

- The successful fabrication of 4 types of collagen-based scaffolds of high porosity (>96%), with a high degree of interconnectivity and mixed size of pores, mainly ranging from 50-200µm (suitable to support cell growth), was achieved and confirmed via FT-IR.
- Degradation analysis showed that the cross-linking of collagen with another substance (CS or HAp) significantly increased the degradation resistance of collagen and that collagen sourced from bovine skin was far better at maintaining structural integrity within physiological conditions over time.
- Scaffolds made from type II collagen and chondroitin sulphate presented the greatest water uptake ability and resistance to bending over all the scaffolds. Pure type I collagen scaffolds had a greater water uptake ability per mass whilst the scaffolds containing HAp had greater water uptake ability per volume and the addition of HAp increased bending modulus.
- Multi-phase scaffolds were successfully formed with great integration and similar properties to each of its constituents.

Due to the limited supply of collagen caused by the pandemic and the closure of the lab at Veterans Affairs Medical, it was not possible to take this work further within the timeframe of this work. Therefore, the next steps for this work would be to develop tri-layered scaffolds and

ultimately 4 layered scaffolds. However, as discussed in Chapter 2, there is a large variability between collagen sources and even between different batches of the same species and location. Therefore, it is highly likely that further results will not perfectly match those obtained in these studies.

5.7. References

- [1] R. J. Kane *et al.*, ‘Hydroxyapatite reinforced collagen scaffolds with improved architecture and mechanical properties’, *Acta Biomater*, vol. 17, pp. 16–25, Apr. 2015, doi: 10.1016/j.actbio.2015.01.031.
- [2] Yang Xu, ‘Development of Tri-layered Biomimetic Atelocollagen Scaffolds with Interfaces for Osteochondral Tissue Engineering’, University of Oxford, 2022.
- [3] J. Glowacki and S. Mizuno, ‘Collagen scaffolds for tissue engineering’, *Biopolymers*, vol. 89, no. 5, pp. 338–344, May 2008, doi: 10.1002/bip.20871.
- [4] S. Mizuno and J. Glowacki, ‘Three-dimensional composite of demineralized bone powder and collagen for in vitro analysis of chondroinduction of human dermal fibroblasts’, *Biomaterials*, vol. 17, no. 18, pp. 1819–1825, Sep. 1996, doi: 10.1016/0142-9612(96)00041-5.
- [5] J. Caballé-Serrano, S. Zhang, A. Sculean, A. Staehli, and D. D. Bosshardt, ‘Tissue Integration and Degradation of a Porous Collagen-Based Scaffold Used for Soft Tissue Augmentation’, *Materials (Basel)*, vol. 13, no. 10, p. 2420, May 2020, doi: 10.3390/ma13102420.
- [6] Y. Xie *et al.*, ‘Interconnected collagen porous scaffolds prepared with sacrificial PLGA sponge templates for cartilage tissue engineering’, *J. Mater. Chem. B*, vol. 9, no. 40, pp. 8491–8500, Oct. 2021, doi: 10.1039/D1TB01559A.
- [7] N. Rashidi, ‘Fabrication of a novel bilayered osteochondral scaffold using a bottom up approach’, Ph.D., University of Oxford, 2018. Accessed: Apr. 17, 2023. [Online]. Available: <http://ora.ox.ac.uk/objects/uuid:586e058f-0c20-41d3-8444-515d62fc8df0>
- [8] C. Z. Liu and J. T. Czernuszka, ‘Development of biodegradable scaffolds for tissue engineering: a perspective on emerging technology’, *Materials Science and Technology*, vol. 23, no. 4, pp. 379–391, Apr. 2007, doi: 10.1179/174328407X177027.

- [9] E. Sachlos, D. Gotor, and J. T. Czernuszka, 'Collagen Scaffolds Reinforced with Biomimetic Composite Nano-Sized Carbonate-Substituted Hydroxyapatite Crystals and Shaped by Rapid Prototyping to Contain Internal Microchannels', *Tissue Engineering*, vol. 12, no. 9, pp. 2479–2487, Sep. 2006, doi: 10.1089/ten.2006.12.2479.
- [10] N. Rashidi, M. Tamaddon, C. Liu, D. D. Brand, and J. Czernuszka, 'A Bilayer Osteochondral Scaffold with Self-Assembled Monomeric Collagen Type-I, Type-II, and Polymerized Chondroitin Sulfate Promotes Chondrogenic and Osteogenic Differentiation of Mesenchymal Stem Cells', *Advanced NanoBiomed Research*, vol. 2, no. 1, p. 2100089, 2022, doi: 10.1002/anbr.202100089.
- [11] H. Semyari *et al.*, 'Fabrication and characterization of collagen–hydroxyapatite-based composite scaffolds containing doxycycline via freeze-casting method for bone tissue engineering', *J Biomater Appl*, vol. 33, no. 4, pp. 501–513, Oct. 2018, doi: 10.1177/0885328218805229.
- [12] Y. Wu and S. Bose, 'Nanocrystalline hydroxyapatite: micelle templated synthesis and characterization', *Langmuir*, vol. 21, no. 8, pp. 3232–3234, Apr. 2005, doi: 10.1021/la046754z.
- [13] N. Rashidi, M. Tamaddon, C. Liu, and J. Czernuszka, 'Polymerization of chondroitin sulfate and its stimulatory effect on cartilage regeneration; a bioactive material for cartilage regeneration', *Polymer Testing*, vol. 116, p. 107796, Dec. 2022, doi: 10.1016/j.polymertesting.2022.107796.
- [14] S. Diermann, M. Lu, G. Edwards, M. Dargusch, and H. Huang, 'In vitro degradation of a unique porous PHBV scaffold manufactured using selective laser sintering', *Journal of Biomedical Materials Research Part A*, Oct. 2018, doi: 10.1002/jbm.a.36543.
- [15] N. Sultana Phd Ceng Csci and T. Khan, 'In Vitro Degradation of PHBV Scaffolds and nHA/PHBV Composite Scaffolds Containing Hydroxyapatite Nanoparticles for Bone

- Tissue Engineering’, *Journal of Nanomaterials*, vol. 2012, Apr. 2012, doi: 10.1155/2012/190950.
- [16] H. Zhu, J. Shen, X. Feng, H. Zhang, Y. Guo, and J. Chen, ‘Fabrication and characterization of bioactive silk fibroin/wollastonite composite scaffolds’, *Materials Science and Engineering: C*, vol. 30, no. 1, pp. 132–140, Jan. 2010, doi: 10.1016/j.msec.2009.09.009.
- [17] M. Kouhi, M. Fathi, M. P. Prabhakaran, M. Shamanian, and S. Ramakrishna, ‘Enhanced proliferation and mineralization of human fetal osteoblast cells on PHBV-bredigite nanofibrous scaffolds’, *Materials Today: Proceedings*, vol. 5, no. 7, Part 3, pp. 15702–15709, Jan. 2018, doi: 10.1016/j.matpr.2018.04.181.
- [18] W.-H. Liang, B. L. Kienitz, K. J. Penick, J. F. Welter, T. A. Zawodzinski, and H. Baskaran, ‘Concentrated Collagen-Chondroitin Sulfate Scaffolds for Tissue Engineering Applications’, *J Biomed Mater Res A*, vol. 94, no. 4, pp. 1050–1060, Sep. 2010, doi: 10.1002/jbm.a.32774.
- [19] C. Vepari and D. L. Kaplan, ‘Silk as a Biomaterial’, *Prog Polym Sci*, vol. 32, no. 8–9, pp. 991–1007, 2007, doi: 10.1016/j.progpolymsci.2007.05.013.
- [20] S. J. Hollister, ‘Porous scaffold design for tissue engineering’, *Nat Mater*, vol. 4, no. 7, pp. 518–524, Jul. 2005, doi: 10.1038/nmat1421.
- [21] F. J. O’Brien, B. A. Harley, M. A. Waller, I. V. Yannas, L. J. Gibson, and P. J. Prendergast, ‘The effect of pore size on permeability and cell attachment in collagen scaffolds for tissue engineering’, *Technol Health Care*, vol. 15, no. 1, pp. 3–17, 2007.
- [22] C. M. Murphy and F. J. O’Brien, ‘Understanding the effect of mean pore size on cell activity in collagen-glycosaminoglycan scaffolds’, *Cell Adh Migr*, vol. 4, no. 3, pp. 377–381, 2010, doi: 10.4161/cam.4.3.11747.

- [23] C. M. Murphy, M. G. Haugh, and F. J. O'Brien, 'The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering', *Biomaterials*, vol. 31, no. 3, pp. 461–466, Jan. 2010, doi: 10.1016/j.biomaterials.2009.09.063.
- [24] F. J. O'Brien, B. A. Harley, I. V. Yannas, and L. J. Gibson, 'The effect of pore size on cell adhesion in collagen-GAG scaffolds', *Biomaterials*, vol. 26, no. 4, pp. 433–441, Feb. 2005, doi: 10.1016/j.biomaterials.2004.02.052.
- [25] M. Cheng *et al.*, 'A novel open-porous magnesium scaffold with controllable microstructures and properties for bone regeneration', *Sci Rep*, vol. 6, no. 1, p. 24134, Apr. 2016, doi: 10.1038/srep24134.
- [26] H. Yu *et al.*, 'Novel porous three-dimensional nanofibrous scaffolds for accelerating wound healing', *Chemical Engineering Journal*, vol. 369, pp. 253–262, Aug. 2019, doi: 10.1016/j.cej.2019.03.091.
- [27] S. H. Oh, I. K. Park, J. M. Kim, and J. H. Lee, 'In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method', *Biomaterials*, vol. 28, no. 9, pp. 1664–1671, Mar. 2007, doi: 10.1016/j.biomaterials.2006.11.024.
- [28] D. J. Griffon, M. R. Sedighi, D. V. Schaeffer, J. A. Eurell, and A. L. Johnson, 'Chitosan scaffolds: interconnective pore size and cartilage engineering', *Acta Biomater*, vol. 2, no. 3, pp. 313–320, May 2006, doi: 10.1016/j.actbio.2005.12.007.
- [29] H. Stenhamre, U. Nannmark, A. Lindahl, P. Gatenholm, and M. Brittberg, 'Influence of pore size on the redifferentiation potential of human articular chondrocytes in poly(urethane urea) scaffolds', *J Tissue Eng Regen Med*, vol. 5, no. 7, pp. 578–588, Jul. 2011, doi: 10.1002/term.350.

- [30] J. H. Nam, S. Y. Lee, G. Khan, and E. S. Park, 'Validation of the optimal scaffold pore size of nasal implants using the 3-dimensional culture technique', *Arch Plast Surg*, vol. 47, no. 4, pp. 310–316, Jul. 2020, doi: 10.5999/aps.2020.00213.
- [31] 'Cryogenic prototyping of chitosan scaffolds with controlled micro and macro architecture and their effect on in vivo neo-vascularization and cellular infiltration - Lim - 2010 - Journal of Biomedical Materials Research Part A - Wiley Online Library'. Accessed: Feb. 20, 2023. [Online]. Available: <https://onlinelibrary.wiley.com/doi/full/10.1002/jbm.a.32747>
- [32] S. M. M. Roosa, J. M. Kemppainen, E. N. Moffitt, P. H. Krebsbach, and S. J. Hollister, 'The pore size of polycaprolactone scaffolds has limited influence on bone regeneration in an in vivo model', *Journal of Biomedical Materials Research Part A*, vol. 92A, no. 1, pp. 359–368, 2010, doi: 10.1002/jbm.a.32381.
- [33] D. D. Deligianni, N. D. Katsala, P. G. Koutsoukos, and Y. F. Missirlis, 'Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength', *Biomaterials*, vol. 22, no. 1, pp. 87–96, Jan. 2000, doi: 10.1016/S0142-9612(00)00174-5.
- [34] K. Nam, T. Kimura, and A. Kishida, 'Controlling coupling reaction of EDC and NHS for preparation of collagen gels using ethanol/water co-solvents', *Macromol Biosci*, vol. 8, no. 1, pp. 32–37, Jan. 2008, doi: 10.1002/mabi.200700206.
- [35] F. Everaerts, M. Torrianni, M. Hendriks, and J. Feijen, 'Biomechanical properties of carbodiimide crosslinked collagen: influence of the formation of ester crosslinks', *J Biomed Mater Res A*, vol. 85, no. 2, pp. 547–555, May 2008, doi: 10.1002/jbm.a.31524.
- [36] H. Staroszczyk, K. Sztuka, J. Wolska, A. Wojtasz-Pająk, and I. Kołodziejaska, 'Interactions of fish gelatin and chitosan in uncrosslinked and crosslinked with EDC

- films: FT-IR study', *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 117, pp. 707–712, Jan. 2014, doi: 10.1016/j.saa.2013.09.044.
- [37] A. Barth, 'Infrared spectroscopy of proteins', *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1767, no. 9, pp. 1073–1101, Sep. 2007, doi: 10.1016/j.bbabo.2007.06.004.
- [38] B. de Campos Vidal and M. L. S. Mello, 'Collagen type I amide I band infrared spectroscopy', *Micron*, vol. 42, no. 3, pp. 283–289, Apr. 2011, doi: 10.1016/j.micron.2010.09.010.
- [39] K. J. Payne and A. Veis, 'Fourier transform ir spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies', *Biopolymers*, vol. 27, no. 11, pp. 1749–1760, 1988.
- [40] L. Rieppo, S. Saarakkala, T. Närhi, H. J. Helminen, J. S. Jurvelin, and J. Rieppo, 'Application of second derivative spectroscopy for increasing molecular specificity of fourier transform infrared spectroscopic imaging of articular cartilage', *Osteoarthritis and Cartilage*, vol. 20, no. 5, pp. 451–459, May 2012, doi: 10.1016/j.joca.2012.01.010.
- [41] M. Jackson, L.-P. Choo, P. H. Watson, W. C. Halliday, and H. H. Mantsch, 'Beware of connective tissue proteins: Assignment and implications of collagen absorptions in infrared spectra of human tissues', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1270, no. 1, pp. 1–6, Jan. 1995, doi: 10.1016/0925-4439(94)00056-V.
- [42] A. Kohler, D. Bertrand, H. Martens, K. Hannesson, C. Kirschner, and R. Ofstad, 'Multivariate image analysis of a set of FTIR microspectroscopy images of aged bovine muscle tissue combining image and design information', *Anal Bioanal Chem*, vol. 389, no. 4, pp. 1143–1153, Oct. 2007, doi: 10.1007/s00216-007-1414-9.

- [43] M. Salarian, M. Solati-Hashjin, S. S. Shafiei, A. Goudarzi, R. Salarian, and A. Nemati, 'Surfactant Assisted Synthesis and Characterization of Hydroxyapatite Nanorods Under Hydrothermal Condition', *Materials Science-Poland*, vol. 27, Jan. 2009.
- [44] B. B. Doyle, E. G. Bendit, and E. R. Blout, 'Infrared spectroscopy of collagen and collagen-like polypeptides', *Biopolymers*, vol. 14, no. 5, pp. 937–957, May 1975, doi: 10.1002/bip.1975.360140505.
- [45] A. Martínez Cortizas and O. López-Costas, 'Linking structural and compositional changes in archaeological human bone collagen: an FTIR-ATR approach', *Sci Rep*, vol. 10, no. 1, Art. no. 1, Oct. 2020, doi: 10.1038/s41598-020-74993-y.
- [46] V. Pucino *et al.*, 'Lactate Buildup at the Site of Chronic Inflammation Promotes Disease by Inducing CD4⁺ T Cell Metabolic Rewiring', *Cell Metabolism*, vol. 30, no. 6, pp. 1055-1074.e8, Dec. 2019, doi: 10.1016/j.cmet.2019.10.004.
- [47] H. K. Makadia and S. J. Siegel, 'Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier', *Polymers (Basel)*, vol. 3, no. 3, pp. 1377–1397, Sep. 2011, doi: 10.3390/polym3031377.
- [48] H. Zhang, L. Zhou, and W. Zhang, 'Control of Scaffold Degradation in Tissue Engineering: A Review', *Tissue Engineering Part B: Reviews*, vol. 20, no. 5, pp. 492–502, Oct. 2014, doi: 10.1089/ten.teb.2013.0452.
- [49] M. Meyer, 'Processing of collagen based biomaterials and the resulting materials properties', *BioMedical Engineering OnLine*, vol. 18, no. 1, p. 24, Mar. 2019, doi: 10.1186/s12938-019-0647-0.
- [50] Y. Sugawara, H. Kamioka, T. Honjo, K. Tezuka, and T. Takano-Yamamoto, 'Three-dimensional reconstruction of chick calvarial osteocytes and their cell processes using confocal microscopy', *Bone*, vol. 36, no. 5, pp. 877–883, May 2005, doi: 10.1016/j.bone.2004.10.008.

- [51] J. Liu *et al.*, ‘Pre-vascularization in fibrin Gel/PLGA microsphere scaffolds designed for bone regeneration’, *NPG Asia Materials*, Aug. 2018, doi: 10.1038/s41427-018-0076-8.
- [52] T. C. Lim, K. S. Chian, and K. F. Leong, ‘Cryogenic prototyping of chitosan scaffolds with controlled micro and macro architecture and their effect on in vivo neo-vascularization and cellular infiltration’, *J Biomed Mater Res A*, vol. 94, no. 4, pp. 1303–1311, Sep. 2010, doi: 10.1002/jbm.a.32747.
- [53] E. Sachlos and J. T. Czernuszka, ‘Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds’, *Eur Cell Mater*, vol. 5, pp. 29–39; discussion 39-40, Jun. 2003, doi: 10.22203/ecm.v005a03.
- [54] H. Schoof, J. Apel, I. Heschel, and G. Rau, ‘Control of pore structure and size in freeze-dried collagen sponges’, *J Biomed Mater Res*, vol. 58, no. 4, pp. 352–357, 2001, doi: 10.1002/jbm.1028.
- [55] ‘P. V. Hobbs Ice physics. Oxford, Clarendon Press, 1974. xvii, 837 p. £29. | Journal of Glaciology | Cambridge Core’. Accessed: Apr. 10, 2023. [Online]. Available: <https://www.cambridge.org/core/journals/journal-of-glaciology/article/p-v-hobbs-ice-physics-oxford-clarendon-press-1974-xvii-837-p-29/CC251D81A7E9AABD75BD6766B825657B>
- [56] H. Liu, L. Zhang, P. Shi, Q. Zou, Y. Zuo, and Y. Li, ‘Hydroxyapatite/polyurethane scaffold incorporated with drug-loaded ethyl cellulose microspheres for bone regeneration’, *J Biomed Mater Res B Appl Biomater*, vol. 95, no. 1, pp. 36–46, Oct. 2010, doi: 10.1002/jbm.b.31680.
- [57] S. Jiang, H. Pan, Y. Chen, X. Xu, and R. Tang, ‘Amorphous calcium phosphate phase-mediated crystal nucleation kinetics and pathway’, *Faraday Discuss*, vol. 179, pp. 451–461, 2015, doi: 10.1039/c4fd00212a.

- [58] Q. Zhang, H. Lu, N. Kawazoe, and G. Chen, ‘Pore size effect of collagen scaffolds on cartilage regeneration’, *Acta Biomater*, vol. 10, no. 5, pp. 2005–2013, May 2014, doi: 10.1016/j.actbio.2013.12.042.
- [59] Y. J. Wu, T. Chen, I.-F. Chen, S. M. Kuo, and C. W. Chuang, ‘Developing highly porous collagen scaffolds by using alginate microsphere porogens for stem cell cultures’, *Materials Letters*, vol. 223, pp. 120–123, Jul. 2018, doi: 10.1016/j.matlet.2018.04.039.
- [60] H. Susi, J. S. Ard, and R. J. Carroll, ‘The infrared spectrum and water binding of collagen as a function of relative humidity’, *Biopolymers*, vol. 10, no. 9, pp. 1597–1604, 1971, doi: 10.1002/bip.360100913.
- [61] S. Chuaychan, S. Benjakul, and H. Kishimura, ‘Characteristics of acid- and pepsin-soluble collagens from scale of seabass (*Lates calcarifer*)’, *LWT - Food Science and Technology*, vol. 63, no. 1, pp. 71–76, Sep. 2015, doi: 10.1016/j.lwt.2015.03.002.
- [62] A. Fikai *et al.*, ‘Colagen / Hydroxyapatite Interactions in Composite Biomaterials’, *Materiale Plastice*, vol. 46, Jan. 2009.
- [63] M. Kikuchi *et al.*, ‘Biomimetic synthesis of bone-like nanocomposites using the self-organization mechanism of hydroxyapatite and collagen’, *Composites Science and Technology*, vol. 64, no. 6, pp. 819–825, May 2004, doi: 10.1016/j.compscitech.2003.09.002.
- [64] S.-H. Rhee, J. D. Lee, and J. Tanaka, ‘Nucleation of Hydroxyapatite Crystal through Chemical Interaction with Collagen’, *Journal of the American Ceramic Society*, vol. 83, no. 11, pp. 2890–2892, 2000, doi: 10.1111/j.1151-2916.2000.tb01656.x.
- [65] C.-H. Fang *et al.*, ‘Biomimetic Synthesis of Nanocrystalline Hydroxyapatite Composites: Therapeutic Potential and Effects on Bone Regeneration’, *Int J Mol Sci*, vol. 20, no. 23, p. 6002, Nov. 2019, doi: 10.3390/ijms20236002.

- [66] K. Gelse, E. Pöschl, and T. Aigner, ‘Collagens--structure, function, and biosynthesis’, *Adv Drug Deliv Rev*, vol. 55, no. 12, pp. 1531–1546, Nov. 2003, doi: 10.1016/j.addr.2003.08.002.
- [67] S. Nomura, A. Hiltner, J. B. Lando, and E. Baer, ‘Interaction of water with native collagen’, *Biopolymers*, vol. 16, no. 2, pp. 231–246, Feb. 1977, doi: 10.1002/bip.1977.360160202.
- [68] M. C. Varley, S. Neelakantan, T. W. Clyne, J. Dean, R. A. Brooks, and A. E. Markaki, ‘Cell structure, stiffness and permeability of freeze-dried collagen scaffolds in dry and hydrated states’, *Acta Biomaterialia*, vol. 33, pp. 166–175, Mar. 2016, doi: 10.1016/j.actbio.2016.01.041.
- [69] K. Sato, Y. Kumagai, and J. Tanaka, ‘Apatite formation on organic monolayers in simulated body environment’, *Journal of Biomedical Materials Research*, vol. 50, no. 1, pp. 16–20, 2000, doi: 10.1002/(SICI)1097-4636(200004)50:1<16::AID-JBM3>3.0.CO;2-G.
- [70] H. Akazawa and Y. Ueno, ‘Control of surface wettability of hydroxyapatite thin films by way of crystal imperfections’, *Appl. Phys. Express*, vol. 8, no. 10, p. 107001, Sep. 2015, doi: 10.7567/APEX.8.107001.
- [71] M. D. Grynpas, D. R. Eyre, and D. A. Kirschner, ‘Collagen type II differs from type I in native molecular packing’, *Biochimica et Biophysica Acta (BBA) - Protein Structure*, vol. 626, no. 2, pp. 346–355, Dec. 1980, doi: 10.1016/0005-2795(80)90129-4.
- [72] E. Wachtel and A. Maroudas, ‘The effects of pH and ionic strength on intrafibrillar hydration in articular cartilage’, *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1381, no. 1, pp. 37–48, Jun. 1998, doi: 10.1016/S0304-4165(97)00158-X.

