Assessment of Crosslink Density in Collagen Models and Ultrafast Laser Crosslinking of Corneal and Cartilage Tissues as Novel Treatment Modalities

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Abstract

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Osteoarthritis (OA) is a progressive and complex joint disease that results from breakdown of articular cartilage and remodeling of underlying bone, which affects millions of Americans. While the expected lifetime of the load-bearing cartilage tissue should coincide with the lifespan of an individual, it has a limited ability to self-repair and the damage to the tissue can accumulate severely. One of the major challenges in OA treatment is its long asymptomatic period. Symptoms usually become noticeable when the disease is reaching advanced stages, and currently there is no effective intervention for early stages of OA. This may be due to lack of a reliable diagnostic method for detecting early OA. While OA is a degenerative joint disorder that may lead to gross cartilage loss and morphological damage to other joint tissues, a lot of subclinical, subtle biochemical changes occur in the early stages of OA progression. The degradation of collagen type II matrix in articular cartilage extracellular matrix (ECM) network may correlate with the progression of cartilage OA. During onset of OA, with the loss of collagen crosslinks, the collagen matrix in cartilage ECM becomes more disorganized and the cartilage can become susceptible to disorder and thus aggravate the degeneration. Raman spectroscopy has been utilized in studies of components of connective tissues, such as OA, and cartilage degradation. Although studies have demonstrated the potential of applying Raman for diagnosing cartilage degeneration, the analysis of Raman spectrum obtained from articular cartilage is rather complicated and so far there is no generally accepted quantitative analysis for diagnosing early stages of OA.
The first stage of this doctoral study aims to extend the capability of Raman spectroscopy to quantitatively characterize collagen network in articular cartilage, to investigate the possible correlation with the degeneration of OA. The first part of this doctoral dissertation is focused on developing a novel, non-destructive, quantitative diagnostic modality, based on Raman spectroscopy that has potential to detect changes in biochemical composition of articular cartilage. The study is focused on the basic research associated with quantification of crosslink density and kinetics of the crosslinking process. A theoretical and computational framework for characterization of collagen crosslinks has been established and applied onto two models, 2-dimensional collagen type I thin films, and immature bovine, proteoglycan depleted, articular cartilage. Glutaraldehyde solution has been applied onto the model as a convenient method to introduce various levels of collagen crosslinks.

Refractive error is a problem with focusing light accurately onto the retina due to the shape or other misfunctioning of the eye, rather than on the retina for the normal vision. The most common types of refractive errors are near-sightedness, far-sightedness, astigmatism, and presbyopia. Refractive errors have become a growing public health problem worldwide. Its incidence has doubled over the last 50 years in the United States and Europe. It is even more significant issue in some East Asian countries, where its prevalence reaches 70 to 90%. Most affected individuals use spectacles or contact lenses, which generally provides adequate refractive error correction. However, both are subject to limitations. Glasses do not work well in the rain and mist may form on them following changes in temperature or humidity. Contact lenses improve the field of vision and acuity, but many people find their presence on ocular surfaces intolerable. Over the last two to three decades, refractive surgery for the permanent correction of vision has thus emerged as an attractive choice for many patients. However, such a surgery is an invasive
procedure that may compromise corneal structure, and postsurgical complications have been reported.

In the second stage of this doctoral work, a novel, non-invasive femtosecond laser collagen crosslinks manipulation method is studied. This laser collagen crosslinking treatment is applied on corneal tissue for vision correction. Two examples of the laser treatment on an ex vivo porcine eyes model are proposed in the study: corneal flattening, which is used to correct refractive errors due to myopia, and corneal steepening, which is used to treat hyperopia. The effective refractive power is used to evaluate the effectiveness of the two different treatments. The depth of the crosslinked region in the cornea is assessed by two-photon autofluorescence (TPF) imaging. TPF imaging can be used to visualize changes induced in the cornea, because collagen is a primary extracellular source of nonlinear emissions. The safety of the proposed treatment methods is examined by haematoxylin and eosin (H&E) stained histological sections of corneas. The ex vivo porcine corneas are also cultured for one week after treatment, to determine whether crosslink density remains stable, and to check for degradation in the crosslinked layers of the stromal matrix, and further prove the safety of the proposed laser treatment method through the evaluation of cell viability after one week of treatment. An in vivo rabbit animal model, widely used as a model for the correction of refractive errors, is further utilized to demonstrate the stability and safety of the induced changes. The effective refractive power of live rabbits is assessed 24 h, seven days, and then weekly up to three months after the laser crosslinking treatment. The safety of the laser treatment is first evaluated by histology staining, and further confirmed by in vivo confocal laser scanning microscopy.