- [73] C. Berthet, D. J. Hulmes, A. Miller, and P. A. Timmins, 'Structure of collagen in cartilage of intervertebral disk', *Science*, vol. 199, no. 4328, pp. 547–549, Feb. 1978, doi: 10.1126/science.622556.
- [74] D. R. Eyre and H. Muir, 'Collagen polymorphism: Two molecular species in pig intervertebral disc', *FEBS Letters*, vol. 42, no. 2, pp. 192–196, Jun. 1974, doi: 10.1016/0014-5793(74)80783-0.
- [75] E. J. Miller, 'Biochemical characteristics and biological significance of the genetically-distinct collagens', *Mol Cell Biochem*, vol. 13, no. 3, pp. 165–192, Dec. 1976, doi: 10.1007/BF01731779.
- [76] C. M. Murphy, A. Matsiko, M. G. Haugh, J. P. Gleeson, and F. J. O'Brien, 'Mesenchymal stem cell fate is regulated by the composition and mechanical properties of collagen–glycosaminoglycan scaffolds', *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 11, pp. 53–62, Jul. 2012, doi: 10.1016/j.jmbbm.2011.11.009.
- [77] B. A. Harley, J. H. Leung, E. C. C. M. Silva, and L. J. Gibson, 'Mechanical characterization of collagen-glycosaminoglycan scaffolds', *Acta Biomater*, vol. 3, no. 4, pp. 463–474, Jul. 2007, doi: 10.1016/j.actbio.2006.12.009.
- [78] B. P. Kanungo and L. J. Gibson, 'Density-property relationships in mineralized collagen-glycosaminoglycan scaffolds', *Acta Biomater*, vol. 5, no. 4, pp. 1006–1018, May 2009, doi: 10.1016/j.actbio.2008.11.029.
- [79] D. W. Weisgerber, D. O. Kelkhoff, S. R. Caliri, and B. a. C. Harley, 'The impact of discrete compartments of a multi-compartment collagen-GAG scaffold on overall construct biophysical properties', *J Mech Behav Biomed Mater*, vol. 28, pp. 26–36, Dec. 2013, doi: 10.1016/j.jmbbm.2013.07.016.

- [80] F. O'Brien, 'Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds.', Jan. 2011.
- [81] C. M. Tierney, M. G. Haugh, J. Liedl, F. Mulcahy, B. Hayes, and F. J. O'Brien, 'The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering', *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 2, pp. 202–209, Apr. 2009, doi: 10.1016/j.jmbbm.2008.08.007.
- [82] M. B. Keogh, F. J. O'Brien, and J. S. Daly, 'Substrate stiffness and contractile behaviour modulate the functional maturation of osteoblasts on a collagen–GAG scaffold', *Acta Biomaterialia*, vol. 6, no. 11, pp. 4305–4313, Nov. 2010, doi: 10.1016/j.actbio.2010.06.001.
- [83] C. G. Finkemeier, 'Bone-grafting and bone-graft substitutes', *J Bone Joint Surg Am*, vol. 84, no. 3, pp. 454–464, Mar. 2002, doi: 10.2106/00004623-200203000-00020.
- [84] R. Langer and N. Peppas, 'Chemical and Physical Structure of Polymers as Carriers for Controlled Release of Bioactive Agents: A Review', *Journal of Macromolecular Science, Part C*, vol. 23, no. 1, pp. 61–126, Jan. 1983, doi: 10.1080/07366578308079439.
- [85] R. Visse and H. Nagase, 'Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry', *Circ Res*, vol. 92, no. 8, pp. 827–839, May 2003, doi: 10.1161/01.RES.0000070112.80711.3D.
- [86] L. Chung *et al.*, 'Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis', *EMBO J*, vol. 23, no. 15, pp. 3020–3030, Aug. 2004, doi: 10.1038/sj.emboj.7600318.

- [87] J. M. Lee, H. H. L. Edwards, C. A. Pereira, and S. I. Samii, 'Crosslinking of tissue-derived biomaterials in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)', *J Mater Sci: Mater Med*, vol. 7, no. 9, pp. 531–541, Sep. 1996, doi: 10.1007/BF00122176.
- [88] I. Yannas, J. Burke, P. Gordon, C. Huang, and R. Rubenstein, 'Design of an artificial skin. II. Control of chemical composition', *Journal of Biomedical Materials Research*, vol. 14, no. 2, pp. 107–132, 1980, doi: 10.1002/jbm.820140203.
- [89] Y. S. Pek, M. Spector, I. V. Yannas, and L. J. Gibson, 'Degradation of a collagen–chondroitin-6-sulfate matrix by collagenase and by chondroitinase', *Biomaterials*, vol. 25, no. 3, pp. 473–482, Feb. 2004, doi: 10.1016/S0142-9612(03)00541-6.

Chapter 6 - In Vitro Evaluation of Scaffolds

6.1. Introduction

In vitro studies were conducted in collaboration with researchers at Kafrelsheikh University. We performed *in vitro* studies to evaluate the clinical potential of the collagen-based scaffolds for the regeneration of the ligament bone interface. Cell studies were first conducted to test biological properties such as biocompatibility and differentiation in the scaffolds *in vitro*.

The study was approved by the Institutional Animal Use and Care Unit of Kafrelsheikh University. Three male Sprague-Dawley rats (age 6 weeks, weight 200 g) were kept in conventional pathogen-free cages. They had *ad libitum* access to water and commercial food. The light cycle of 12 h light and 12 h darkness was maintained and humidity of 40-60% was adjusted.

Phosphate buffer saline (PBS) and complete mesenchymal stem cells medium were purchased from Thermofisher, USA. CD44P-E, CD45-PE, CD105 AlexaFluor 555, CD11b-PE, CD90-FITC, CD73 AlexaFluor 555, Ki67-FITC and Hoechst 33342 were purchased from Invitrogen, USA.

Mesenchymal stem cells (MSCs) are multipotent precursor cells. MSCs possess the ability to self-renew and differentiate into several lineages, these include osteoblasts, chondrocytes and fibroblasts which are the main cell types present throughout the ligament-bone enthesis (see Section 2.3.2). Furthermore, their relative abundance and ease of isolation make them an attractive cell source for *in vitro* study [1], [2].

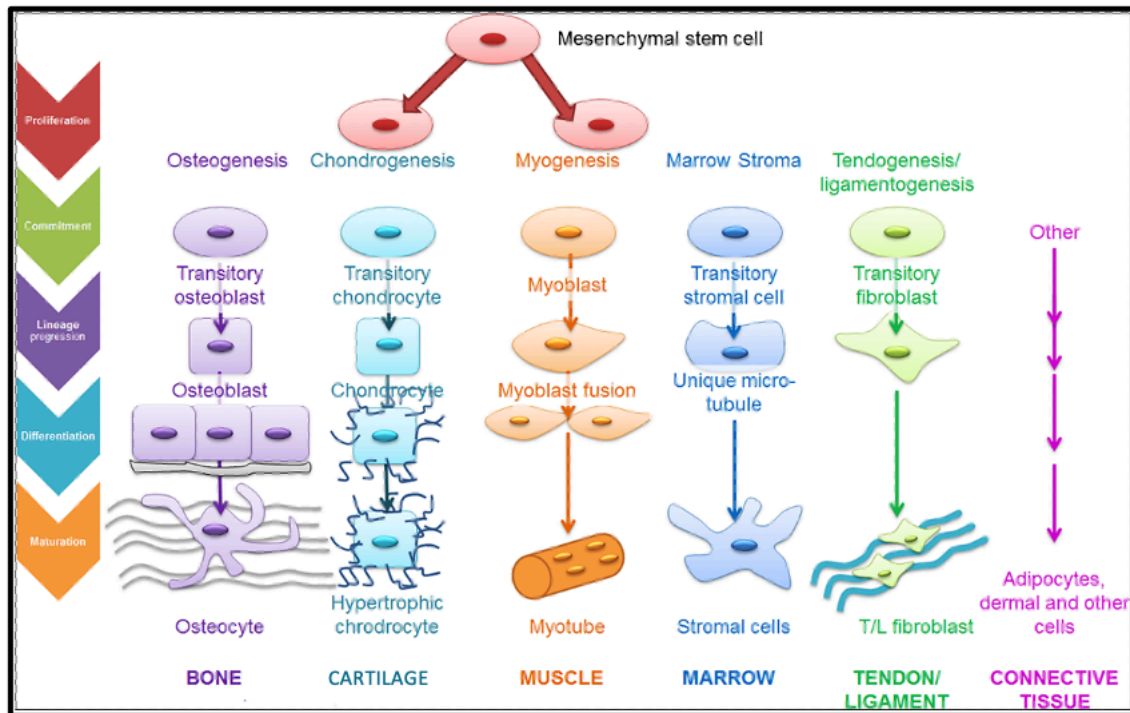


Figure 6.1: The differentiation lineages of mesenchymal stem cells [3]

This chapter will focus on the beginnings of *in vitro* studies to evaluate the effectiveness of the designed scaffolds for tissue regeneration of the enthesis and will outline ongoing and future plans. Preliminary cell studies were conducted to test the biological properties of scaffolds, such as biocompatibility and cell adhesion.

6.2. Techniques

6.2.1. Immunophenotyping and Flow Cytometry

Immunophenotyping is the analysis of the types of antigens or “markers” on the surface of the cells to identify the presence and proportions of the various populations of interest. Antibodies are used to identify cells by detecting specific antigens expressed by the cells. These markers are usually functional membrane proteins involved in cell communication, adhesion, or metabolism.

Cell markers are a very useful way to identify a specific cell population. However, often more than one cell type will express each marker, therefore, it is important to test for two or more antibodies simultaneously for immunophenotyping cells. By evaluating the unique repertoire of cell markers using several antibodies together, each coupled with different fluorochromes, a given population can be identified and quantified. A common immune cell marker is a CD marker, which stands for "cluster of differentiation". CDs are commonly used for detection in flow cytometry of specific immune cell (leukocytes) populations and subpopulations.

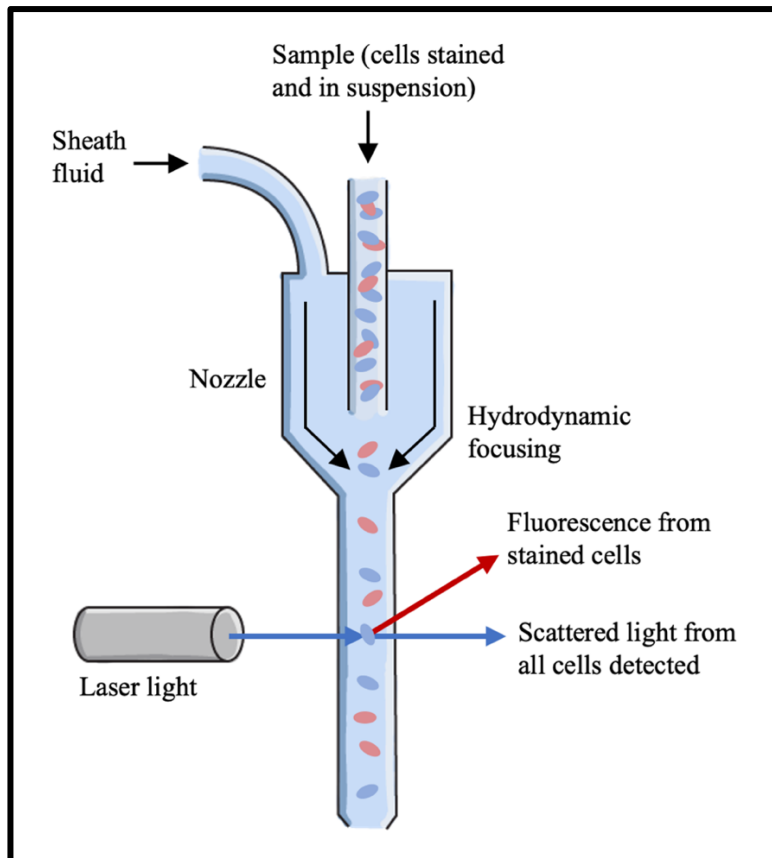


Figure 6.2: Flow cytometry schematic.

Flow cytometry is a cell analysis technique that is used to simultaneously measure and then analyse multiple physical characteristics of single particles, usually cells, as they flow past a laser while suspended in a buffered salt solution [4].

The fluidics system, illustrated in Figure 6.2, transports the particles in a single file stream to the laser beam for interrogation. The lasers illuminate the particles in the sample stream and optical filters direct the resulting light signals to the appropriate detectors. The system uses an optical-to-electronic coupling system to record how the cells or particles detected scatter incident laser light and emit fluorescence. This allows for properties such as a particle's relative size, granularity, internal complexity, and relative fluorescence intensity to be determined.

6.2.2. Confocal Microscopy

Similar to the fluorescence microscope, the confocal uses fluorescence optics, however, instead of illuminating the whole sample at once, laser light is focused onto a defined spot at a specific depth within the sample. This leads to the emission of fluorescent light at exactly this point. Pinholes inside the optical pathway cut off signals that are out of focus, thus allowing only the fluorescence signals from the illuminated spot to enter the light detector. By scanning the specimen in a raster pattern, images of a single optical plane are created, and 3D objects can be visualised by scanning several planes and stacking them. It is also possible to analyse multicolour immunofluorescence staining using several lasers and emission/ excitation filters.

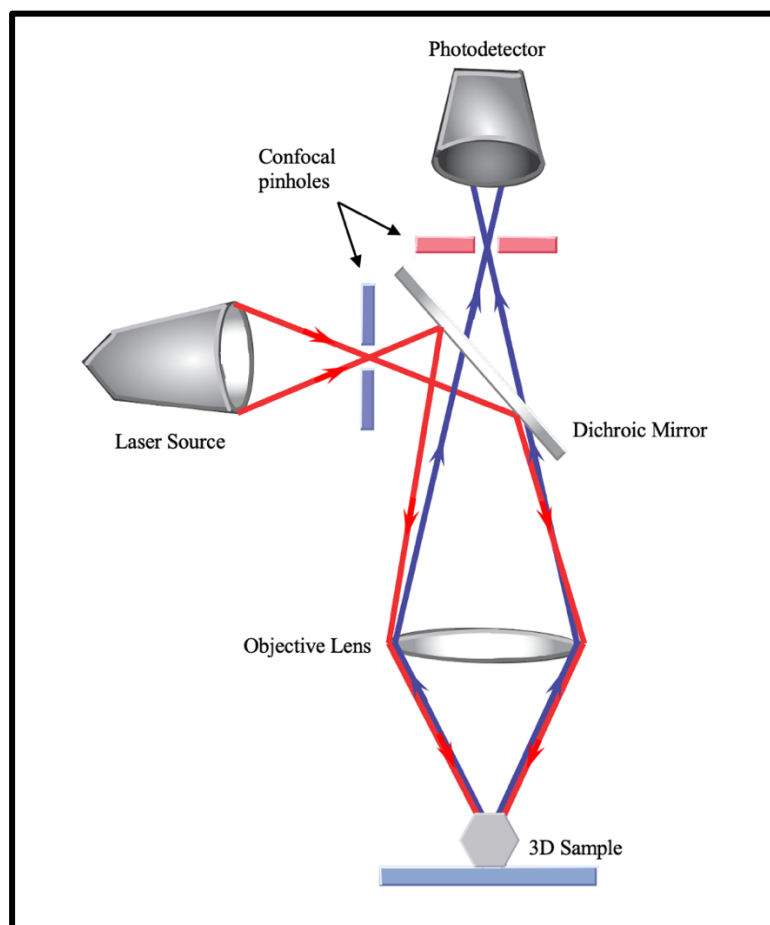


Figure 6.3: Confocal Microscopy schematic.

6.3. Methods

6.3.1. Scaffold Preparation

Three types of mono-layer scaffolds were prepared: Pure collagen type I (soft tissue layer), Collagen type II + chondroitin sulphate (uncalcified fibrocartilage layer) and Collagen type I + HAp (HAp: Collagen = 7:3, calcified tissue). Suspensions of collagen-based scaffolds from monomeric atelocollagen for *in vitro* studies were prepared as described in Chapter 5.

For monolayer scaffold fabrication for *in vitro* studies, individual collagen solutions were cast into cylindrical PTFE moulds of diameter 2mm using a syringe, frozen at -20°C and then lyophilised to obtain the porous scaffolds.

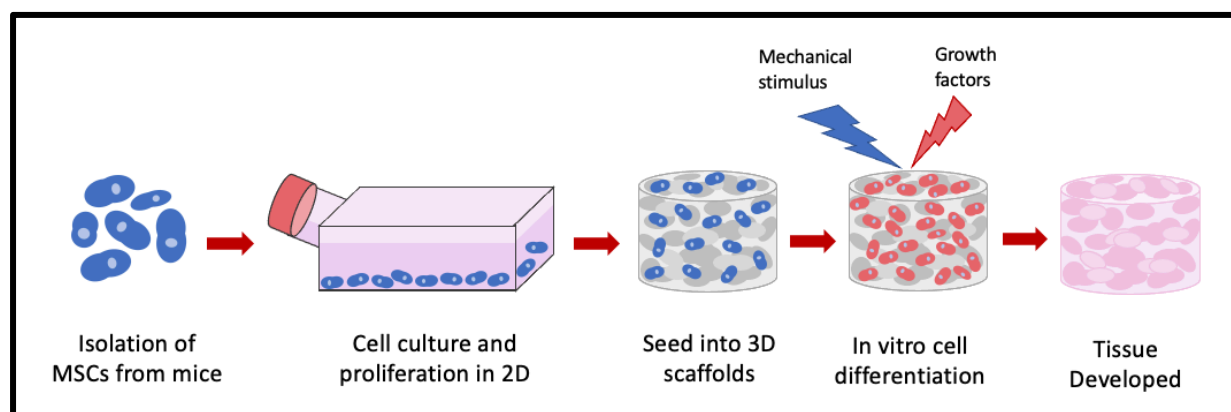


Figure 6.4: Schematic of *in vitro* tissue development.

6.3.2. Cell Isolation and Culture

After the rats were euthanised by cervical dislocation, bone marrow aspirate was extracted from the femora and tibia bone, under aseptic conditions, the aspirate was resuspended in PBS and cells were collected by centrifugation for 5 min at 3000 rpm using Velab Centrifuge (VE-4000) and discarding of the supernatant.

The cells were resuspended with complete MSC medium, transferred to sterile culture flasks and cultured at 37°C and 5% CO₂. Suspended cells were regarded as aspirated and media was replaced with fresh every 3, 8, 12, 24, 48 and 72 hrs of culture [5].

6.3.3. Characterisation of Mesenchymal Stem Cells

The adherent cells were dissociated by trypsinisation. Flow cytometry using FACSCalibur flow cytometer (BD Biosciences) was applied to quantify the expression of the following CDs: CD44, CD45, CD11b, CD105, CD90 and CD73. At least 1×10^4 cells were examined per assay and data were analysed using Flowing Software 2.5.1 (Turku Center for Biotechnology, Turku University, Finland). The Geometrical Means (Geomean) of the respective histograms were automatically calculated after gating the desired population [6].

6.3.4. Biocompatibility of Scaffolds

Mesenchymal stem cells (2×10^5) were cultured with each mono-layered cylindrical scaffold (soft tissue, uncalcified fibrocartilage and calcified layer), 2 mm in diameter and 2 mm in thickness for 6 weeks in a 24-well plate. At the end of this duration, cells adherent to the scaffold were stained with the fluorescently labelled antibody Ki67-FITC to detect living cells [7].

6.3.5. Adhesion and Infiltration Evaluation of Mesenchymal Stem Cells on Scaffolds

To assess cellular adhesion, scaffold surfaces were stained with Hoechst 33342 to detect nuclei of cells attached to the scaffold surface [8]. Samples were examined by confocal laser scanning microscopy (Zeiss LSM710, Jena, Germany, excitation at 488 nm and emission at 521 nm). The cell infiltration into the scaffolds was assessed by sectioning the scaffolds 1 μ m down and staining the exposed surface with Hoechst 33342.

6.4. Results and Discussion

6.4.1. Scaffold Preparation

It was not possible to send samples of all 4 types of collagen-based scaffolds discussed in Chapter 5 mimicking each distinct region of the enthesis due to restrictions in materials, resources, and funding. Scaffolds of HAp: Collagen ratio 7:3 were chosen as the calcified tissue layer over 4:1, as scaffolds of 7:3 ratio were able to withstand degradation longer than those of 4:1 ratio. Scaffolds of a 7:3 ratio were chosen as they would be able to withstand the demands of shipping and culturing in media for months. From here on out, collagen scaffolds with a ratio of collagen type I to hydroxyapatite will be referred to as Col I + HAp.

6.4.2. Mesenchymal Stem Cells' Expression of Surface Markers

MSCs are recognised by the expression of unique cell surface markers. Therefore, cells were tested for expression of CD44, CD45, CD11b, CD73, CD90 and CD105 (Figure 6.5-6.9). Expression of surface molecules CD44, CD73, and CD90 typical of stem and progenitor mesenchymal cells were observed. Whereas expression of hematopoietic stem cell marker CD45 and myeloid-derived suppressor cell marker CD11b were not detected. These results are in agreement with published data as a confirmation for mesenchymal stem cells [9].

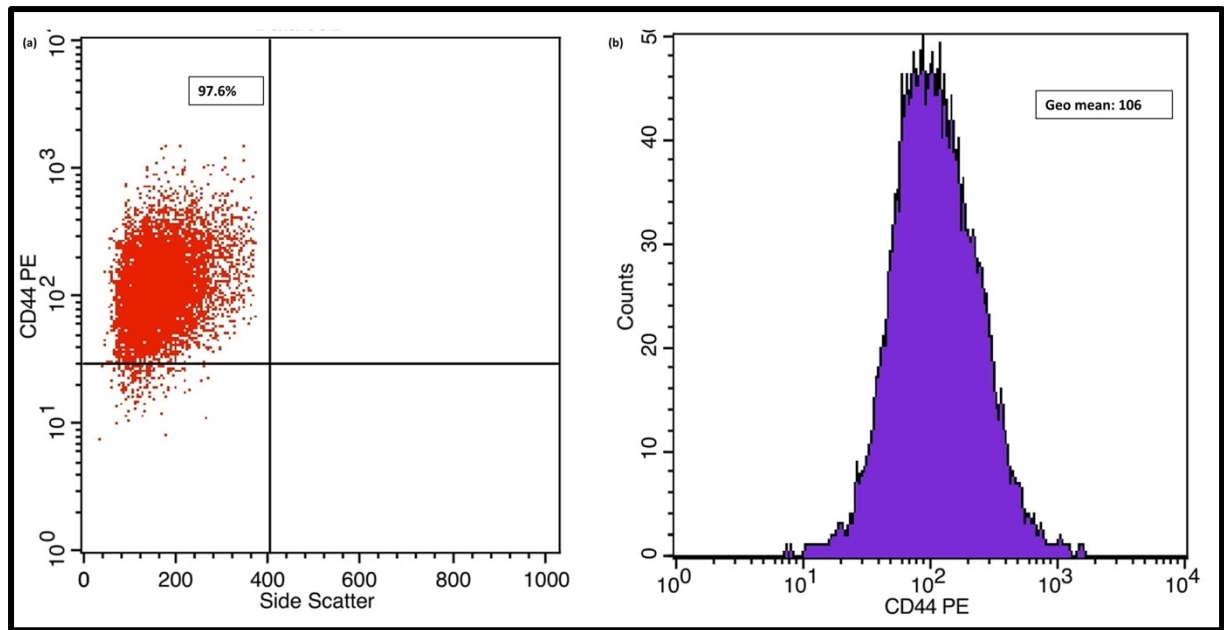


Figure 6.5: (a) representative dot plot from flow cytometry analysed for CD44 surface expression with (b) Geometric mean fluorescence intensity of CD44 markers.

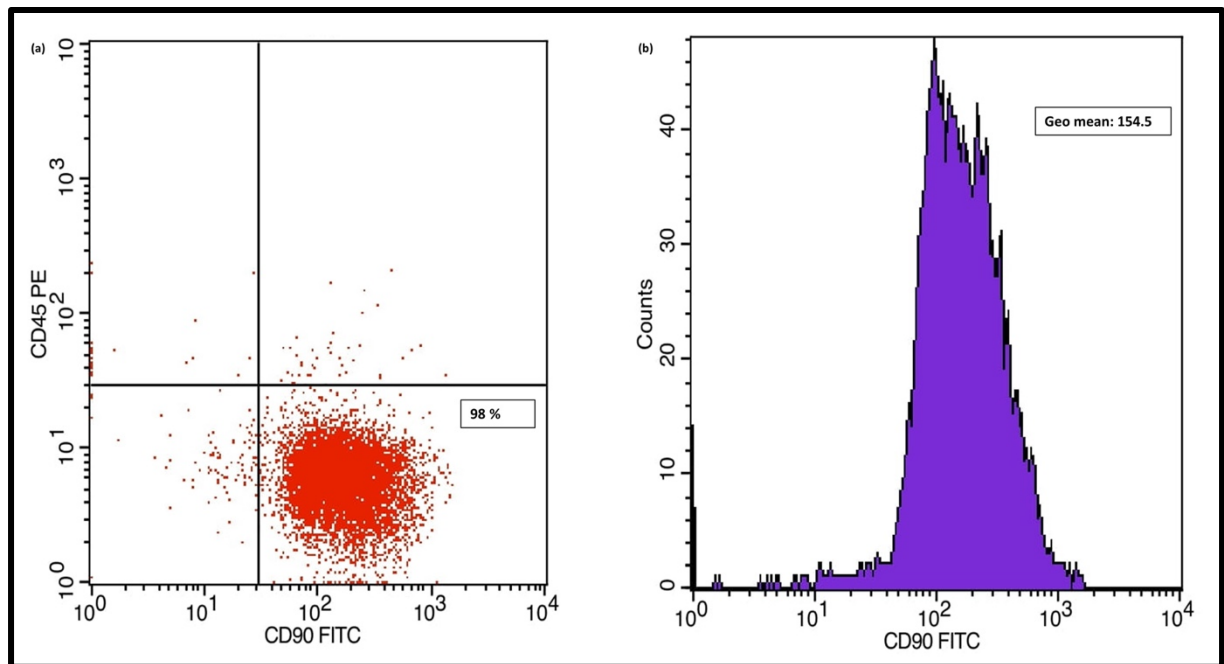


Figure 6.6: (a) representative dot plot from flow cytometry analysed for CD45 and CD90 surface expressions with (b) Geometric mean fluorescence intensity of CD90 markers.

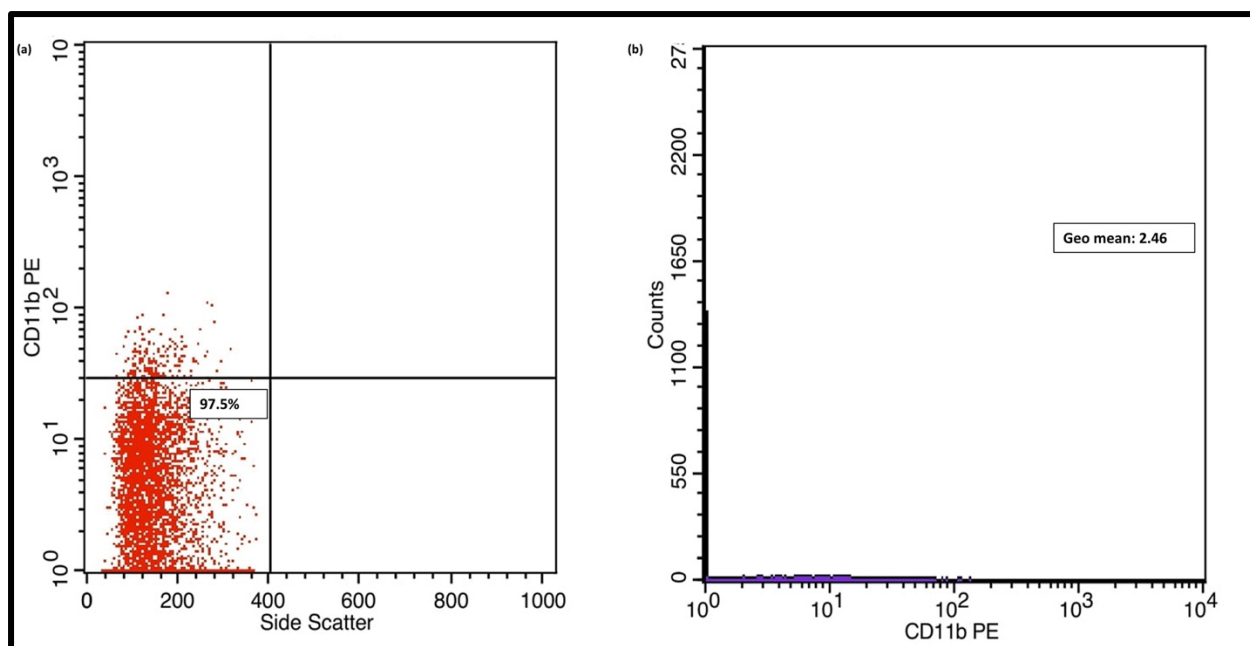


Figure 6.7: (a) representative dot plot from flow cytometry analysed for CD11b surface expression with (b) Geometric mean fluorescence intensity of CD11b markers.

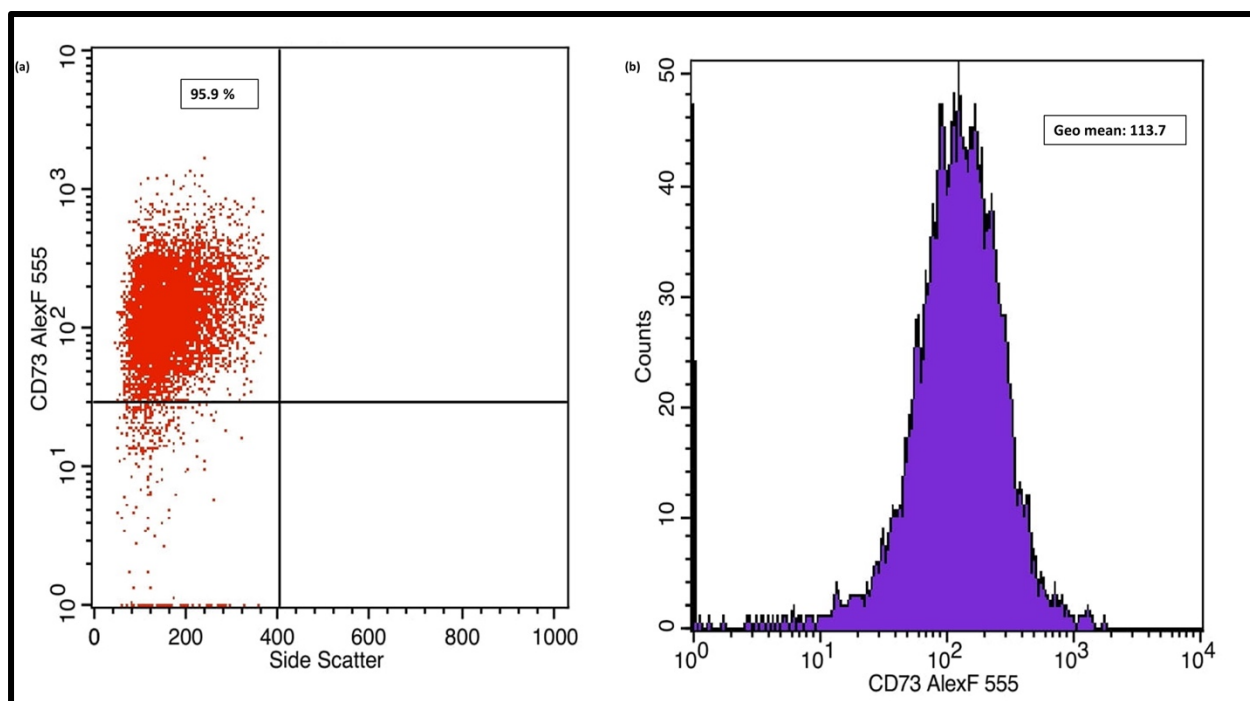


Figure 6.8: (a) representative dot plot from flow cytometry analysed for CD73b surface expression with (b) Geometric mean fluorescence intensity of CD73 markers.

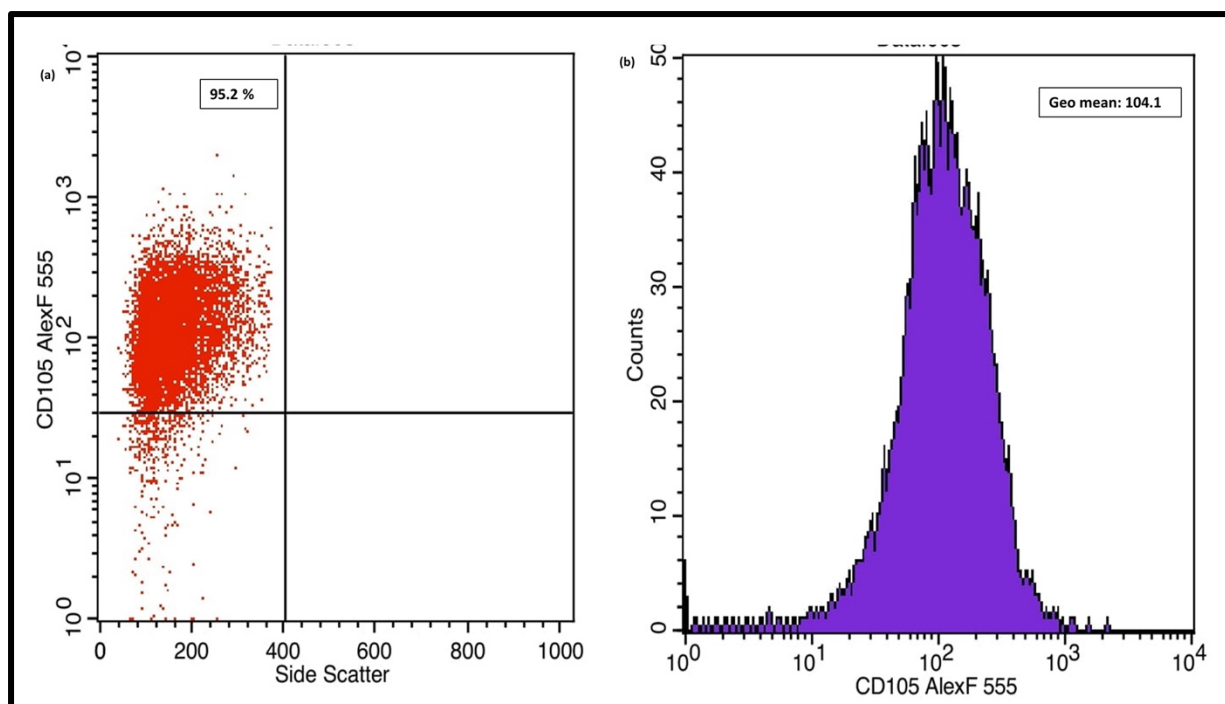


Figure 6.9: (a) representative dot plot from flow cytometry analysed for CD105 surface expression with (b) Geometric mean fluorescence intensity of CD105 markers.

6.4.3. Scaffolds Biocompatibility

Ki67 is a nuclear antigen that is an excellent marker of active cell proliferation. All 3 collagen-based scaffolds presented positive cells for marker Ki67 of 80% or greater, illustrated in Figures 6.10-6.12. This shows the active proliferation of the MSCs and indicates that the scaffolds are not exerting a cytotoxic effect on the cells.

These results demonstrate the biocompatible nature of the collagen-based scaffolds and confirm that there are no toxic residual solvents or chemicals being released during the degradation of the scaffolds. Our findings are in agreement with previous reports about the biocompatibility of collagen-based scaffolds [10], [11].

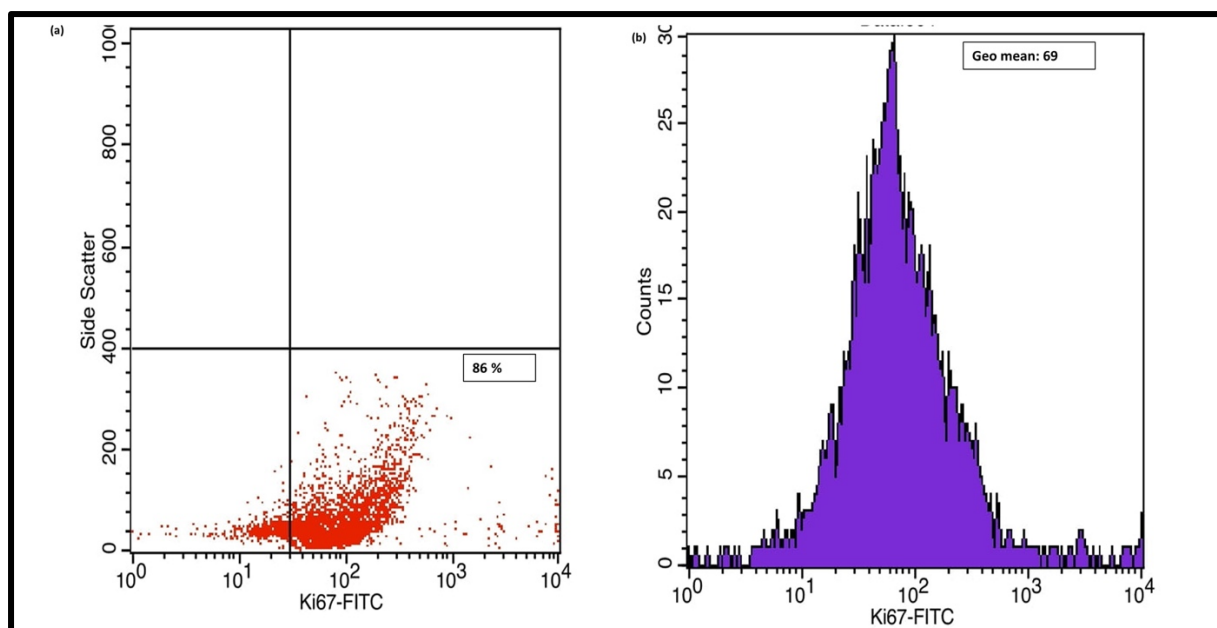


Figure 6.10: Flow-cytometric analysis of soft tissue layer collagen-based scaffold (a)representative dot plot from flow cytometry analysed for Ki67 antibody stained against FITC surface expressions with (b) Geometric mean fluorescence intensity of Ki67-FITC.

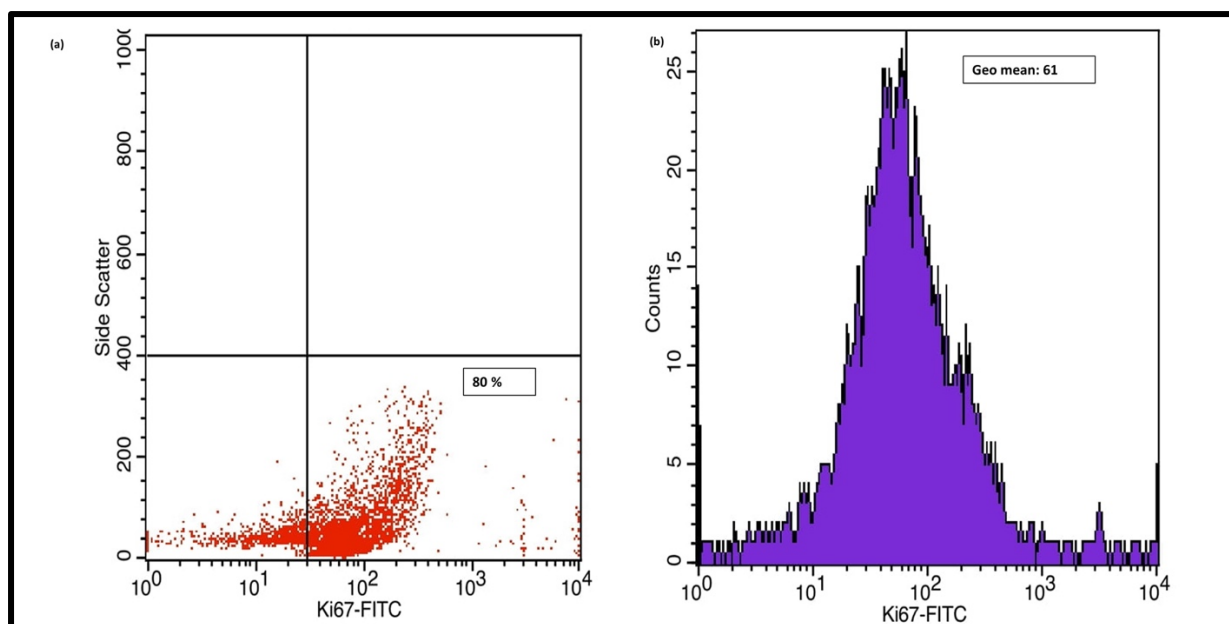


Figure 6.11: Flow-cytometric analysis of uncalcified fibrocartilage layer collagen-based scaffold (a)representative dot plot from flow cytometry analysed for Ki67 antibody stained against FITC surface expressions with (b) Geometric mean fluorescence intensity of Ki67-FITC.

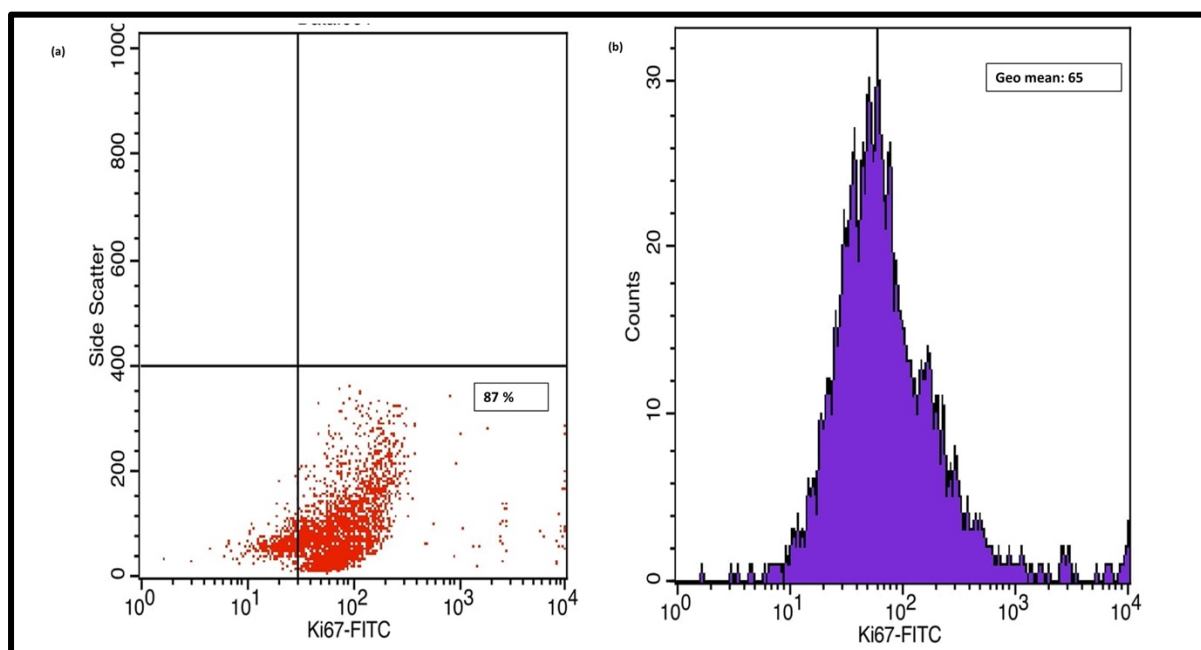


Figure 6.12: Flow-cytometric analysis of calcified tissue layer collagen-based scaffold (a)representative dot plot from flow cytometry analysed for Ki67 antibody stained against FITC surface expressions with (b) Geometric mean fluorescence intensity of Ki67-FITC.

6.4.4. Scaffold-Mesenchymal Stem Cell Interaction

Hoechst 33342 is a blue-emitting fluorescent DNA dye which permeates cell membranes and binds to the minor groove of A/T-rich dsDNA sequences, thus preferentially staining nuclei. It is commonly used to detect dividing cells.

Hoechst 33342 stained scaffolds were positive indicating the adhesion of cells to the scaffolds. Presented Figures 6.13-6.16 are topological views of the scaffolds. Sections taken at a depth of 1 μm (Figure 6.16) show the presence of cells which indicate that the cells had infiltrated the scaffolds and were within the matrix. Similar results were found for all scaffold types. This adhesion is a pre-requisite for mesenchymal stem cell nesting, propagation and differentiation [11].

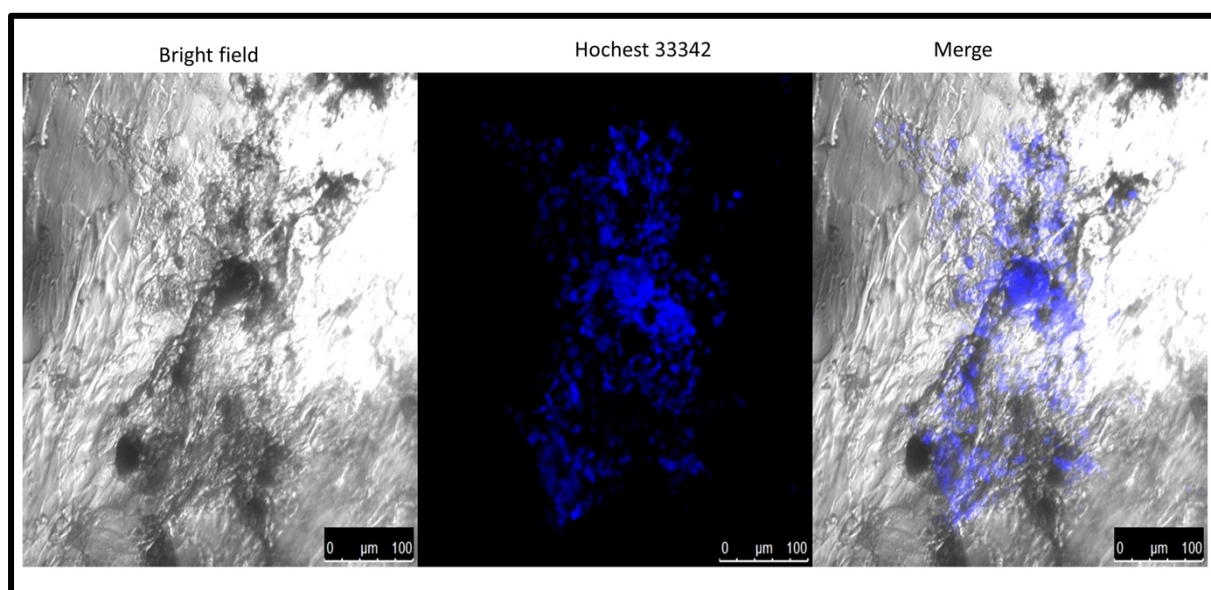


Figure 6.13: Representative confocal imaging of Hoechst 33342 stained Col I sample surface.

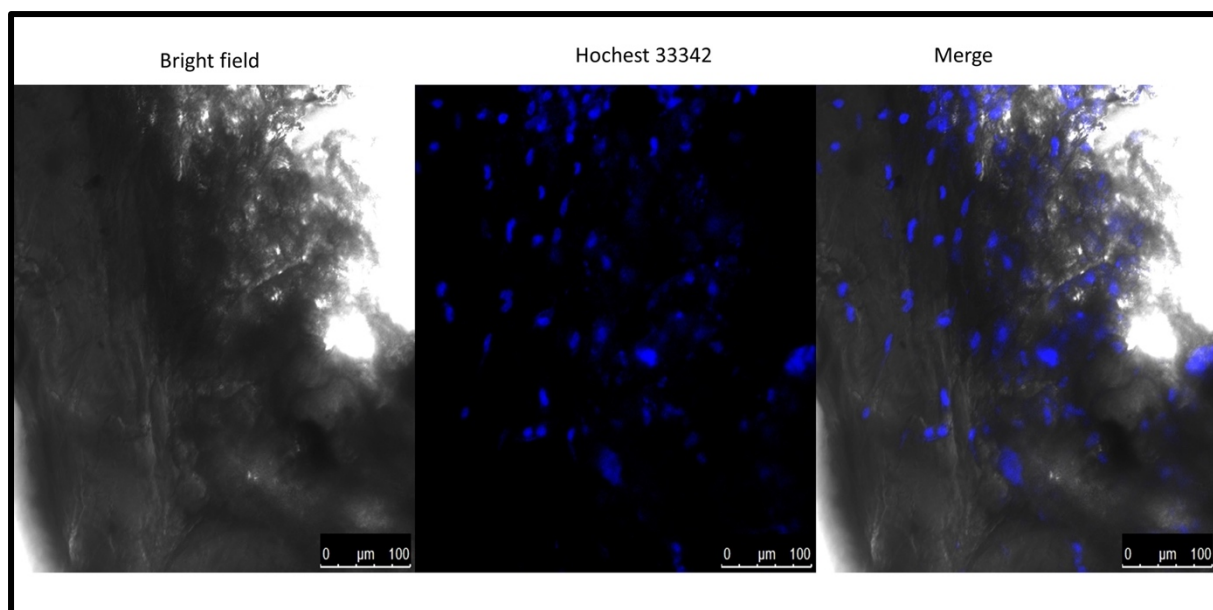


Figure 6.14: Representative confocal imaging of Hoechst 33342 stained Col II + CS sample surface.

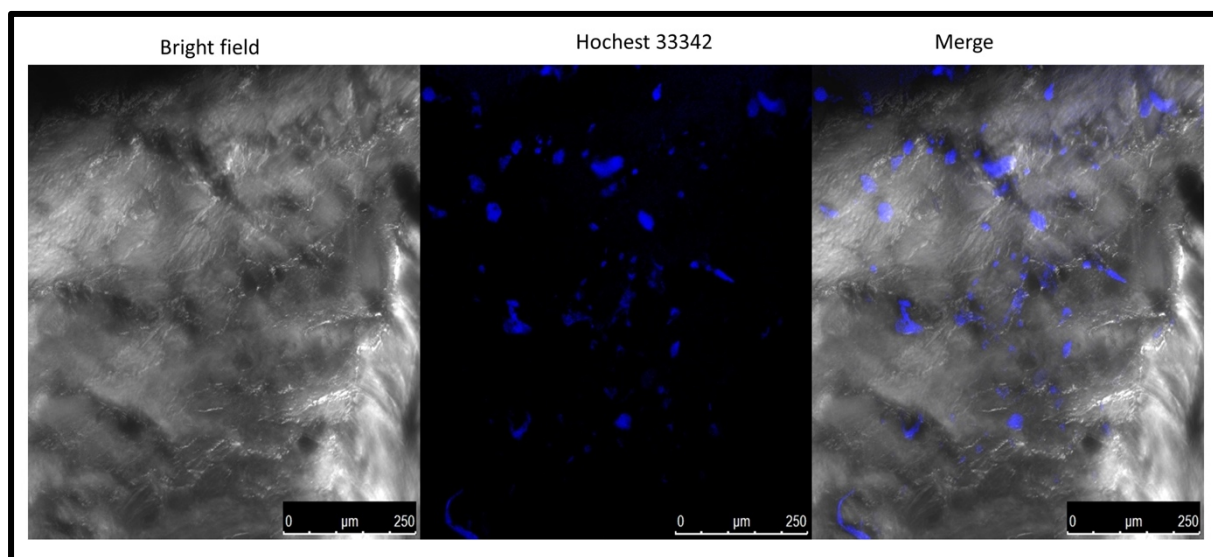


Figure 6.15: Representative confocal imaging of Hoechst 33342 stained Col I + HAp sample surface.

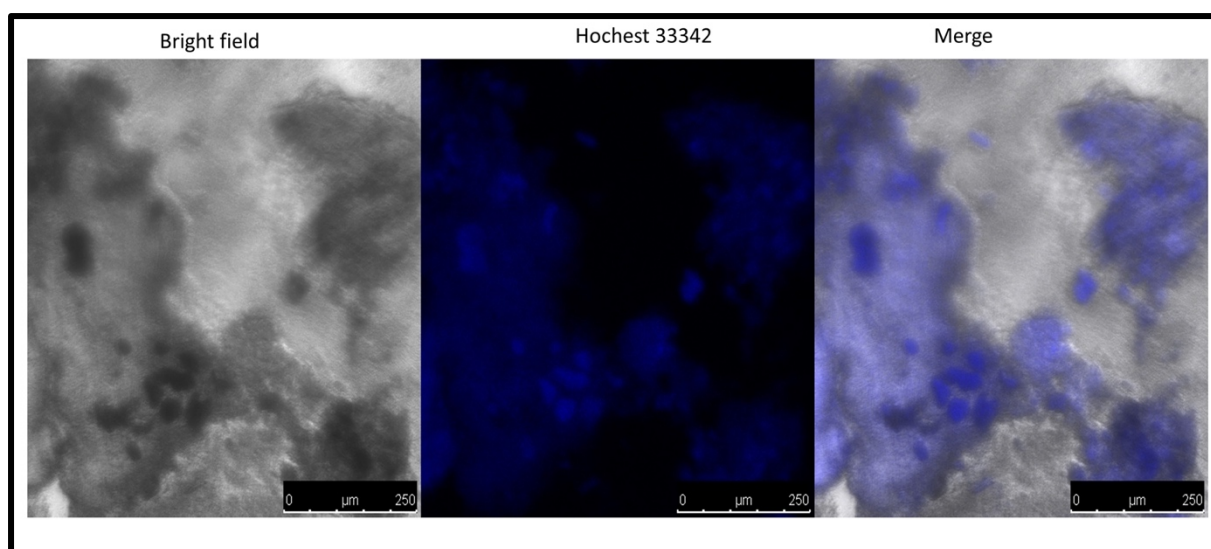


Figure 6.16: Representative confocal imaging of Hoechst 33342 stained Col I sample 1 micrometre depth.

6.5. What Does This Mean and Future Work

Overall, the biocompatible nature of all 3 collagen-based scaffolds has been shown through the 80% or greater result readings for nuclear antigen marker Ki67, indicating active proliferation of MSCs and that the scaffolds are not exerting a cytotoxic effect on the cells, and positive staining of Hoechst 33342, demonstrating cell adhesion of cells to scaffolds and migration of the cells towards the centre of the scaffolds. These results are a strong indication of the biocompatibility of the designed scaffolds and are the first steps in showing the viability of these scaffolds as an implantable device for tissue growth. The next steps would be to identify the differentiation and proliferation potential of these scaffolds.

Currently, MSCs are being differentiated into 3 different cell lines: fibroblasts, chondrocytes, and osteoblasts. These are the most common types of cells found in the different regions of the ligament to bone enthesis. All 3 differentiated cell lines will be implanted into the 3 single-layer scaffold types. It is hypothesised that greater proliferation of cells will be observed on their corresponding scaffolds. The next step will be to implant undifferentiated MSCs onto the single-layer scaffolds to monitor MSC lineages and determine whether the scaffold composition and microstructure can influence stem cell differentiation. As discussed in Section 1.8, cell behaviour is influenced via mechanotransduction, and reports have shown that matrix elasticity, which influences cell-ECM contact, can regulate how MSCs differentiate into distinct lineages by regulating MSC destiny and direct gene expression [12]–[14]. For instance, the matrix is deformed as a result of MSC's interaction with elastic ECM, a stiffer matrix efficiently resists the forces generated by cells and stimulates spreading promoting differentiation of MSCs towards osteogenic lineage [14]–[17]. Furthermore, chemical differences between the scaffolds should also influence cell lineage. Chondroitin sulphate is known to promote chondrogenic cell lineage [18], [19], whilst hydroxyapatite is osteogenic

[20], [21]. Therefore, we expect to find that MSCs implanted onto the 3 types of single-layer scaffolds (col I, Col II + CS and Col I + HAp) will differentiate towards their respective lineages through the influence of the chemical and mechanical environments of the scaffolds alone, without the need for growth factors.

Following on from there, we will endeavour to use bi-layered scaffolds to differentiate each region's respective cell type on their corresponding layer on a single scaffold. Before moving on to using tri-layered scaffolds to develop the entire bone-to-ligament interface in a single scaffold. The 2 types of bi-layered scaffolds, shown in Figure 7.17, have been produced with great structural integrity and porosity, as discussed in Chapter 6.

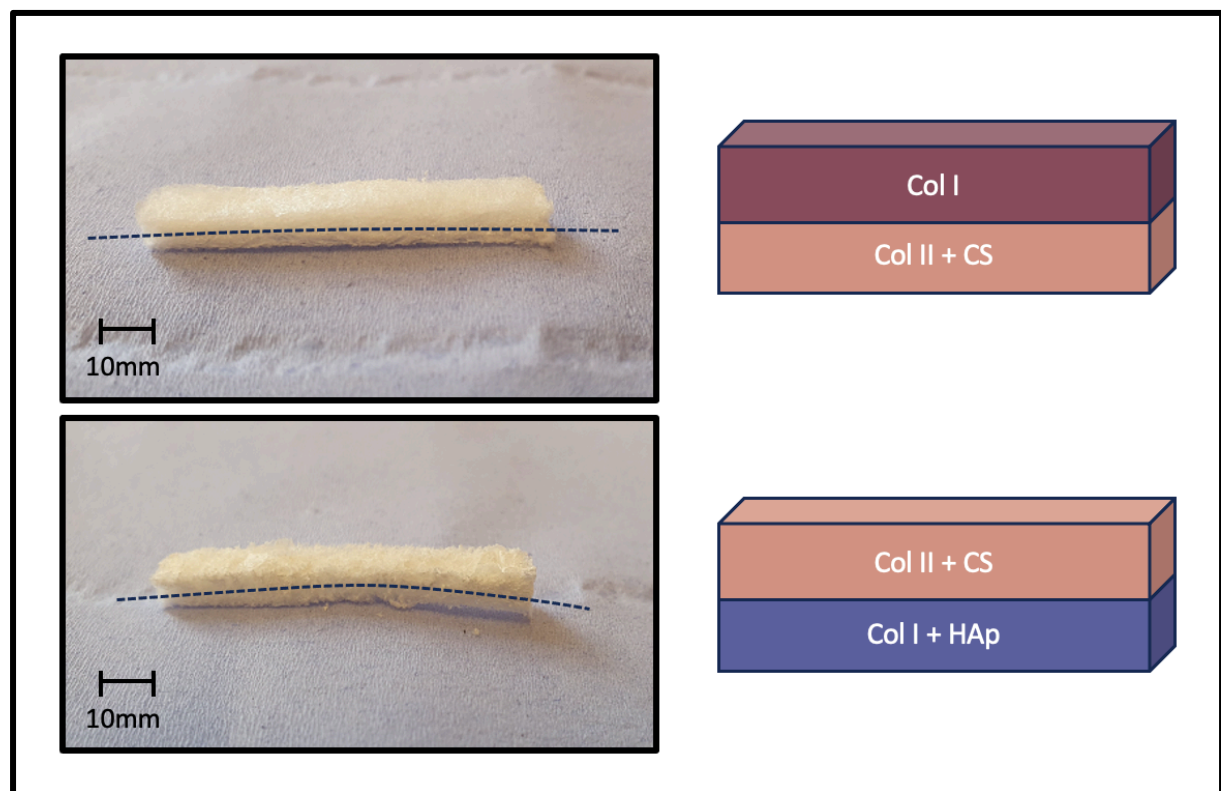


Figure 6.17: Bi-layered scaffolds for in vitro studies. Figures on the left are images of the physical bi-layered scaffolds whilst figures on the right are illustrations of the corresponding

scaffolds. Above, collagen type I layer on top, with collagen type II and chondroitin sulphate. Below, collagen type II and chondroitin sulphate on top, with collagen I and hydroxyapatite. Dashed lines indicate the intersection of the two layers. Scale bars 10mm.

6.6. References

- [1] M. Cucchiarini, J. K. Venkatesan, M. Ekici, G. Schmitt, and H. Madry, ‘Human mesenchymal stem cells overexpressing therapeutic genes: From basic science to clinical applications for articular cartilage repair’, *Bio-Medical Materials and Engineering*, vol. 22, no. 4, pp. 197–208, Jan. 2012, doi: 10.3233/BME-2012-0709.
- [2] T. D. Bornes, A. B. Adesida, and N. M. Jomha, ‘Mesenchymal stem cells in the treatment of traumatic articular cartilage defects: a comprehensive review’, *Arthritis Research & Therapy*, vol. 16, no. 5, p. 432, Sep. 2014, doi: 10.1186/s13075-014-0432-1.
- [3] M. Shariatzadeh, J. Song, and S. Wilson, ‘The efficacy of different sources of mesenchymal stem cells for the treatment of knee osteoarthritis’, *Cell and Tissue Research*, vol. 378, Dec. 2019, doi: 10.1007/s00441-019-03069-9.
- [4] K. M. McKinnon, ‘Flow Cytometry: An Overview’, *Curr Protoc Immunol*, vol. 120, p. 5.1.1-5.1.11, Feb. 2018, doi: 10.1002/cpim.40.
- [5] A. Smajilagić, M. Aljičević, A. Redžić, S. Filipović, and A. Lagumdžija, ‘Rat bone marrow stem cells isolation and culture as a bone formative experimental system.’, *Bosnian journal of basic medical sciences*, vol. 13, no. 1, pp. 27–30, Feb. 2013, doi: 10.17305/bjbms.2013.2409.
- [6] S. A. Abdel Gaber *et al.*, ‘ABCG2-mediated suppression of chlorin e6 accumulation and photodynamic therapy efficiency in glioblastoma cell lines can be reversed by KO143’, *Journal of Photochemistry and Photobiology B: Biology*, vol. 178, pp. 182–191, Jan. 2018, doi: 10.1016/j.jphotobiol.2017.10.035.
- [7] Y.-L. Wu *et al.*, ‘Mesenchymal stem cell-derived CCN2 promotes the proliferation, migration and invasion of human tongue squamous cell carcinoma cells’, *Cancer Science*, vol. 108, no. 5, pp. 897–909, 2017, doi: 10.1111/cas.13202.

- [8] M. A. Goodell, ‘Stem cell identification and sorting using the Hoechst 33342 side population (SP).’, *Current protocols in cytometry*, vol. Chapter 9, p. Unit9.18, Nov. 2005, doi: 10.1002/0471142956.cy0918s34.
- [9] M. Dominici *et al.*, ‘Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.’, *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006, doi: 10.1080/14653240600855905.
- [10] Yang Xu, ‘Development of Tri-layered Biomimetic Atelocollagen Scaffolds with Interfaces for Osteochondral Tissue Engineering’, University of Oxford, 2022.
- [11] P. Sananta *et al.*, ‘Collagen scaffold for mesenchymal stem cell from stromal vascular fraction (biocompatibility and attachment study): Experimental paper.’, *Annals of medicine and surgery (2012)*, vol. 59, pp. 31–34, Nov. 2020, doi: 10.1016/j.amsu.2020.07.055.
- [12] Y.-K. Wang and C. S. Chen, ‘Cell adhesion and mechanical stimulation in the regulation of mesenchymal stem cell differentiation’, *Journal of Cellular and Molecular Medicine*, vol. 17, no. 7, pp. 823–832, 2013, doi: 10.1111/jcmm.12061.
- [13] A. A. Saidova and I. A. Vorobjev, ‘Lineage Commitment, Signaling Pathways, and the Cytoskeleton Systems in Mesenchymal Stem Cells’, *Tissue Engineering Part B: Reviews*, vol. 26, no. 1, pp. 13–25, Feb. 2020, doi: 10.1089/ten.teb.2019.0250.
- [14] M. Sun *et al.*, ‘Effects of Matrix Stiffness on the Morphology, Adhesion, Proliferation and Osteogenic Differentiation of Mesenchymal Stem Cells’, *Int J Med Sci*, vol. 15, no. 3, pp. 257–268, Jan. 2018, doi: 10.7150/ijms.21620.
- [15] C.-M. Lo, H.-B. Wang, M. Dembo, and Y. Wang, ‘Cell Movement Is Guided by the Rigidity of the Substrate’, *Biophysical Journal*, vol. 79, no. 1, pp. 144–152, Jul. 2000, doi: 10.1016/S0006-3495(00)76279-5.

- [16] T. Yeung *et al.*, ‘Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion’, *Cell Motility*, vol. 60, no. 1, pp. 24–34, 2005, doi: 10.1002/cm.20041.
- [17] M. L. Knothe Tate, T. D. Falls, S. H. McBride, R. Atit, and U. R. Knothe, ‘Mechanical Modulation of Osteochondroprogenitor Cell Fate’, *Int J Biochem Cell Biol*, vol. 40, no. 12, pp. 2720–2738, 2008, doi: 10.1016/j.biocel.2008.05.011.
- [18] D. Bosnakovski, M. Mizuno, G. Kim, S. Takagi, M. Okumura, and T. Fujinaga, ‘Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis’, *Biotechnol Bioeng*, vol. 93, no. 6, pp. 1152–1163, Apr. 2006, doi: 10.1002/bit.20828.
- [19] S. Varghese, N. S. Hwang, A. C. Canver, P. Theprungsirikul, D. W. Lin, and J. Elisseeff, ‘Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells’, *Matrix Biol*, vol. 27, no. 1, pp. 12–21, Jan. 2008, doi: 10.1016/j.matbio.2007.07.002.
- [20] K. J. L. Burg, S. Porter, and J. F. Kellam, ‘Biomaterial developments for bone tissue engineering’, *Biomaterials*, vol. 21, no. 23, pp. 2347–2359, Dec. 2000, doi: 10.1016/S0142-9612(00)00102-2.
- [21] H. Zhou and J. Lee, ‘Nanoscale hydroxyapatite particles for bone tissue engineering’, *Acta Biomaterialia*, vol. 7, no. 7, pp. 2769–2781, Jul. 2011, doi: 10.1016/j.actbio.2011.03.019.

Chapter 7 - Thesis Summary and Future Work

7.1. Thesis Summary

Tissue engineering of the ligament-bone enthesis seeks to improve the repair and regeneration of damaged or diseased tissue which current treatments cannot completely achieve and in turn, eliminate graft drawbacks and the need for further intervention. This study aimed to develop multiphasic composite scaffolds from soluble monomeric collagens, chondroitin sulphate (CS) and hydroxyapatite (HAp), based on a biomimetic approach.

The main findings of this thesis are summarised as follows:

$\Rightarrow$ *Collagen*

The behaviour of collagen can vary vastly depending on the source and method of extraction. Since the monomeric collagens utilised throughout this work are scarcely used (the extraction process is a method developed by Dr David Brand who kindly donates his work to the Biomaterials group), this study was able to gain further insight into their behaviour and cross-linking routes. Differences in the triple helical α -domains of collagen dictate their chemical and mechanical behaviour. Bovine skin was found to not be suitable for fibre formation but had excellent degradation resistance. This knowledge was then effectively utilised in the creation of various forms of collagen-based scaffolds.

$\Rightarrow$ *Biological Study*

The ligament-bone enthesis consists of 4 distinct tissue types: ligament, uncalcified fibrocartilage, calcified fibrocartilage, and bone. Although distinct, biological studies showed that the bounds between the different layers are not distinct and that microstructural changes are gradual. The ECM of the enthesis was found to be mostly formed from collagen and fibre orientation changed from parallelly aligned fibres in ligaments to a random orientation in bone. Aggrecan, a major component of articular cartilage, was present within

the fibrocartilage and calcium was found within the calcified tissues with the presence of a calcification “tidemark” separating the two fibrocartilage regions. Nanoindentation testing confirmed that the stiffness of soft tissue is highly related to calcification from the macro down to the nano-scale.

⇒ *Fibre Scaffolds*

Although open porous and interconnected networks are essential for cell nutrition, proliferation, and migration for the formation of new tissue, sponge-like porous scaffolds typically have less than desirable mechanical properties. Collagen which had been formed into thin fibres was developed to improve the mechanical properties of porous scaffolds. Fibres formed strong interfacial bonding with the porous scaffold matrix and were effective in creating scaffolds with improved mechanical behaviour.

⇒ *Porous Scaffolds*

Although multi-layered scaffolds have been developed by quite a few researchers, limited studies have focused on enthesis tissue and even fewer that aimed to engineer the enthesis between ligament and bone. 4 types of collagen-based scaffolds of high porosity were successfully designed and fabricated to correspond with each of the distinct regions present in the enthesis along with their combination to form two-phase scaffolds. Multi-phase scaffolds were successfully formed with great integration and similar properties to each of its constituents.

⇒ *In vitro Evaluation of Scaffolds*

In the tissue engineering process, it is critical to check the toxicity and biocompatibility of developed scaffolds. Preliminary *in vitro* studies showed that porous scaffolds showed active proliferation of cells and excellent cell adhesion and migration. Furthermore, scaffolds were not exerting a cytotoxic effect on the implanted cells. A promising start to showing the viability of the designed scaffolds as implantable devices.

7.2. Future Work

Sadly, the COVID-19 pandemic was extremely disruptive and limited certain aspects of this study which were lightly mentioned throughout this thesis. The aspect of this work which provided a significant novelty, was ultimately the greatest limiting factor. The scarcity of monomeric collagen posed a huge challenge and greatly dictated the direction of the study. Although this study yielded significant results, the following aspects of this study can be investigated further:

⇒ In vitro

In vitro studies for this work are still ongoing. Cells differentiated into 3 different cell lines: fibroblasts, chondrocytes, and osteoblasts are being seeded onto the designed scaffolds to assess whether variations in scaffold composition encourage greater proliferation of specific cell types. Moving forward, undifferentiated stem cells will be implanted onto the scaffolds to assess whether the scaffolds alone can drive the differentiation of stem cells into certain lineages. The ultimate goal would be to use a multiphase scaffold and undifferentiated stem cells to engineer the enthesis with a gradient of tissue in a single piece.

⇒ Multiphasic Scaffolds

Due to limitations, it was not possible to take this work further than two-phase scaffolds. The next steps would be to try and develop three and then four-phase scaffolds to mimic the entire enthesis. Although two-phase scaffolds assessed in this work presented excellent integration, it is hypothesised that avoiding delamination of layers becomes more challenging with more layers and reducing the thickness of each layer.

⇒ Reinforced Scaffolds

Combining the fibre work with the multi-phasic scaffolds has the potential to produce scaffolds with highly desirable and novel behaviour. However, it is hypothesised that the presence of extruded collagen fibres could encourage the differentiation of stem cells into highly aligned fibroblasts, which although desirable for the ligament region, is undesired in the other regions. Therefore, an understanding of how extruded collagen fibres affect cell behaviour would be important before attempting to merge the two ideas.

Supplementary Material

S.1. Collagen

S.1.1. Biosynthesis of Collagens

The biosynthesis of collagens, starting with gene transcription of the genes within the nucleus to the aggregation of collagen heterotrimers into large fibrils, is a complex multistep process Fig 1. Since most of our knowledge of these mechanisms is based on fibril-forming collagens, this discussion will mostly focus on type I collagen. It is likely that the basic mechanisms of triple helix formation and processing will also apply to other collagen types.

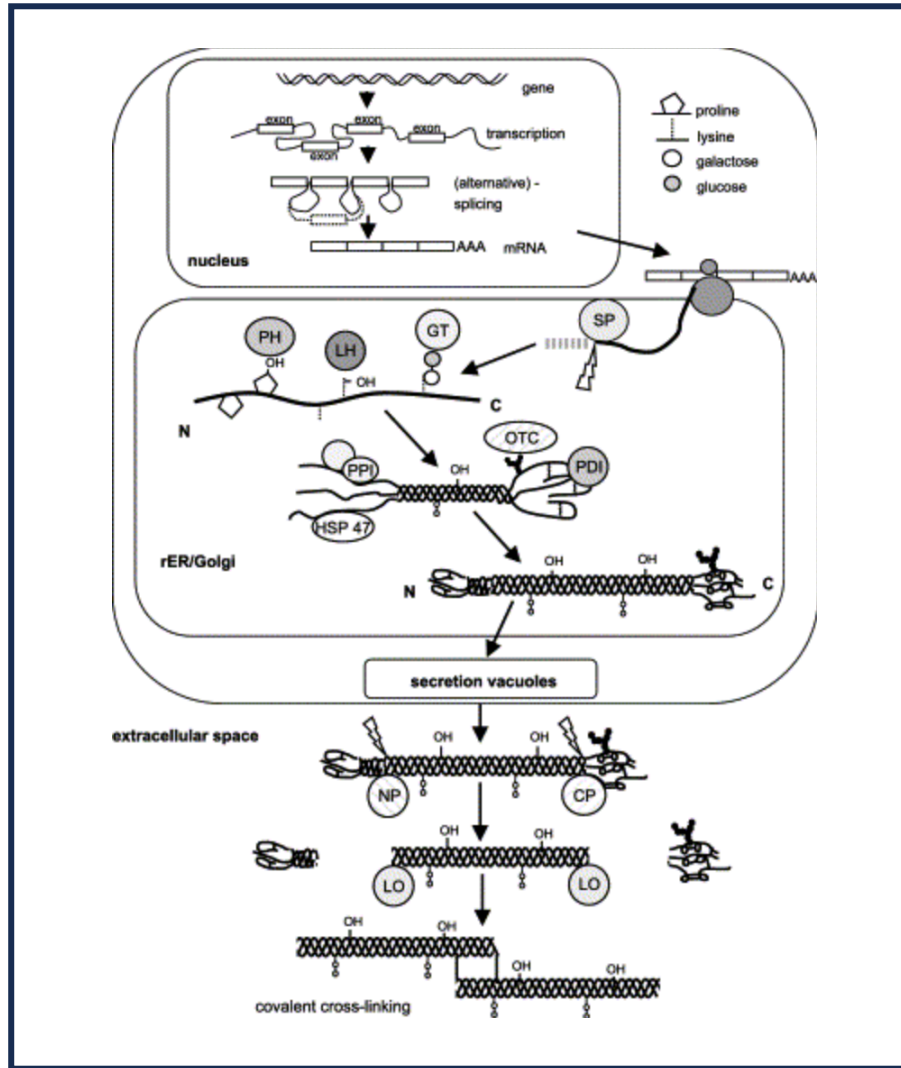


Figure S1: Schematic representation of collagen synthesis starting from the nuclear transcription of the collagen genes, mRNA processing, ribosomal protein synthesis (translation) and post-translational modifications, secretion and the final steps of fibril formation [1]. (SP: signal peptidase; GT: hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase; LH: lysyl hydroxylase; PH: prolyl hydroxylase; OTC: oligosaccharyl transferase complex; PDI: protein disulphide isomerase; PPI: peptidyl-prolyl cis-trans-isomerase; NP: procollagen N-proteinase; CP: procollagen C-proteinase; LO: lysyl oxidase; HSP47: heat shock protein 47, colligin1).

S.1.1.1. Transcription and Translation

Collagen synthesis begins with transcription from DNA into a new molecule of messenger RNA (mRNA) within the fibroblast nucleus. Genes; sections of the DNA, for pro- α 1 and pro- α 2 chains are transcribed. Transcription begins when an enzyme called RNA polymerase attaches to the double helix of the DNA and begins mRNA synthesis. After matching complementary bases to the original DNA strand this is not the final mRNA but rather the pre-mRNA. The pre-mRNA is a pattern of exons-introns. Certain proteins come along and remove the introns from the strand of pre-mRNA whilst the exons are sliced together into a long chain of mature mRNA. Most collagen genes revealed a complex exon–intron pattern, ranging from 3 to 117 exons, with the mature mRNAs of fibrillar collagens encoded by more than 50 exons. Therefore, in many cases, different mRNA species could be detected, caused by multiple transcription initiation sites, alternative splicing of exons or a combination of both. In addition to splicing, the pre-mRNA undergoes capping at the 5'end and polyadenylation at the 3'end. The mature mRNA is translated into pre-pro-polypeptide chains by moving into the cytoplasm and interacting with ribosomes.

S.1.1.2. Posttranslational Modifications of Collagen

After translation, this chain then travels to the endoplasmic reticulum (ER) for post-translational processing where three major modifications are made for the pre-pro-polypeptide to become pro-collagen:

1. The signal peptide on the N-terminal is removed
2. Hydroxylation of proline and lysine residues catalysed by enzymes prolyl 3-hydroxylase, prolyl 4-hydroxylase, and lysyl hydroxylase, respectively. The hydroxylase enzymes require ferrous ions, 2-oxoglutarate, molecular oxygen, and ascorbate as cofactors.

Note: propyl 4-hydroxylase catalyses the formation of 4-hydroxyproline. The presence of 4-hydroxyproline is essential for intramolecular hydrogen bonds and thus contributes to the thermal stability of the triple helical domain, and therefore also to the integrity of the monomer and collagen fibril. The function of 3-hydroxyproline is not known [2]. The extent of lysine hydroxylation also varies between tissues and collagen types [3].

3. Glycosylation of the selected hydroxyl groups on lysine with galactose and glucose b; this is catalysed by the enzymes hydroxyllysyl galactosyltransferase and galactosylhydroxyllysyl-glucosyltransferase, respectively.

Following hydroxylation, the polypeptide chains aggregate into a triple-helix molecule, procollagen. The globular structure of the C-propeptides is stabilized by intrachain disulphide bonds and a N-linked carbohydrate group is added by the oligosaccharyl transferase complex. The C-terminal domains of three α -chains align and initiate the formation of the triple helix, progressing to the N-terminus. The triple helix configuration is 3 left-handed helices twisted into a right-handed coil.

S.1.1.3. Secretion of collagens

After processing, the pro-collagen molecules are packed within the Golgi compartment for final modifications assembled into secretory vesicles and released into the extracellular space. Following the secretion, the pro-collagen trimers are processed depending on the collagen type. Enzymes known as collagen peptidases; procollagen N-proteinase and procollagen C-proteinase, perform propeptide cleavage and remove the C-propeptides and N-propeptides. The molecule becomes tropocollagen.

S.1.1.4. Extracellular processing and modification

Collagen fibril assembly is a complex process and current understanding is largely on in vitro experiments. The fibril-forming collagens spontaneously aggregate after processing of procollagens into ordered fibrillar structures in vitro. The ability for the “self-assembly” is encoded in the structure of the collagens and several models describe the mechanism for the periodic fibrillar assembly. The collagen monomers (tropocollagen) align in a quarter-staggered array due to hydrophobic and electrostatic interactions, which may aggregate into five-stranded fibrils and subsequently into larger fibrils [4], [5]. The molecular arrangement into fibrils is initially united by hydrogen bonding.

The copper-dependent enzyme, lysyl oxidase, catalyses the formation of aldehydes from lysine and hydroxylysine residues in the telopeptides, which interact with adjacent residues from adjacent molecules to form cross-links, which contribute to the mechanical resilience of collagen fibrils. Most cross-links have been shown to be located at the overlap position connecting the N- or C-telopeptides with specific residues within the helical part of adjacent molecules [6].

These intermolecular cross-links are prerequisites for the physical and mechanical properties of collagen fibrils and a stable network formation.

Note: The formed fibrils can be orientated differently in distinct types of tissues. In tendons, the type I collagen fibrils align parallel to each other and form bundles or fibres, whereas in the skin, the orientation is more random with the formation of a complex network of interlaced fibrils [2]. Furthermore, the fibril formation is influenced by the propeptides of procollagen molecules. Thus, the cleavage of the C-propeptides of type I collagen is an essential step for regulating fibril formation, but the function of the N-terminal propeptides in this process is still

not fully understood and may differ between collagen types. It has been suggested that they may regulate the diameter of the forming fibrils and their removal from type I procollagen influences the regular fibril morphology [2] [7].

Type	Composition	Distribution
Fibrillar		
I	$\alpha 1[\text{I}]2\alpha 2[\text{I}]$	bone, dermis, tendon, ligament, cornea
II	$\alpha 1[\text{II}]3$	Cartilage, vitreous body, nucleus pulposus
III	$\alpha 1[\text{III}]3$	Skin, blood vessels, reticular fibres of most tissues
V	$\alpha 1[\text{V}]3$, $\alpha 1[\text{V}]2\alpha 2[\text{V}]$, $\alpha 1[\text{V}]\alpha 2[\text{V}]\alpha 3[\text{V}]$	lung, bone, dermis, cornea, placenta, fetal membranes; together with type I collagen
XI	$\alpha 1[\text{V}]2\alpha 2[\text{V}]$	Dermis, cornea, cartilage, vitreous body
Network		
IV	$\alpha 1[\text{IV}]2\alpha 2[\text{IV}]$; $\alpha 3[\text{IV}]\alpha 4[\text{IV}]\alpha 5[\text{IV}]$; $\alpha 5[\text{IV}]2\alpha 6[\text{IV}]$	Basement membranes
VI	$\alpha 1[\text{VI}]\alpha 2[\text{VI}]\alpha 3[\text{VI}]$; $\alpha 1[\text{VI}]\alpha 2[\text{VI}]\alpha 4[\text{VI}]$	Bone, cartilage, cornea, dermis, placenta, lungs, blood vessel, intervertebral disc
VIII	$\alpha 1[\text{VIII}]3$; $\alpha 2[\text{VIII}]3$; $\alpha 1[\text{VIII}]2\alpha 2[\text{VIII}]$	endothelial cells, Descemet's membrane
X	$\alpha 1[\text{X}]3$	hypertrophic Cartilage
Anchoring fibrils		
VII	$\alpha 1[\text{VII}]2\alpha 2[\text{VII}]$	skin, dermal–epidermal junctions; oral mucosa, cervix,
FACIT		
IX	$\alpha 1[\text{IX}]\alpha 2[\text{IX}]\alpha 3[\text{IX}]$	Cartilage, cornea, vitreous humor
XII	$\alpha 1[\text{XII}]3$	perichondrium, ligaments, tendon
XIV	$\alpha 1[\text{XIV}]3$	dermis, tendon, vessel wall, placenta, lungs, liver
XIX	$\alpha 1[\text{XIX}]3$	human rhabdomyosarcoma

XX	$\alpha 1[XX]3$	corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	$\alpha 1[XXI]3$	blood vessel wall
MACIT		
XII	$\alpha 1[XIII]3$	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	$\alpha 1[XVII]3$	dermal epidermal junctions
MULTIPLEXIN		
XV	$\alpha 1[XV]3$	fibroblasts, smooth muscle cells, kidney, pancreas
XVI	$\alpha 1[XVI]3$	fibroblasts, amnion, keratinocytes
XVIII	$\alpha 1[XVIII]3$	lungs, liver

Table S1: Given are the molecular composition, the genomic localization of the different chains as well as the basic tissue distribution. MACIT, membrane-associated collagen with interrupted triple helices. MULTIPLEXIN, multiple triple helix domains and interruptions.

S.1.2. Degradation of Interstitial Collagens

In the course of physiological remodelling and pathological states, the collagen of the extracellular matrix is degraded and resorbed. Collagen resorption is an important process during the growth of most organs in which the matrix must be removed to allow for size increments. The tightly packed helical structure of collagens makes them resilient to most means of proteolytic degradation. Therefore, once adulthood is reached, collagen resorption takes place very slowly in most tissues. One exception is bone, where remodelling is a continuous process.

The major pathway identified in the turnover of collagen in normal physiological processes involves a group of secreted or membrane-associated matrix metalloproteases (collagenases) and is believed to take place within the pericellular/extracellular environment. Due to the

resilience of collagen to proteolytic attack, a highly sophisticated means of degradation is required. The initial translation product in the collagen biosynthesis process is susceptible to several proteases (e.g., an N-terminal signal-peptide protease, the N- and C-terminal procollagen peptidases, and various collagenases) that are capable of cleavage and degrading collagen to small peptides or amino acids.

The degradation of interstitial collagens begins with the cleavage of tropocollagen under pathological and physiological conditions by a subfamily of Zn^{2+} - and Ca^{2+} -containing enzymes, known as collagenases, at a single site characterised by the tripeptide sequence G-[I/L]-[A/L] that is located approximately three-fourths from the N-terminus of the protein. Collagenases belong to the larger matrix metalloproteinase (MMP) family [8] [9], [10]. Fibril-forming collagens I, II, and III are cleaved by MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase), MMP-13 (collagenase 3) that generate three-quarter and one-quarter sized fragments, and by membrane-anchored MMP-14. MMP-2 is also able to cleave collagen I [11]. Collagen II is a preferential substrate of MMP-13, whereas collagens I and III are preferentially cleaved by MMP-1 and MMP-8 [11].

The unique MMP cleavage site characterised by a covalent bond between a glycine residue and a leucine or isoleucine residue is known as the scissile peptide bond. Protease substrates are labelled by increasing distance from the scissile peptide bond, with the C-terminal side “primed” and N-terminal side “unprimed”: $\text{P}_4\text{-P}_3\text{-P}_2\text{-P}_1\sim\text{P}_1^{\text{I}}\text{-P}_2^{\text{I}}\text{-P}_3^{\text{I}}\text{-P}_4^{\text{I}}$; where $\sim$ denotes the scissile peptide bond. The active site of MMPs is funnel-shaped with a wide end on the N-terminal side, and a narrow end on the C-terminal side.

Tropocollagen has a diameter of $\sim 13\text{\AA}$ while the catalytic domain of MMPs (on the C-terminal side) is only $6\text{\AA}$ wide. Suggesting that the folded structure of collagen cannot fit into the catalytic site. Furthermore, the scissile peptide bond is buried within the folded triple helical structure of collagen. As the catalytic site of MMPs cannot accommodate the folded structure of collagen, and the collagenase scissile bond is hidden within the triple-helix structure, the precise mechanism of collagen cleavage by MMPs remains unclear. Therefore, it has been long assumed that the collagen triple helix must partially unwind such that individual chains would be able to fit into the collagenase active site and the scissile bond would be solvent exposed.

There have been two hypothetical molecular mechanisms for collagenolysis put forward to explain how this unfolding occurs. One hypothesis is that collagen exists in a well-folded triple helical state and that collagenases unwind the triple helix upon binding the coordinated action of the catalytic and hemopexin-like domains in a process known as “molecular tectonics”. This active unwinding of the triple helix would not only separate the three chains but also expose and subsequently enable the cleavage of the scissile bond. The problem with this mechanism is that molecular tectonics requires different MMP domains to undergo significant structural rearrangement that in principle requires large amounts of energy. Collagenolysis, however, does not require energy input. Therefore, an alternative hypothesis is that the conformational ensemble of collagen in the vicinity of the collagenase cleavage site includes relatively low-energy structures that are partially unfolded conformers. This hypothesis corresponds to a conformational selection mechanism in that the degradation-prone conformations of collagen exist regardless of whether the enzyme is present and that the enzyme “selects” the appropriate conformers for degradation.

It is possible that proteases other than collagenases have a synergistic role in the degradation of the fibrils (and fibres) by cleaving peptide bonds in the telopeptide regions adjacent to sites of intermolecular crosslinks [12].

The different physiological events in which collagenolysis occurs have been grouped by Woessner [13] into three classes: normal, pathological, and induced. Normal resorption of collagen occurs, for example, during tissue remodelling with growth, especially embryogenesis, when there is an overall net synthesis of new fibrous tissue. Various pathological disorders such as rheumatoid arthritis, cause the abnormal resorption of collagen. Finally, collagen degradation is an essential part of the healing process and is induced in response to trauma such as skin wounds, burns and bone fractures.

NOTE: During wound healing and in other locations where the resorption is of an induced nature, the stimulus for collagenolysis occurs locally within the traumatised area. Wound repair is a complex process and is broadly categorised into the following four phases: hemostasis, inflammation, proliferation and remodelling (maturation). During the inflammation phase, soluble fragments from collagen degradation stimulate macrophage activation, which is responsible for the removal of microbes and devitalised tissue and is related to the development of the inflammatory reaction. Furthermore, the degradation of collagen contributes to the release of bioactive fragments (matricrytins) which enables the re-establishment of the tissue architecture during healing [14] [15].

A balance of enzyme activity and inhibition is required for normal wound healing and is under tight regulatory control. Wounds infected with microbes that produce collagen-degrading enzymes add to the imbalance and lead to chronic wounds.

In other circumstances, there is an abundant and continuous synthesis of new collagen which does not form mature and stable fibres, causing scar tissue to become hypertrophic or the formation of keloid.

S.1.3. Mechanical Behaviour of Collagen Hierarchy

Most natural biological materials have a hierarchical structure from the molecular to the macroscopic level [16]. Their mechanical response and fracture properties arise from the properties of the individual elements at each level in the hierarchy as well as from the interaction between these elements across the different length scales. Therefore, there is a fundamental need to determine the mechanics at different hierarchical levels and their interplay from a biochemical and molecular level upward.

By observing the deformation of collagen at the micro- and nano-scale, studies by Gupta *et al.* have demonstrated that mechanical strain is distributed over distinct hierarchical levels (molecules, fibrils, fibres)[17]–[19]. The stretching of collagen-based tissue involves concurrent deformation at each scale.

In recent years, significant efforts have been made to characterise the mechanical properties of hierarchically structured collagen using experimental, computational, and theoretical approaches. Earlier work primarily focused on the macroscopic, overall mechanical properties of collagen fibres and related tissues to elate the structure-mechanical relations at each of the hierarchical levels of the organisation [18] [20]–[22]. While significant progress has been made in the mechanical testing of micrometre and millimetre-sized biological specimens, there is still a lack of quantitative reproducible experimental techniques for making mechanical measurements on specimens with characteristic dimensions in the smaller range of 10-100nm.

However, there have been studies which have tried to bridge this gap. Some works have focused on the mechanical properties of individual tropocollagen molecules [23]–[25]. Whilst other have focused on the mechanical properties of individual collagen fibrils to provide valuable insight into the Young's modulus and their nonlinear deformation behaviour [26]–[28]. However, most of these have yet to relate the behaviour of the individual molecules to the overall material response.

It was the pioneering works by Sasaki and Odajima and by Fratzl *et al.* who used X-ray scattering to propose models to describe how collagen molecules in fibrils are elongated and rearranged under external forces to produce the properties of the larger tissue[29]–[31]. By using samples of tendon, they described how at small strains there is a straightening of kinks in the collagen structure, first at the fibrillar and then at the molecular level, higher strains lead to molecular gliding within the fibrils and ultimately to a disruption of the fibril structure (figure S2). They also presented the idea of additional gliding occurring at the interfibrillar level due to considerably lower strains being observed within collagen fibrils than in the whole tissue.

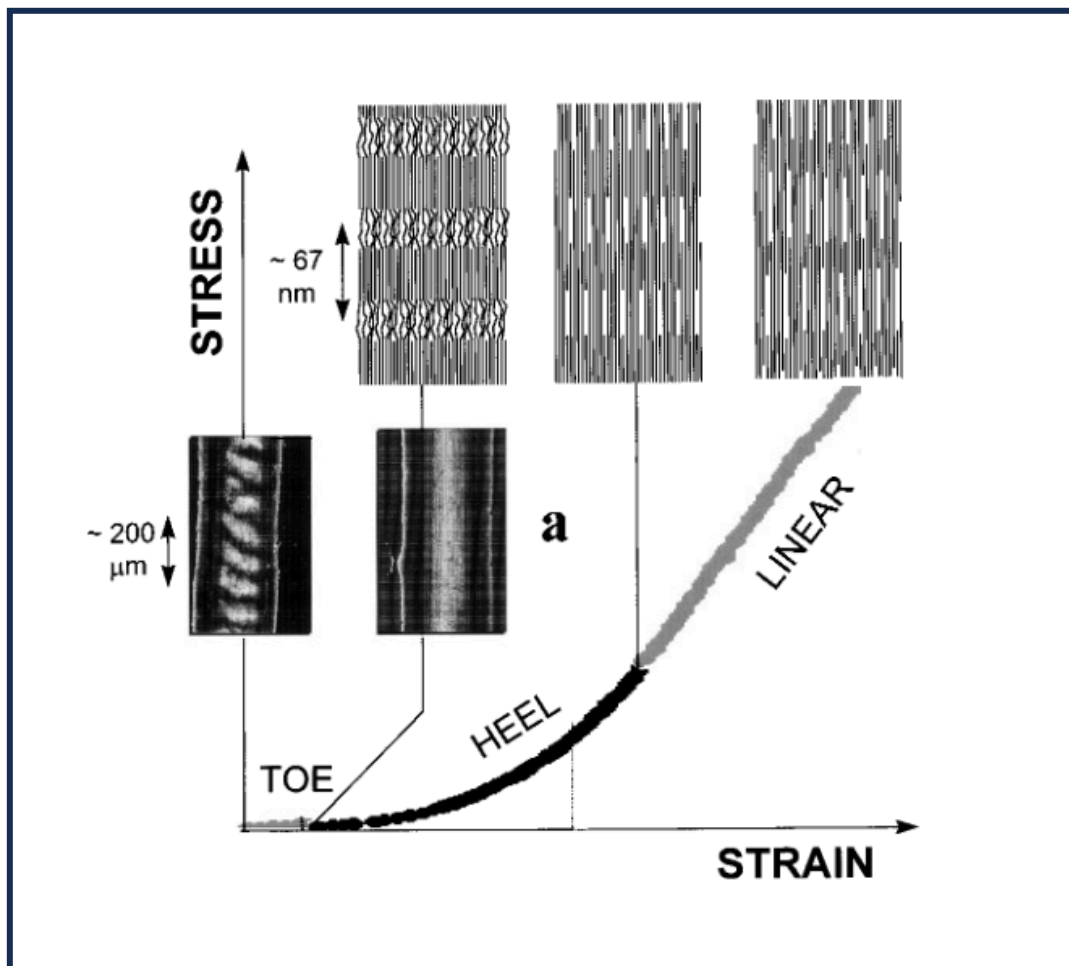


Figure S2: Typical stress-strain curve of a rat tail tendon. Toe region- The macroscopic crimp of the fibrils is removed. Heel region- straightening of molecular kinks in the gap. Linear region- gliding of molecules [29].

Hierarchical level or state	Young's modulus (GPa)
Collagen molecule	5.1 [32] 0.35-12 [24]
Fibrils wet	0.86± 0.45 GPa [26] 0.2.-0.8 [28]
Fibrils dry	2-7 [28]

Table S2: Comparison of Young's modulus of collagen molecule and collagen fibrils.

S.1.4. Crosslinking Routes

S.1.4.1. Physical Crosslinking

Dehydrothermal treatment (DHT), involves a condensation reaction between -COOH and NH₂ group in neighbouring collagen-chains by removal of water molecules from desired materials under high temperature conditions(>90°C) while under vacuum for several days, resulting in the formation of interchain links [33] [34]. Experimental parameters such as temperature, exposure duration and vacuum levels have been found to control the mechanical properties of collagen fibres [7]. Higher DHT temperatures result in higher cross-linking density, thus greater compressive and tensile strength, up to several folds in collagen-GAG scaffolds.

The major advantages of DHT treatment are that it does not involve the use of cytotoxic reagents and the sterilisation provided by the high temperature and exposure periods necessary. However, along with forming cross-links, it has been shown that the high temperatures used during DHT cross-linking also denature collagen [8]. Which is generally considered undesirable due to its tendency to disrupt the conformation of native tissue, however, in some cases may reduce the inflammation response and increase cellular infiltration *in vivo* [37]. Furthermore, DHT is a slow and time-consuming process which typically takes anywhere from 3-5 days depending on the DHT temperature [38]. Generally, the degree of cross-linking using DHT is considerably lower than obtained by chemical methods [39]. Sometimes, DHT treatments are followed by a chemical treatment (cyanamide) to increase the stability of the treated material [40].

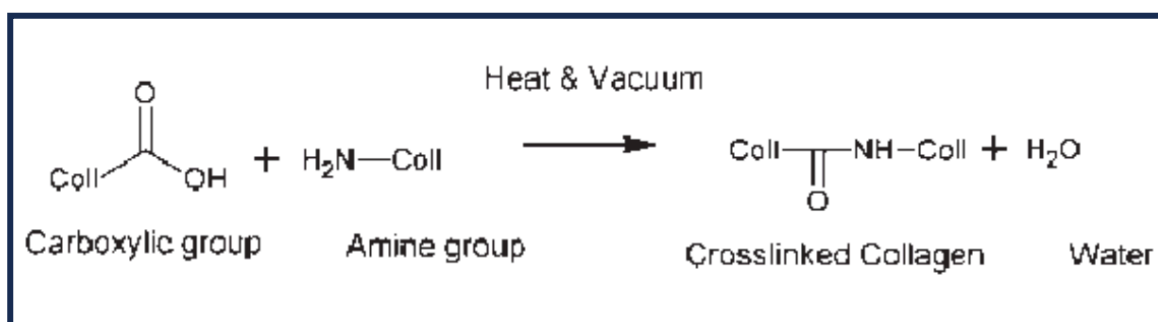


Figure S3: DHT cross-linking route. Crosslink formation between adjacent carboxylic and amine groups through a condensation reaction[41].

When collagen is exposed to irradiations from gamma (γ) rays, physical crosslinks are formed through the replacement of intermolecular forces by a covalent bond [42] [43]. The extent of cross-linking achieved through irradiation techniques depend on the γ -ray dosage (100-400 Gy). Ionising irradiation is widely used as a terminal sterilization method for biomedical devices. However, at higher dosages ($> 1\text{kGy}$), the triple helix structure of collagen degrades through peptide bond cleaving [44], ionising irradiation severely damaging tissue ECM and other collagen-based materials. Therefore, whilst the use of low-intensity γ -rays are still used to sterilise collagen scaffolds, their use for cross-linking has been abandoned as the risk of collagen chain scission cannot be completely avoided even at dosages lower than the dose required for sterilisation[45].

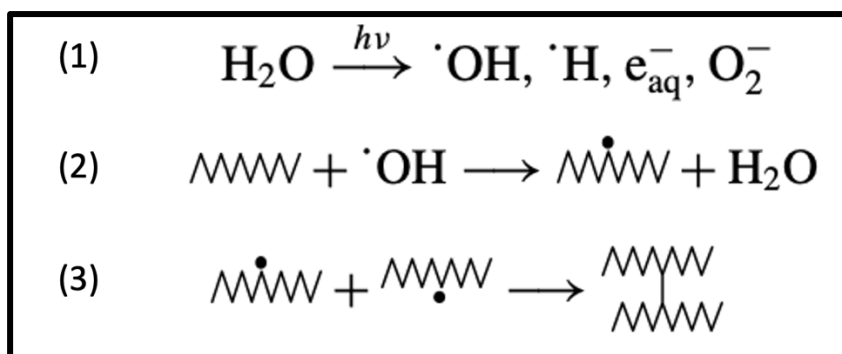


Figure S4: The reaction mechanism via formation of hydroxyl radicals irradiated with gamma-rays [42].

As a physical cross-linker gamma radiation can be used. However, the energy of gamma radiation can destroy the native structure of the protein. An alternative physical cross-linking agent to gamma radiation is UV irradiation. Short wavelength Ultra-violet (UV) irradiation (254nm) modifies aromatic side chains such as tyrosine and phenylalanine to form crosslinks through free-radical mechanism [46]–[49]. UV intensity and exposure time are the parameters which control the degree of crosslinking. However, the number of crosslinks formed is limited by the number of available aromatic groups [50]. UV crosslinking offers many advantages such as avoiding the need for toxic chemicals, ease of treatment and sterility [51]. Furthermore, UV crosslinking of collagen scaffolds showed improved physical properties (increase in tensile strength and stiffness), and resistance to enzymatic degradation and did not adversely affect cell ligation [46]. However, these need to be balanced against potential collagen denaturing and fragmentation which could lead to the release of pro-inflammatory peptides [35] [39] [52]. Although, less harmful to proteins than gamma, UV can also destroy the protein structure.

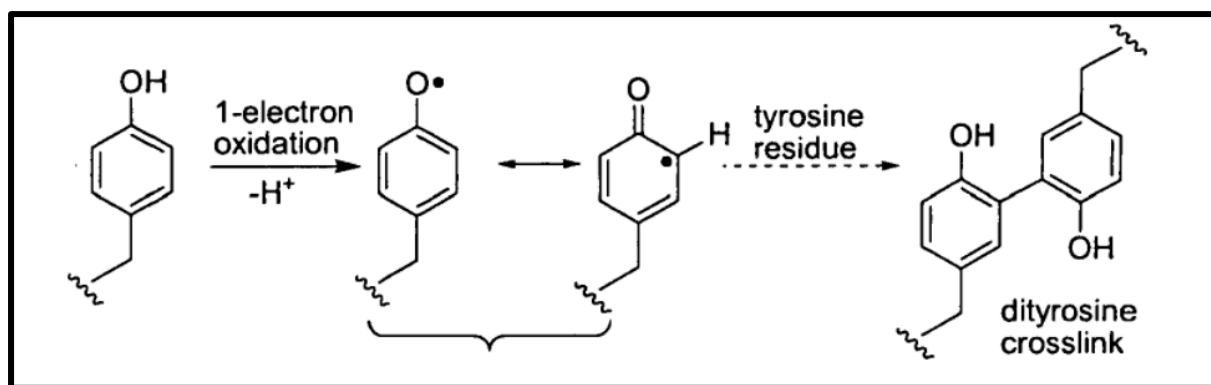


Figure S5: UV-assisted cross-linking mechanism. Crosslinking between two tyrosine free radicals [53].

	Advantages	Disadvantages
Dehydrothermal treatment	-Additional sterilisation unnecessary -Time consuming	-high temperatures denature collagen - Additional chemical cross-linking may be necessary
Gamma irradiation	-Additional sterilisation unnecessary	-Risk of damaging tissue ECM and other collagen-based materials
UV irradiation	-Less risk of damaging tissue -Additional sterilisation unnecessary.	-Limited cross-links formed -Potential collagen denaturing and fragmentation

Table S3: Advantages and disadvantages of Physical crosslinkers.

S.1.4.2. Chemical Crosslinking

Present chemical cross-linking procedures often use bifunctional reagents containing reactive groups which form bridges between two amino acid side chains of adjacent protein molecules. Glutaraldehyde (GTA) is the most frequently used cross-linking agent since it is easily available, inexpensive and forms aqueous solutions that can easily cross-link tissue in a relatively short period. GTA is a five-carbon aliphatic molecule with an aldehyde at each end

of the chain rendering it bifunctional. Aqueous solutions of the aldehyde consist of mixtures of free aldehyde, mono- and dehydrated monomeric GTA, monomeric and polymeric cyclic hemiacetals and various, β -unsaturated polymers (Figure S6). GTA reacts with the amine and hydroxyl functional groups of proteins and polymers, respectively through Schiff-base reaction and connects the biopolymeric chains via intra- or intermolecular interactions. All the free amine groups which are available react with the GTA to form a more strongly crosslinked network [54] [55]. However, the situation is complicated by the fact that it polymerises and is rarely in its monomeric form in aqueous solutions [56]. The polymerisation of GTA takes place especially fast under alkaline pH conditions, but even at pH5, aqueous solutions of GTA contain polymeric moieties. As the concentration of GTA increases, GTA is substituted as a pendant group on the backbone chain of collagen rather than as a crosslinking agent. This increase in GTA concentration switches the crosslinking mechanism in collagen from intrafibrillar to inter-fibrillar [57].

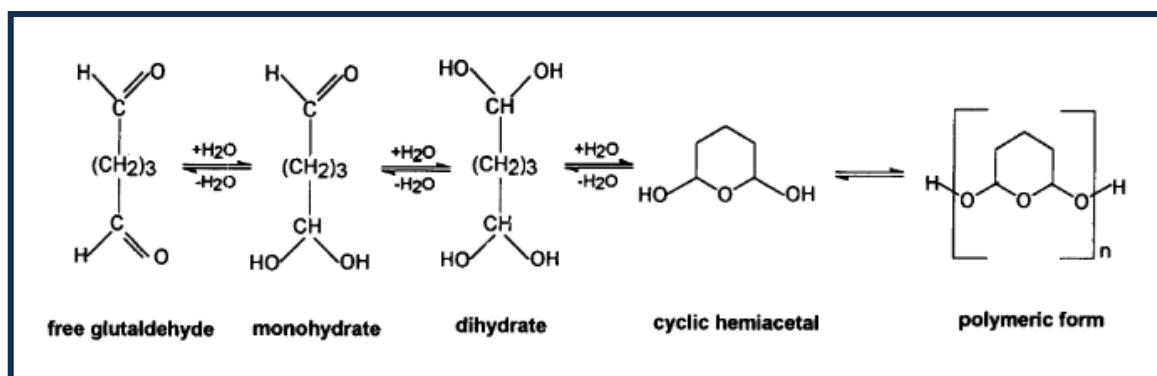


Figure S6: Forms Glutaraldehyde takes in aqueous media.

At low concentrations GTA produces intramolecular crosslinks in collagen, while at higher concentrations GTA forms long polymeric chains which produce intermolecular crosslinks [58]. At high concentrations, the reaction of GTA with collagen is reversible [58]. After implantation of GTA crosslinked collagenous biomaterials, large polymers of GTA are

continuously hydrolysed [59] resulting in the release of free monomeric GTA into surrounding tissue [60]. Studies have shown that cytotoxicity and slow growth of granulation tissue are drawbacks associated with materials crosslinked via GTA[61], [62]. Furthermore, the colour of crosslinked scaffolds has a yellow hue.

Schiff base structures have been shown to increase mechanical strength (elastic modulus increase from 20 MPa to 60MPa) of collagen structures and to enhance thermal strength (thermal shrinkage temperature increased from 56°C to 78°C)[63]. Furthermore, along with an increase in tensile strength, bone tissue scaffolds which were cross-linked using GTA indicated a significant reduction in antigenicity [64]. The functional groups of GTA, aldehyde groups, are toxic to cells and cause severe inflammation in the body; therefore, detoxifying strategies need to be implemented to increase the biocompatibility and viability of GTA-crosslinkers.

To overcome the adverse effects of GTA, several alternative cross-linking methods have been developed. Alternative acyl-azide cross-linking methods involve the transformation of lateral carboxyl groups of collagen chains in acyl-azide, via the formation of hydrazides (hydrazine method [65]) or by using diphenylphosphoryl azide (DPPA method [66]). It has been shown that the treatment of collagen-rich tissue with acyl-azides results in a material with thermal stability and resistance to collagenase digestion similar to those obtained after GTA treatment [67]. The hydrazine method has been used previously for forming the peptide bond in peptide synthesis and for enzyme immobilization on collagen membranes [68]. Schematically, in the hydrazine method carboxyl groups are transformed via a three-step reaction (methylation, hydrazine formation and acyl-azide formation) and are then allowed to react with the amino group of an adjacent chain of collagen. While the DPPA method involves the reagent reacting with carboxylic groups of aspartic and glutamic acid residues on collagen lateral chains to form

an acyl azide, which would then react with the lateral amino groups of collagens (chiefly lysine and hydroxylysine). Of the two acyl azide methods, the DPPA method presents the greater cytocompatibility [66], furthermore, this method is a simpler, faster way to prepare acyl azide groups and to cross-link collagen and therefore is more suitable for cross-linking collagen-based materials.

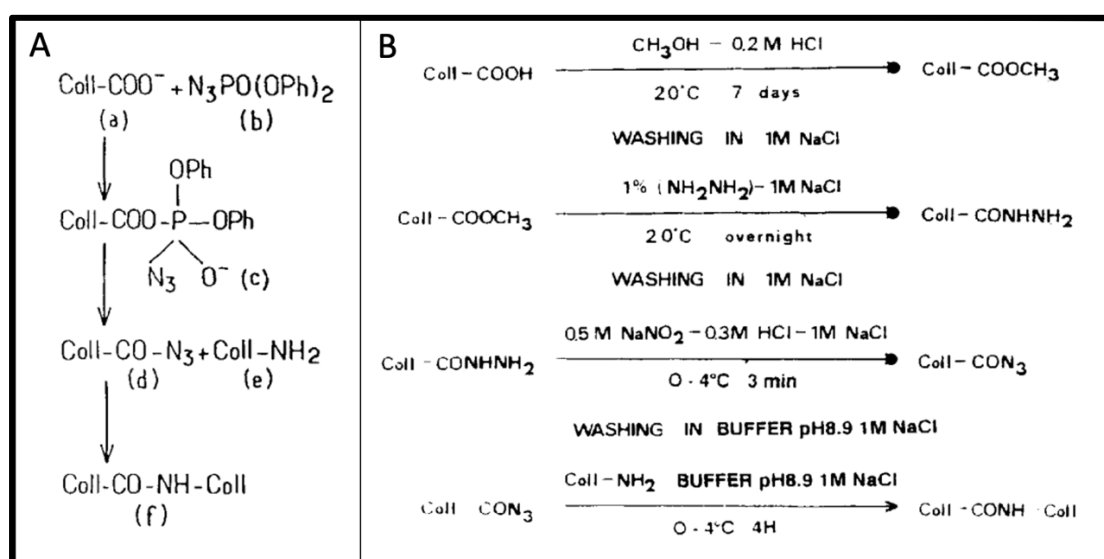


Figure S7: Chemical Pathway of collagen cross-linking by (A) DPPA method [66],
(B) Hydrazine method [65]. 5/1/25 3:53:00 PM

A carbodiimide functional group is a chemical group consisting of a C=N=C bond. 1-Ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC) fixation involves the activation of carboxylic groups in the peptide chain and the formation of O-acylisourea intermediate which can be nucleophilic attacked by free amino groups of lysine and hydroxyl lysine residues thus forming an amide bond[69]. EDC Crosslinking forms a network of crosslinking effectively increasing the mechanical stability of collagen material and preventing movement of macromolecules and infiltration of water molecules[70]. N-hydroxysuccinimide, an affinity reagent, added to the EDC solution will activate carboxylic groups, which are less susceptible to hydrolysis and

effectively increase the number of cross-links [71]. EDC-crosslinked ECM-derived scaffolds are soft and similar to natural tissue materials, reveal low residual toxicity and have been shown to resist enzymatic degradation of collagen matrix in vitro [69] [72]. An advantage of EDC/NHS is that all of the residues are water soluble, and can easily be washed out of the scaffold construct by distilled water after the cross-linking reaction. This crosslinker has been used in several studies on bone tissue regeneration due to its low cytotoxicity in comparison to GTA[73]. EDC-cross-linked materials have poor biomechanical properties and more rapid degradation and biodegradation profiles, compared to GTA-cross-linked ones. However, mechanical strength in carbodiimide cross-linked collagenous scaffolds were shown to increase 4 folds as compared to the physically cross-linked (DHT) scaffolds (2.0 kPa, 0.5kPa respectively) in compressive modulus[41].

Various studies have shown that EDC/NHS crosslinking increases the mechanical strength and denaturation temperature of collagenous scaffolds and improves their resistance against enzymatic degradation [74]. Furthermore, they are non-toxic and exhibit a higher cell attachment potential and controlled swelling characteristics with the least cytotoxicity compared to GA. Furthermore, unlike its counterparts GTA and Genipin, EDC/NHS crosslinking forms zero-length cross-links with water-soluble by-products. A major advantage of this treatment is non-toxicity since neither EDC nor NHS are incorporated into the material and are easily removed by washing [69] [71] [75]. Unlike GTA, the use of carbodiimides has not been shown to produce any by-products which may induce cytotoxicity [76]. Carbodiimide crosslinked collagenous scaffolds presented improved resistance against thermal treatment and enzymatic degradation. Non-toxic and improved cell proliferation [77]–[79]. Careful control over the cross-linking parameters allows refinement of the mechanical properties and degradation kinetics of the resultant scaffold [80].

A combination of physical and chemical cross-linking methods have been carried out. A combination of DHT followed by chemical cross-linking using EDC/NHS or GTA indicated a higher cross-linking degree since, whilst physical cross-linking resulted in a low cross-linking extent due to cross-linking only occurring at the surface, a combination of both methods allowed for both the surface and bulk of the material to be cross-linked [81]–[83].

In a study by Rault et al. [67] which studied the thermal stability and collagenase susceptibilities of various chemical cross-linkers including GTA, EDC, hydrazine and DPPA, showed that GTA was the most effective reagent which required the lowest concentration to produce collagen-based structures with the highest thermal stability. Additionally, the treatment of collagen films with acyl azides results in a material with thermal stability and resistance to collagenase similar to those obtained after GTA treatment. However, although GTA is the most efficient chemical agent and thus most widely used, it has been shown to elicit a cytotoxic effect which limits fibroblast growth and consequently decreases the benefit of the use of collagen [65]. Long-term in vivo biocompatibility experiments, in which acyl azide- and GTA-treated pericardium were implanted in a circulatory model in young rats for 6- 24 months, it was observed that acyl azide treatment allowed a more rapid, extensive development of endothelial-like cells than GTA treatment [84].

In an organotypic culture model by Petite et al. [84] and also those of van Wachem et al. [85] with a methyl cellulose cell culture system and after subcutaneous implantation in rats demonstrate that collagen biomaterials treated with acyl azide methods or with carbodiimide (EDC) have better biocompatibility than the same biomaterials treated with GTA. As acyl azide and carbodiimide methods do not result in the presence of unreacted functional groups in the

material and can be used to couple other molecules containing carboxylic groups (such as glycosaminoglycans).

Formaldehyde, being a reactive electrophilic species, reacts readily with various functional groups of biological macromolecules in a cross-linking fashion. It involves a two-step process. First, the formaldehyde reacts with available free amine in the protein to generate a Schiff base; the subsequent cross-linking occurs by the formation of a covalent bond between the protein. Studies indicate that the most frequent type of cross-link formed by formaldehyde in collagen is between the nitrogen atom at the end of the side-chain of lysine and the nitrogen atom of a peptide linkage and the number of such cross-links increases with time [86], [87].

In contrast to most other cross-linking molecules (except for carbodiimide derivatives, formaldehyde is very small, containing only four atoms, and exhibits a relatively short spacer arm (in the range of 2.3–2.7 Å). Therefore, only closely associated proteins can be cross-linked due to the small size of formaldehyde. The application of formaldehyde as a cross-linker has several advantages. The reactions with formaldehyde, generally under mild conditions and in dilute solution, are reversible [88], simple washing or steam distillation results in quantitative recovery of the formaldehyde. Formaldehyde is also thought to allow very fast cross-linking and the stabilization of transient interactions [88]. Furthermore, formaldehyde is available in almost every laboratory at costs that amount to only a fraction of other cross-linkers. Formaldehyde cross-linking is relatively inefficient, primarily because on storage it is converted to the less reactive paraformaldehyde, $\text{OH-CH}_2(\text{CHOH})_2\text{-CHO}$. Furthermore, epitopes recognized by antibodies raised against endogenous proteins could be destroyed by formaldehyde modification, which would prevent their application [89]. Similarly, the physiological environment of a protein of interest, and the type and extent of its interactions may also affect the experimental outcome.



Figure S8: Mechanism of formaldehyde cross-linking [90].

Genipin (GP) is a plant-derived, non-toxic water-soluble crosslinking agent. GP is a hydrolytic product of geniposide extracted from the fruit of *Gardenia jasminoides Ellis* [91]. It is an iridoid compound which can bridge free amino groups of lysine or hydroxylsine residues of different polypeptide chains by monomeric or oligomeric cross-links in collagen [92]–[94]. GP is classed as a green chemical or natural crosslinking agent and shows superiority in many aspects, especially in terms of having lower cytotoxicity and in vivo immunogenicity as compared with GTA-treated counterparts [95]–[98]. GP is an active colourless material that characteristically gives a deep blue colour to primary amino acids by spontaneously reacting with amino acids to form blue pigments [99]. However, the dark blue appearance of GP-cross-linked ECM-derived scaffolds, together with the exorbitant price due to the complex extraction process and low cross-linking density greatly limits its applications.

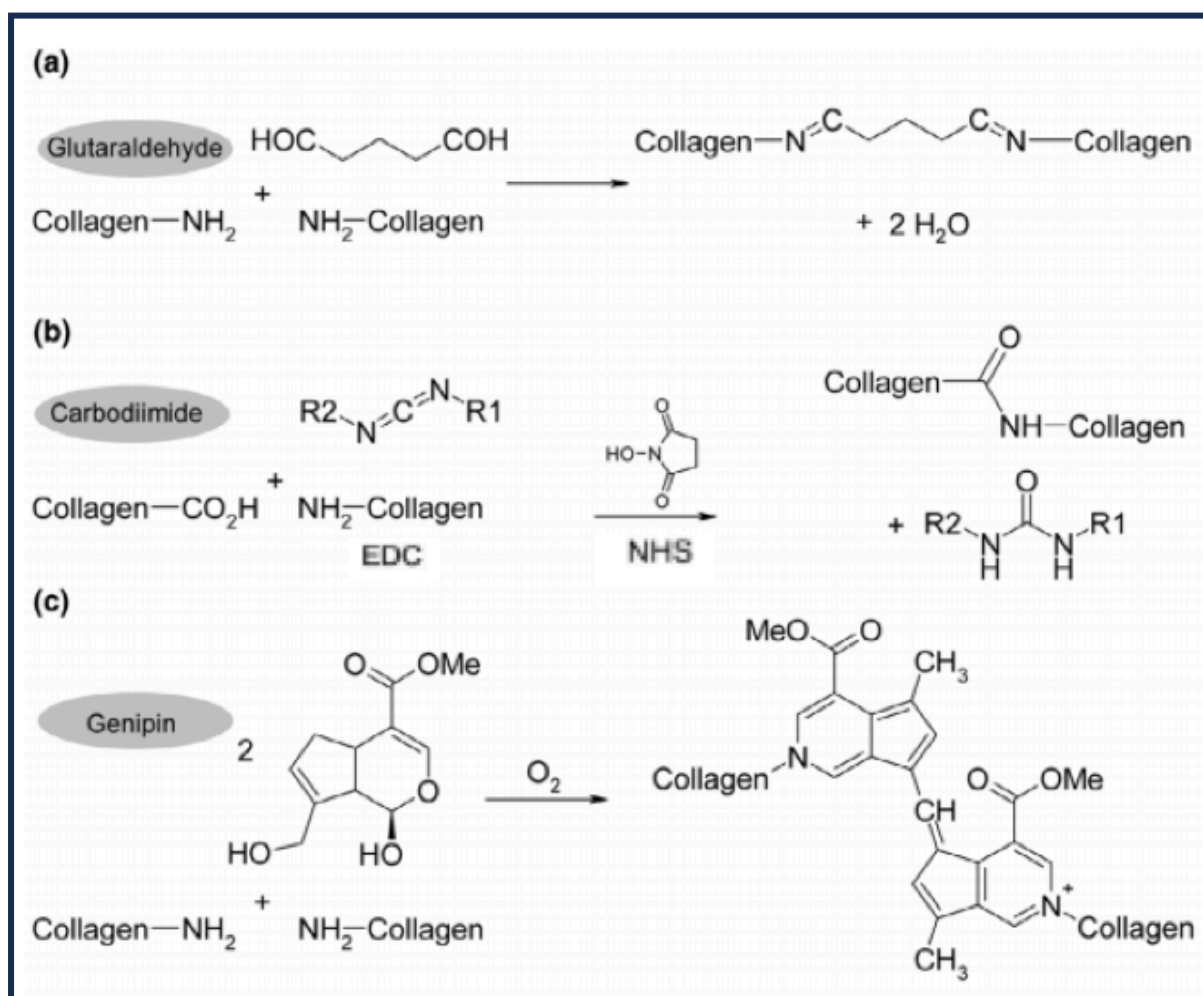


Figure S9: Chemical crosslinking routes of collagen (a) GTA (b) EDC/NHS (c) GP.

S.1.4.3. Enzymatic Crosslinking

Enzymatic crosslinking agents have been increasingly used to crosslink [100]; transglutaminase (TGase), lysyl oxidase, and horseradish peroxidase (HRP) are non-toxic and biocompatible enzyme crosslinkers.

One enzymatic chemical crosslinking strategy is the use of transglutaminases (TGase; protein-glutamine gamma-glutamyltransferase) in the fabrication of different types of biopolymeric scaffolds and hydrogels [101]. TGase are a wide family of thiol enzymes that catalyse post-translational protein modification mainly by inducing isopeptide bond formation, but also through the covalent conjugation of glutamine and lysine residues [102]. Transglutaminases

are a mild alternative to chemical crosslinking, Once formed, these bonds are highly resistant to proteolytic degradation. Consequently, stable polymeric networks are assembled, without the addition of co-factors.

Broderick et al [103]. treated three-dimensional hydroxyapatite/collagen composites with nGTase and found that crosslinking improved the mechanical strength and thermal stability of the scaffolds. Furthermore, cultures of the osteoblast-like cells and human umbilical vein endothelial cells, have shown good adhesion and proliferation, good viability, and good differentiation in such biopolymeric scaffolds[104]. Treatment of native collagen type I, derived from the bovine dermis layer, with mTGase, has been found to enhance attachment, proliferation and differentiation of human osteoblasts and fibroblasts [105].

Lysyl oxidase (LOX) is a key component in the formation and repair of the native extracellular matrix. This ubiquitous enzyme oxidizes primary amines of lysines to aldehydes. The formed reactive aldehydes react further to crosslink the extracellular matrix [106]. Lysyl oxidase is responsible for the covalent cross-linkages which stabilize collagen and elastin fibrous proteins. Consequently, lysyl oxidase is involved in the morphogenesis and regeneration potential of many connective tissues including skeleton, respiratory tract and cardiovascular tissue [107]. Interestingly, this enzyme can be used as a matrix crosslinker, not only to improve tissue or biomaterial strength over time but also to enhance matrix formation [108] [109]. The cross-linking of elastin by LOX family members is essential for the integrity and elasticity of elastic fibres, which play an important role in the characteristic resilience of various tissues. Furthermore, the cross-linking of tropoelastin molecules by LOX occurs concurrently with elastin deposition onto the microfibrils [110].

Bakota *et al.* [106], used lysyl oxidase for the cross-linking of a multidomain peptide hydrogel. They found that this method increased the storage modulus, G' , to over 400Pa without changes in hydrogel concentration and dramatic changes in nanostructural architecture. Furthermore, unlike other hydrogel systems that degrade over time, the hydrogels formed by this method become more robust due to the continuous activity of lysyl oxidase, which leads to a continuous increase in the mechanical stability of hydrogels composed of biopolymers rich in lysine. Lysyl oxidase-mediated cross-linking shows great value in tissue engineering using lysine-rich peptide-based hydrogels [106] [111].

Horseradish peroxidase (HRP) is a single-chain β -type hemoprotein responsible for the catalysis of the conjugation of phenol and aniline derivatives in the presence of H_2O_2 and has been employed to form chemically crosslinked hydrogels [112]. It has attracted attention in biomedical applications due to fast gelation, controllable crosslinking density, and gelation rate. Despite the cytocompatibility of HRP substrates involving in vivo applications, a major concern is potential immunogenicity as HRP is not a mammalian-derived peroxidase [113].

Horseradish cross-linkable dextran-tyramine hydrogels also in combination with hyaluronic acid and heparin have recently shown high potential as artificial extracellular matrixes for cartilage tissue engineering [101], [114], [115]. These polysaccharide hybrids were designed to mimic the molecular structure of the extracellular matrix of native cartilage.

Although enzyme-mediated cross-linking shows great value in tissue engineering using hydrogels its application in 3D tissue engineering scaffolds has yet to be explored.

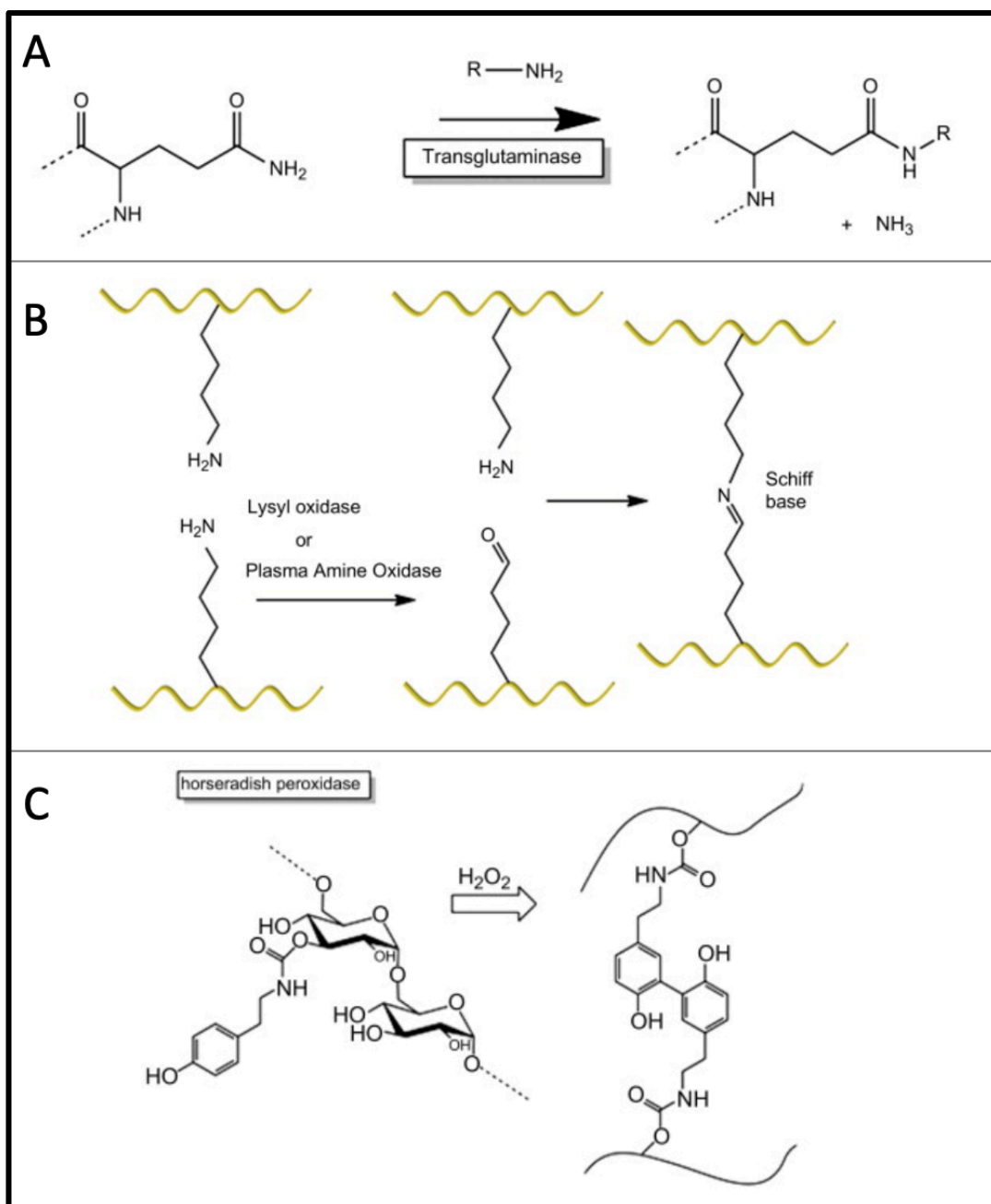


Figure S10: Mechanism of (A) TGase-assisted, (B) lysyl oxidase-assisted, (C) Horseradish peroxidase-assisted cross-linking reactions [113].

	Advantages	Disadvantages
Transglutaminase	-Acts as “biological glue”, participation in cell-matrix interactions and general maintenance of tissue integrity. -highly cytocompatibility	-Short-lived proteins (half-life of type 2 tissue transglutaminase is about 11 hours) -Large enzymes, difficult to produce recombinantly -Involvement in chronic inflammatory diseases of the joint, activating pro-inflammatory cytokines that might lead to mineralisation and disease progression -Poor mechanical properties
Lysyl Oxidase	-Involved in native cross-linking of extracellular matrix components -Cross-linked structures become more robust in time due to continuous crosslinking	-Lysyl oxidase is not commercially available -Copper-dependent
Horseradish peroxidase	-Peroxidases are involved in several processes in our body and are used in a wide range of biotechnological tools -highly stable -No co-factors are necessary - highly cytocompatible and -High mechanical strength	-Despite the several in vitro applications or horseradish peroxidase, the in vivo role is not fully elucidated.

Table S4: Advantages and disadvantages of Enzymatic crosslinkers [113].

S.2. References

- [1] K. Gelse, E. Pöschl, and T. Aigner, ‘Collagens—structure, function, and biosynthesis’, *Advanced Drug Delivery Reviews*, vol. 55, no. 12, pp. 1531–1546, Nov. 2003, doi: 10.1016/j.addr.2003.08.002.
- [2] S. Ricard-Blum, F. Ruggiero, and M. van der Rest, ‘The Collagen Superfamily’, in *Collagen: Primer in Structure, Processing and Assembly*, J. Brinckmann, H. Notbohm, and P. K. Müller, Eds., in Topics in Current Chemistry. , Berlin, Heidelberg: Springer, 2005, pp. 35–84. doi: 10.1007/b103819.
- [3] K. I. Kivirikko, L. Ryhänen, H. Anttinen, P. Bornstein, and D. J. Prockop, ‘Further hydroxylation of lysyl residues in collagen by procollagen lysyl hydroxylase in vitro’, *Biochemistry*, vol. 12, no. 24, pp. 4966–4971, Nov. 1973, doi: 10.1021/bi00748a023.
- [4] D. Silver, J. Miller, R. Harrison, and D. J. Prockop, ‘Helical model of nucleation and propagation to account for the growth of type I collagen fibrils from symmetrical pointed tips: a special example of self-assembly of rod-like monomers.’, *Proc Natl Acad Sci U S A*, vol. 89, no. 20, pp. 9860–9864, Oct. 1992.
- [5] A. Veis, ‘Collagen Fibrillogenesis’, *Connective Tissue Research*, vol. 10, no. 1, pp. 11–24, Jan. 1982, doi: 10.3109/03008208209034402.
- [6] M. J. Seibel, S. P. Robins, and J. P. Bilezikian, *Dynamics of Bone and Cartilage Metabolism: Principles and Clinical Applications*. Elsevier, 2006.
- [7] J. Rossert and B. de Crombrughe, ‘Chapter 12 - Type I Collagen: Structure, Synthesis, and Regulation’, in *Principles of Bone Biology (Second Edition)*, J. P. Bilezikian, L. G. Raisz, and G. A. Rodan, Eds., San Diego: Academic Press, 2002, pp. 189–XVIII. doi: 10.1016/B978-012098652-1.50114-1.
- [8] J. Gross, ‘Collagen biology: structure, degradation, and disease’, *Harvey Lect.*, vol. 68, pp. 351–432, 1974.

- [9] J. B. Weiss, 'Enzymic degradation of collagen', *Int Rev Connect Tissue Res*, vol. 7, pp. 101–157, 1976, doi: 10.1016/b978-0-12-363707-9.50009-5.
- [10] E. D. Harris and S. M. Krane, 'Collagenases', *New England Journal of Medicine*, vol. 291, no. 13, pp. 652–661, Sep. 1974, doi: 10.1056/NEJM197409262911305.
- [11] T. Klein and R. Bischoff, 'Physiology and pathophysiology of matrix metalloproteases', *Amino Acids*, vol. 41, no. 2, pp. 271–290, Jul. 2011, doi: 10.1007/s00726-010-0689-x.
- [12] P. M. Starkey, A. J. Barrett, and M. C. Burleigh, 'The degradation of articular collagen by neutrophil proteinases', *Biochim. Biophys. Acta*, vol. 483, no. 2, pp. 386–397, Aug. 1977, doi: 10.1016/0005-2744(77)90066-3.
- [13] W. J. 'Biological mechanisms of collagen resorption', *Treatise on Collagen*, vol. 2, pp. 149–245, 1968.
- [14] I. Kareva, A. Abou-Slaybi, O. Dodd, O. Dashevsky, and G. L. Klement, 'Normal Wound Healing and Tumor Angiogenesis as a Game of Competitive Inhibition', *PLoS One*, vol. 11, no. 12, p. e0166655, 2016, doi: 10.1371/journal.pone.0166655.
- [15] D. Wenzel *et al.*, 'Endostatin, the proteolytic fragment of collagen XVIII, induces vasorelaxation', *Circ Res*, vol. 98, no. 9, pp. 1203–1211, May 2006, doi: 10.1161/01.RES.0000219899.93384.ed.
- [16] J. D. Currey, 'MATERIALS SCIENCE: Hierarchies in Biomineral Structures', *Science*, vol. 309, no. 5732, pp. 253–254, Jul. 2005, doi: 10.1126/science.1113954.
- [17] H. S. Gupta, P. Messmer, P. Roschger, S. Bernstorff, K. Klaushofer, and P. Fratzl, 'Synchrotron Diffraction Study of Deformation Mechanisms in Mineralized Tendon', *Phys. Rev. Lett.*, vol. 93, no. 15, p. 158101, Oct. 2004, doi: 10.1103/PhysRevLett.93.158101.
- [18] H. S. Gupta, J. Seto, W. Wagermaier, P. Zaslansky, P. Boesecke, and P. Fratzl, 'Cooperative deformation of mineral and collagen in bone at the nanoscale',

- Proceedings of the National Academy of Sciences*, vol. 103, no. 47, pp. 17741–17746, Nov. 2006, doi: 10.1073/pnas.0604237103.
- [19] H. S. Gupta *et al.*, ‘Nanoscale Deformation Mechanisms in Bone’, *Nano Lett.*, vol. 5, no. 10, pp. 2108–2111, Oct. 2005, doi: 10.1021/nl051584b.
- [20] N. M. Pugno, ‘Mimicking nacre with super-nanotubes for producing optimized super-composites’, *Nanotechnology*, vol. 17, no. 21, pp. 5480–5484, Oct. 2006, doi: 10.1088/0957-4484/17/21/031.
- [21] S. Weiner and H. D. Wagner, ‘THE MATERIAL BONE: Structure-Mechanical Function Relations’, *Annual Review of Materials Science*, vol. 28, no. 1, pp. 271–298, 1998, doi: 10.1146/annurev.matsci.28.1.271.
- [22] P. Fratzl, H. S. Gupta, E. P. Paschalis, and P. Roschger, ‘Structure and mechanical quality of the collagen–mineral nano-composite in bone’, *J. Mater. Chem.*, vol. 14, no. 14, pp. 2115–2123, Jul. 2004, doi: 10.1039/B402005G.
- [23] M. J. Buehler and S. Y. Wong, ‘Entropic elasticity controls nanomechanics of single tropocollagen molecules’, *Biophys. J.*, vol. 93, no. 1, pp. 37–43, Jul. 2007, doi: 10.1529/biophysj.106.102616.
- [24] Y.-L. Sun, Z.-P. Luo, A. Fertala, and K.-N. An, ‘Direct quantification of the flexibility of type I collagen monomer’, *Biochem. Biophys. Res. Commun.*, vol. 295, no. 2, pp. 382–386, Jul. 2002, doi: 10.1016/s0006-291x(02)00685-x.
- [25] L. Bozec and M. Horton, ‘Topography and mechanical properties of single molecules of type I collagen using atomic force microscopy’, *Biophys. J.*, vol. 88, no. 6, pp. 4223–4231, Jun. 2005, doi: 10.1529/biophysj.104.055228.
- [26] Z. L. Shen, M. R. Dodge, H. Kahn, R. Ballarini, and S. J. Eppell, ‘Stress-strain experiments on individual collagen fibrils’, *Biophys. J.*, vol. 95, no. 8, pp. 3956–3963, Oct. 2008, doi: 10.1529/biophysj.107.124602.

- [27] S. J. Eppell, B. N. Smith, H. Kahn, and R. Ballarini, ‘Nano measurements with micro-devices: mechanical properties of hydrated collagen fibrils’, *J R Soc Interface*, vol. 3, no. 6, pp. 117–121, Feb. 2006, doi: 10.1098/rsif.2005.0100.
- [28] J. A. J. van der Rijt, K. O. van der Werf, M. L. Bennink, P. J. Dijkstra, and J. Feijen, ‘Micromechanical testing of individual collagen fibrils’, *Macromol Biosci*, vol. 6, no. 9, pp. 697–702, Sep. 2006, doi: 10.1002/mabi.200600063.
- [29] P. Fratzl, K. Misof, I. Zizak, G. Rapp, H. Amenitsch, and S. Bernstorff, ‘Fibrillar Structure and Mechanical Properties of Collagen’, *Journal of Structural Biology*, vol. 122, no. 1, pp. 119–122, Jan. 1998, doi: 10.1006/jsbi.1998.3966.
- [30] P. Fratzl, S. Schreiber, and K. Klaushofer, ‘Bone mineralization as studied by small-angle x-ray scattering’, *Connect. Tissue Res.*, vol. 34, no. 4, pp. 247–254, 1996, doi: 10.3109/03008209609005268.
- [31] N. Sasaki and S. Odajima, ‘Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy’, *Journal of Biomechanics*, vol. 29, no. 9, pp. 1131–1136, Sep. 1996, doi: 10.1016/0021-9290(96)00024-3.
- [32] S. Cusack and A. Miller, ‘Determination of the elastic constants of collagen by Brillouin light scattering’, *Journal of Molecular Biology*, vol. 135, no. 1, pp. 39–51, Nov. 1979, doi: 10.1016/0022-2836(79)90339-5.
- [33] I. V. Yannas and A. V. Tobolsky, ‘Cross-linking of gelatine by dehydration’, *Nature*, vol. 215, no. 5100, pp. 509–510, Jul. 1967, doi: 10.1038/215509b0.
- [34] K. Weadock, R. M. Olson, and F. H. Silver, ‘Evaluation of Collagen Crosslinking Techniques’, *Biomaterials, Medical Devices, and Artificial Organs*, vol. 11, no. 4, pp. 293–318, Jan. 1983, doi: 10.3109/10731198309118815.
- [35] K. S. Weadock, E. J. Miller, L. D. Bellincampi, J. P. Zawadsky, and M. G. Dunn, ‘Physical crosslinking of collagen fibers: Comparison of ultraviolet irradiation and

- dehydrothermal treatment', *Journal of Biomedical Materials Research*, vol. 29, no. 11, pp. 1373–1379, Nov. 1995, doi: 10.1002/jbm.820291108.
- [36] S. Yunoki, T. Suzuki, and M. Takai, 'Stabilization of low denaturation temperature collagen from fish by physical cross-linking methods', *Journal of Bioscience and Bioengineering*, vol. 96, no. 6, pp. 575–577, Jan. 2003, doi: 10.1016/S1389-1723(04)70152-8.
- [37] M. Koide *et al.*, 'A new type of biomaterial for artificial skin: Dehydrothermally cross-linked composites of fibrillar and denatured collagens', *Journal of Biomedical Materials Research*, vol. 27, no. 1, pp. 79–87, 1993, doi: 10.1002/jbm.820270111.
- [38] M. C. Wang, G. D. Pins, and F. H. Silver, 'Collagen fibres with improved strength for the repair of soft tissue injuries', *Biomaterials*, vol. 15, no. 7, pp. 507–512, Jun. 1994, doi: 10.1016/0142-9612(94)90016-7.
- [39] K. S. Weadock, E. J. Miller, E. Keuffel, and M. G. Dunn, 'Effect of physical crosslinking methods on collagen-fiber durability in proteolytic solutions.', *Journal of biomedical materials research*, 1996, doi: 10.1002/(SICI)1097-4636(199610)32:2<221::AID-JBM11>3.0.CO;2-M.
- [40] J. K. Law, J. R. Parsons, F. H. Silver, and A. B. Weiss, 'An evaluation of purified reconstituted type 1 collagen fibers', *J. Biomed. Mater. Res.*, vol. 23, no. 9, pp. 961–977, Sep. 1989, doi: 10.1002/jbm.820230902.
- [41] M. Haugh, C. Murphy, R. McKiernan, C. Altenbuchner, and F. O'Brien, 'Crosslinking and Mechanical Properties Significantly Influence Cell Attachment, Proliferation, and Migration Within Collagen Glycosaminoglycan Scaffolds', *Tissue engineering. Part A*, vol. 17, pp. 1201–8, May 2011, doi: 10.1089/ten.TEA.2010.0590.

- [42] N. Inoue, M. Bessho, M. Furuta, T. Kojima, S. Okuda, and M. Hara, 'A novel collagen hydrogel cross-linked by gamma-ray irradiation in acidic pH conditions', *J Biomater Sci Polym Ed*, vol. 17, no. 8, pp. 837–858, 2006, doi: 10.1163/156856206777996835.
- [43] M. P. Ohan and M. G. Dunn, 'Glucose stabilizes collagen sterilized with gamma irradiation', *J Biomed Mater Res A*, vol. 67, no. 4, pp. 1188–1195, Dec. 2003, doi: 10.1002/jbm.a.20018.
- [44] L. M. Roa, Ed., *XIII Mediterranean Conference on Medical and Biological Engineering and Computing 2013: MEDICON 2013, 25-28 September 2013, Seville, Spain*. in IFMBE Proceedings. Springer International Publishing, 2014. doi: 10.1007/978-3-319-00846-2.
- [45] W. Q. Sun and P. Leung, 'Calorimetric study of extracellular tissue matrix degradation and instability after gamma irradiation', *Acta Biomaterialia*, vol. 4, no. 4, pp. 817–826, Jul. 2008, doi: 10.1016/j.actbio.2008.02.006.
- [46] N. Davidenko *et al.*, 'Optimisation of UV irradiation as a binding site conserving method for crosslinking collagen-based scaffolds', *Journal of Materials Science: Materials in Medicine*, vol. 27, no. 1, p. 14, 2016, doi: 10.1007/s10856-015-5627-8.
- [47] D. V. Bent and E. Hayon, 'Excited state chemistry of aromatic amino acids and related peptides. III. Tryptophan', *J. Am. Chem. Soc.*, vol. 97, no. 10, pp. 2612–2619, May 1975, doi: 10.1021/ja00843a004.
- [48] D. V. Bent and E. Hayon, 'Excited state chemistry of aromatic amino acids and related peptides. I. Tyrosine', *J. Am. Chem. Soc.*, vol. 97, no. 10, pp. 2599–2606, May 1975, doi: 10.1021/ja00843a002.
- [49] H. Niknejad and R. Mahmoudzadeh, 'Comparison of Different Crosslinking Methods for Preparation of Docetaxel-loaded Albumin Nanoparticles', *Iran J Pharm Res*, vol. 14, no. 2, pp. 385–394, 2015.

- [50] ‘Polymeric Biomaterials: Structure and Function, Volume 1’, CRC Press. Accessed: Mar. 26, 2020. [Online]. Available: <https://www.crcpress.com/Polymeric-Biomaterials-Structure-and-Function-Volume-1/Dumitriu-Popa/p/book/9781420094701>
- [51] Z. Dai, J. Ronholm, Y. Tian, B. Sethi, and X. Cao, ‘Sterilization techniques for biodegradable scaffolds in tissue engineering applications’, *J Tissue Eng*, vol. 7, p. 2041731416648810, May 2016, doi: 10.1177/2041731416648810.
- [52] N. M. Weathington *et al.*, ‘A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation’, *Nat Med*, vol. 12, no. 3, pp. 317–323, Mar. 2006, doi: 10.1038/nm1361.
- [53] T. Scheibel, ‘Recombinant mussel byssus protein’, EP1787995A1, May 23, 2007 Accessed: Jul. 06, 2020. [Online]. Available: <https://patents.google.com/patent/EP1787995A1/en>
- [54] J. H. Bowes and C. W. Cater, ‘The interaction of aldehydes with collagen’, *Biochimica et Biophysica Acta (BBA) - Protein Structure*, vol. 168, no. 2, pp. 341–352, Oct. 1968, doi: 10.1016/0005-2795(68)90156-6.
- [55] H. Chajra *et al.*, ‘Collagen-based biomaterials and cartilage engineering. Application to osteochondral defects’, *Bio-Medical Materials and Engineering*, vol. 18, no. s1, pp. 33–45, Jan. 2008.
- [56] E. A. Woodroof, ‘The chemistry and biology of aldehyde treated tissue heart halve xenografts’, *M. Ionescu (Ed.), Tissue Heart Valves, Butterworths, London*, pp. 347–362, 1979.
- [57] M. Kikuchi, H. N. Matsumoto, T. Yamada, Y. Koyama, K. Takakuda, and J. Tanaka, ‘Glutaraldehyde cross-linked hydroxyapatite/collagen self-organized nanocomposites’, *Biomaterials*, vol. 25, no. 1, pp. 63–69, Jan. 2004, doi: 10.1016/s0142-9612(03)00472-1.

- [58] M. E. Nimni, 'The Cross-Linking and Structure Modification of the Collagen Matrix in the Design of Cardiovascular Prosthesis', *Journal of Cardiac Surgery*, vol. 3, no. 4, pp. 523–533, 1988, doi: 10.1111/j.1540-8191.1988.tb00446.x.
- [59] U. K. Laemmli, 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', *Nature*, vol. 227, no. 5259, Art. no. 5259, Aug. 1970, doi: 10.1038/227680a0.
- [60] D. P. Speer, M. Chvapil, C. D. Eskelson, and J. Ulreich, 'Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials', *J. Biomed. Mater. Res.*, vol. 14, no. 6, pp. 753–764, Nov. 1980, doi: 10.1002/jbm.820140607.
- [61] N. J. Smart, I. R. Daniels, and S. Marquez, 'Supplemental cross-linking in tissue-based surgical implants for abdominal wall repair', *International Journal of Surgery*, vol. 10, no. 9, pp. 436–442, Jan. 2012, doi: 10.1016/j.ijssu.2012.07.010.
- [62] A. Cooke, R. F. Oliver, and M. Edward, 'An in vitro cytotoxicity study of aldehyde-treated pig dermal collagen.', *Br J Exp Pathol*, vol. 64, no. 2, pp. 172–176, Apr. 1983.
- [63] L. H. H. Olde Damink, P. J. Dijkstra, M. J. A. Van Luyn, P. B. Van Wachem, P. Nieuwenhuis, and J. Feijen, 'Glutaraldehyde as a crosslinking agent for collagen-based biomaterials', *J Mater Sci: Mater Med*, vol. 6, no. 8, pp. 460–472, Aug. 1995, doi: 10.1007/BF00123371.
- [64] E. Jorge-Herrero, P. Fernández, C. Escudero, J. M. García-Páez, and J. L. Castillo-Olivares, 'Calcification of pericardial tissue pretreated with different amino acids', *Biomaterials*, vol. 17, no. 6, pp. 571–575, Mar. 1996, doi: 10.1016/0142-9612(96)88707-2.
- [65] H. Petite, I. Rault, A. Huc, P. Menasche, and D. Herbage, 'Use of the acyl azide method for cross-linking collagen-rich tissues such as pericardium', *Journal of Biomedical Materials Research*, vol. 24, no. 2, pp. 179–187, 1990, doi: 10.1002/jbm.820240205.

- [66] H. Petite, V. Frei, A. Huc, and D. Herbage, 'Use of diphenylphosphorylazide for cross-linking collagen-based biomaterials', *Journal of Biomedical Materials Research*, vol. 28, no. 2, pp. 159–165, 1994, doi: 10.1002/jbm.820280204.
- [67] I. Rault, V. Frei, D. Herbage, N. Abdul-Malak, and A. Huc, 'Evaluation of different chemical methods for cross-linking collagen gel, films and sponges', *J Mater Sci: Mater Med*, vol. 7, no. 4, pp. 215–221, Apr. 1996, doi: 10.1007/BF00119733.
- [68] P. R. Coulet and D. C. Gautheron, 'Enzymes immobilized on collagen membranes: A tool for fundamental research and enzyme engineering', *Journal of Chromatography A*, vol. 215, pp. 65–72, Oct. 1981, doi: 10.1016/S0021-9673(00)81386-2.
- [69] L. H. Olde Damink, P. J. Dijkstra, M. J. van Luyn, P. B. van Wachem, P. Nieuwenhuis, and J. Feijen, 'Cross-linking of dermal sheep collagen using a water-soluble carbodiimide', *Biomaterials*, vol. 17, no. 8, pp. 765–773, Apr. 1996, doi: 10.1016/0142-9612(96)81413-x.
- [70] H. Cao and S.-Y. Xu, 'EDC/NHS-crosslinked type II collagen-chondroitin sulfate scaffold: characterization and in vitro evaluation', *J Mater Sci: Mater Med*, vol. 19, no. 2, pp. 567–575, Feb. 2008, doi: 10.1007/s10856-007-3281-5.
- [71] J. S. Pieper, T. Hafmans, J. H. Veerkamp, and T. H. van Kuppevelt, 'Development of tailor-made collagen–glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects', *Biomaterials*, vol. 21, no. 6, pp. 581–593, Mar. 2000, doi: 10.1016/S0142-9612(99)00222-7.
- [72] A. Jayakrishnan and S. R. Jameela, 'Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices', *Biomaterials*, vol. 17, no. 5, pp. 471–484, Jan. 1996, doi: 10.1016/0142-9612(96)82721-9.
- [73] J. Kozłowska and A. Sionkowska, 'Effects of different crosslinking methods on the properties of collagen–calcium phosphate composite materials', *International Journal*

- of Biological Macromolecules*, vol. 74, pp. 397–403, Mar. 2015, doi: 10.1016/j.ijbiomac.2014.12.023.
- [74] C. Yang, ‘Enhanced physicochemical properties of collagen by using EDC/NHS-crosslinking’, *Bull Mater Sci*, vol. 35, no. 5, pp. 913–918, Oct. 2012, doi: 10.1007/s12034-012-0376-5.
- [75] J. S. Pieper, A. Oosterhof, P. J. Dijkstra, J. H. Veerkamp, and T. H. van Kuppevelt, ‘Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate’, *Biomaterials*, vol. 20, no. 9, pp. 847–858, May 1999, doi: 10.1016/s0142-9612(98)00240-3.
- [76] J. C. Sheehan and J. J. Hlavka, ‘The Use of Water-Soluble and Basic Carbodiimides in Peptide Synthesis’, *J. Org. Chem.*, vol. 21, no. 4, pp. 439–441, Apr. 1956, doi: 10.1021/jo01110a017.
- [77] L. Calderon, E. Collin, D. Velasco-Bayon, M. Murphy, D. O’Halloran, and A. Pandit, ‘Type II collagen-hyaluronan hydrogel--a step towards a scaffold for intervertebral disc tissue engineering’, *Eur Cell Mater*, vol. 20, pp. 134–148, Sep. 2010, doi: 10.22203/ecm.v020a12.
- [78] J. H. Shepherd, S. Ghose, S. J. Kew, A. Moavenian, S. M. Best, and R. E. Cameron, ‘Effect of fiber crosslinking on collagen-fiber reinforced collagen-chondroitin-6-sulfate materials for regenerating load-bearing soft tissues’, *J Biomed Mater Res A*, vol. 101, no. 1, pp. 176–184, Jan. 2013, doi: 10.1002/jbm.a.34317.
- [79] B. Hafemann, K. Ghofrani, H. G. Gattner, H. Stieve, and N. Pallua, ‘Cross-linking by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) of a collagen/elastin membrane meant to be used as a dermal substitute: effects on physical, biochemical and biological features in vitro’, *J Mater Sci Mater Med*, vol. 12, no. 5, pp. 437–446, May 2001, doi: 10.1023/a:1011205221972.

- [80] N. Davidenko *et al.*, ‘Control of crosslinking for tailoring collagen-based scaffolds stability and mechanics’, *Acta Biomaterialia*, vol. 25, pp. 131–142, Oct. 2015, doi: 10.1016/j.actbio.2015.07.034.
- [81] R. A. Hortensius and B. A. C. Harley, ‘The use of bioinspired alterations in the glycosaminoglycan content of collagen–GAG scaffolds to regulate cell activity’, *Biomaterials*, vol. 34, no. 31, pp. 7645–7652, Oct. 2013, doi: 10.1016/j.biomaterials.2013.06.056.
- [82] B. A. Harley, J. H. Leung, E. C. C. M. Silva, and L. J. Gibson, ‘Mechanical characterization of collagen-glycosaminoglycan scaffolds’, *Acta Biomater*, vol. 3, no. 4, pp. 463–474, Jul. 2007, doi: 10.1016/j.actbio.2006.12.009.
- [83] E. Quinlan, A. López-Noriega, E. M. Thompson, A. Hibbitts, S. A. Cryan, and F. J. O’Brien, ‘Controlled release of vascular endothelial growth factor from spray-dried alginate microparticles in collagen-hydroxyapatite scaffolds for promoting vascularization and bone repair’, *J Tissue Eng Regen Med*, vol. 11, no. 4, pp. 1097–1109, 2017, doi: 10.1002/term.2013.
- [84] H. Petite, J. L. Duval, V. Frei, N. Abdul-Malak, M. F. Sigot-Luizard, and D. Herbage, ‘Cytocompatibility of calf pericardium treated by glutaraldehyde and by the acyl azide methods in an organotypic culture model’, *Biomaterials*, vol. 16, no. 13, pp. 1003–1008, Sep. 1995, doi: 10.1016/0142-9612(95)94908-4.
- [85] P. B. van Wachem, M. J. van Luyn, L. H. Olde Damink, P. J. Dijkstra, J. Feijen, and P. Nieuwenhuis, ‘Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen’, *J. Biomed. Mater. Res.*, vol. 28, no. 3, pp. 353–363, Mar. 1994, doi: 10.1002/jbm.820280310.

- [86] J. T. Mason and T. J. O’Leary, ‘Effects of formaldehyde fixation on protein secondary structure: a calorimetric and infrared spectroscopic investigation’, *J. Histochem. Cytochem.*, vol. 39, no. 2, pp. 225–229, Feb. 1991, doi: 10.1177/39.2.1987266.
- [87] C. H. Fox, F. B. Johnson, J. Whiting, and P. P. Roller, ‘Formaldehyde fixation’, *J. Histochem. Cytochem.*, vol. 33, no. 8, pp. 845–853, Aug. 1985, doi: 10.1177/33.8.3894502.
- [88] B. W. Sutherland, J. Toews, and J. Kast, ‘Utility of formaldehyde cross-linking and mass spectrometry in the study of protein–protein interactions’, *Journal of Mass Spectrometry*, vol. 43, no. 6, pp. 699–715, 2008, doi: 10.1002/jms.1415.
- [89] M. Lindskog, J. Rockberg, M. Uhlén, and F. Sterky, ‘Selection of protein epitopes for antibody production’, *BioTechniques*, vol. 38, no. 5, pp. 723–727, May 2005, doi: 10.2144/05385ST02.
- [90] R. G. Paul and A. J. Bailey, ‘Chemical Stabilisation of Collagen as a Biomimetic’, *The Scientific World JOURNAL*, vol. 3, pp. 138–155, 2003, doi: 10.1100/tsw.2003.13.
- [91] B. Ma, X. Wang, C. Wu, and J. Chang, ‘Crosslinking strategies for preparation of extracellular matrix-derived cardiovascular scaffolds’, *Regen Biomater*, vol. 1, no. 1, pp. 81–89, Nov. 2014, doi: 10.1093/rb/rbu009.
- [92] H.-W. Sung, R.-N. Huang, L. L. H. Huang, C.-C. Tsai, and C.-T. Chiu, ‘Feasibility study of a natural crosslinking reagent for biological tissue fixation’, *Journal of Biomedical Materials Research*, vol. 42, no. 4, pp. 560–567, 1998, doi: 10.1002/(SICI)1097-4636(19981215)42:4<560::AID-JBM12>3.0.CO;2-I.
- [93] D. Macaya, K. K. Ng, and M. Spector, ‘Injectable Collagen–Genipin Gel for the Treatment of Spinal Cord Injury: In Vitro Studies’, *Advanced Functional Materials*, vol. 21, no. 24, pp. 4788–4797, 2011, doi: 10.1002/adfm.201101720.

- [94] H. W. Sung, I. L. Liang, C. N. Chen, R. N. Huang, and H. F. Liang, 'Stability of a biological tissue fixed with a naturally occurring crosslinking agent (genipin)', *J Biomed Mater Res*, vol. 55, no. 4, pp. 538–546, Jun. 2001, doi: 10.1002/1097-4636(20010615)55:4<538::aid-jbm1047>3.0.co;2-2.
- [95] J. W. Wassenaar, R. L. Braden, K. G. Osborn, and K. L. Christman, 'Modulating in vivo degradation rate of injectable extracellular matrix hydrogels', *J. Mater. Chem. B*, vol. 4, no. 16, pp. 2794–2802, Apr. 2016, doi: 10.1039/C5TB02564H.
- [96] H.-W. Sung, R.-N. Huang, L. L. H. Huang, and C.-C. Tsai, 'In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation', *Journal of Biomaterials Science, Polymer Edition*, vol. 10, no. 1, pp. 63–78, Jan. 1999, doi: 10.1163/156856299X00289.
- [97] T. Jiang, X.-J. Ren, J.-L. Tang, H. Yin, K.-J. Wang, and C.-L. Zhou, 'Preparation and characterization of genipin-crosslinked rat acellular spinal cord scaffolds', *Materials Science and Engineering: C*, vol. 33, no. 6, pp. 3514–3521, Aug. 2013, doi: 10.1016/j.msec.2013.04.046.
- [98] Y. Wang *et al.*, 'Genipin crosslinking reduced the immunogenicity of xenogeneic decellularized porcine whole-liver matrices through regulation of immune cell proliferation and polarization', *Scientific Reports*, vol. 6, no. 1, Art. no. 1, Apr. 2016, doi: 10.1038/srep24779.
- [99] C. Djerassi, J. D. Gray, and F. A. Kincl, 'Naturally Occurring Oxygen Heterocyclics. IX.1 Isolation and Characterization of Genipin²', *The Journal of Organic Chemistry*, vol. 25, no. 12, pp. 2174–2177, 1960, doi: 10.1021/JO01082A022.
- [100] F. Chen *et al.*, 'An Injectable Enzymatically Crosslinked Carboxymethylated Pullulan/Chondroitin Sulfate Hydrogel for Cartilage Tissue Engineering', *Scientific Reports*, vol. 6, no. 1, Art. no. 1, Jan. 2016, doi: 10.1038/srep20014.

- [101] R. Jin *et al.*, ‘Enzymatically Crosslinked Dextran-Tyramine Hydrogels as Injectable Scaffolds for Cartilage Tissue Engineering’, *Tissue Engineering Part A*, vol. 16, no. 8, pp. 2429–2440, Mar. 2010, doi: 10.1089/ten.tea.2009.0764.
- [102] T. A. E. Ahmed, E. V. Dare, and M. Hincke, ‘Fibrin: A Versatile Scaffold for Tissue Engineering Applications’, *Tissue Engineering Part B: Reviews*, vol. 14, no. 2, pp. 199–215, Jun. 2008, doi: 10.1089/ten.teb.2007.0435.
- [103] E. P. Broderick, D. M. O’Halloran, Y. A. Rochev, M. Griffin, R. J. Collighan, and A. S. Pandit, ‘Enzymatic stabilization of gelatin-based scaffolds’, *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 72B, no. 1, pp. 37–42, 2005, doi: 10.1002/jbm.b.30119.
- [104] G. Ciardelli *et al.*, ‘Enzymatically crosslinked porous composite matrices for bone tissue regeneration’, *Journal of Biomedical Materials Research Part A*, vol. 92A, no. 1, pp. 137–151, 2010, doi: 10.1002/jbm.a.32344.
- [105] R. A. de Carvalho and C. R. F. Grosso, ‘Characterization of gelatin based films modified with transglutaminase, glyoxal and formaldehyde’, *Food Hydrocolloids*, vol. 18, no. 5, pp. 717–726, Sep. 2004, doi: 10.1016/j.foodhyd.2003.10.005.
- [106] E. L. Bakota, L. Aulisa, K. M. Galler, and J. D. Hartgerink, ‘Enzymatic cross-linking of a nanofibrous peptide hydrogel’, *Biomacromolecules*, vol. 12, no. 1, pp. 82–87, Jan. 2011, doi: 10.1021/bm1010195.
- [107] H. M. Kagan and W. Li, ‘Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell’, *J Cell Biochem*, vol. 88, no. 4, pp. 660–672, Mar. 2003, doi: 10.1002/jcb.10413.
- [108] Y.-K. I. Lau, A. M. Gobin, and J. L. West, ‘Overexpression of Lysyl Oxidase to Increase Matrix Crosslinking and Improve Tissue Strength in Dermal Wound Healing’, *Ann*

- Biomed Eng*, vol. 34, no. 8, pp. 1239–1246, Aug. 2006, doi: 10.1007/s10439-006-9130-8.
- [109] C. R. Kothapalli and A. Ramamurthi, ‘Lysyl oxidase enhances elastin synthesis and matrix formation by vascular smooth muscle cells’, *Journal of Tissue Engineering and Regenerative Medicine*, vol. 3, no. 8, pp. 655–661, 2009, doi: 10.1002/term.214.
- [110] F. Sato, R. Seino-Sudo, M. Okada, H. Sakai, T. Yumoto, and H. Wachi, ‘Lysyl Oxidase Enhances the Deposition of Tropoelastin through the Catalysis of Tropoelastin Molecules on the Cell Surface’, *Biol. Pharm. Bull.*, vol. 40, no. 10, pp. 1646–1653, 2017, doi: 10.1248/bpb.b17-00027.
- [111] S. E. Grieshaber *et al.*, ‘Assembly Properties of an Alanine-Rich, Lysine-Containing Peptide and the Formation of Peptide/Polymer Hybrid Hydrogels’, *Macromolecular Chemistry and Physics*, vol. 212, no. 3, pp. 229–239, 2011, doi: 10.1002/macp.201000446.
- [112] S. Kobayashi, ‘Enzymatic Polymerization’, in *Encyclopedia of Polymer Science and Technology*, American Cancer Society, 2011. doi: 10.1002/0471440264.pst118.pub2.
- [113] L. S. Moreira Teixeira, J. Feijen, C. A. van Blitterswijk, P. J. Dijkstra, and M. Karperien, ‘Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering’, *Biomaterials*, vol. 33, no. 5, pp. 1281–1290, Feb. 2012, doi: 10.1016/j.biomaterials.2011.10.067.
- [114] R. Jin, L. S. Moreira Teixeira, P. J. Dijkstra, C. A. van Blitterswijk, M. Karperien, and J. Feijen, ‘Enzymatically-crosslinked injectable hydrogels based on biomimetic dextran–hyaluronic acid conjugates for cartilage tissue engineering’, *Biomaterials*, vol. 31, no. 11, pp. 3103–3113, Apr. 2010, doi: 10.1016/j.biomaterials.2010.01.013.
- [115] R. Jin, L. S. Moreira Teixeira, P. J. Dijkstra, C. A. van Blitterswijk, M. Karperien, and J. Feijen, ‘Chondrogenesis in injectable enzymatically crosslinked heparin/dextran

hydrogels', *Journal of Controlled Release*, vol. 152, no. 1, pp. 186–195, May 2011, doi:
10.1016/j.jconrel.2011.01.031.