This laser treatment approach could expand the pool of patients eligible for permanent vision correction, while simultaneously eliminating the adverse effects associated with current
forms of surgery. Furthermore, the approach described is also suitable for the treatment of other disease for collagenous tissues. The last chapter of in this doctoral dissertation have discussed the results of applying this laser treatment techniques for the treatment of progressive OA.

Finally, in a preliminary study, the proposed femtosecond laser treatment modality developed for corneal tissue has been applied onto articular cartilage towards slowing down or retarding progression of early osteoarthritis. We hypothesize that degradation of the articular cartilage extracellular matrix can be slowed down or reversed in the collagen network crosslinked with a femtosecond laser. We further theorize that the crosslinking mechanism introduced in the corneal tissue, which relies on laser ionization and dissociation of the tissue interstitial water to produce of refractive oxygen species, can increase crosslink density of collagen network in an articular cartilage. In the study, the treatment has been applied onto devitalized and live immature bovine cartilage explants, as well as cartilage plugs obtained from OA afflicted human cadaver joints. The preliminary results have shown that the proposed treatment has potential to enhance tissue mechanical properties, and increase wear resistance, an important factor in slowing down the progression of OA. Furthermore, preliminary imaging of live/dead stained tissue has shown that the laser treatment has minimal adverse effects up to two weeks after the laser irradiation.
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For all my family members, my parents, my sister and brother-in-law, my niece and nephew.
Chapter 1: Introduction

1.1 Collagen

Collagen (COL) is the major extracellular structural protein in the body. As the main component of connective tissues, corneas, blood vessels and muscle tissues, it is the most abundant protein in animals. Approximately 30% protein content of the body is COL. It consists of polypeptide chains made of amino acids bonded together. Three polypeptide chains coiling around each other form the triple helix fibrillar structure with two terminals at opposite ends, N-terminal and C-terminal (Figure 1.1) [1-3].

![Figure 1.1: The triple helix structure of collagen [1-3]. N-terminal is on the left-, and C-terminal on the right-hand side.](image)

Different COL types were first discovered by Miller and Matukas in the late 60s [4]. They isolated the COL from chick cartilage and using chromatography found a different COL chain compared to previous findings. The sequence of amino acids and the spatial alignment conformational state of polypeptide chains produce different types of COL. More than 20 various types of COL have been discovered since then, promoted by the development of molecular biology and gene cloning. Types of COL can be divided into several groups according to the structure they form [1]. Fibrillar form includes type I, II, V and XI. Fibril associated collagens with interrupted triple helix form are type IX, XII, XIV, XIX, XXI. Short chain non-fibrillar form have type VIII and X. Type IV COL has a form of non-fibrillar basement membrane. Non-fibrillar multiple triple
helix domains with interruptions form includes type XV and XVIII. Membrane associated COL with interrupted triple helices form have type XIII and XVII. And two types are not defined in any forms, they are type VI and VII. Among different types of COL those with fibrillar form are most common with type I being over 90% of the COL in human body. Different tissues may contain different major COL components. For example, dominant COL found in corneal tissue is type I [5], whereas cartilage is type II, with many other types of COL present [1, 2, 6-10]. For those two tissues, more than two-thirds of the dry weight is counted as COL [11, 12].

1.1.1 Corneal collagen and structure

Cornea is on the superficial layer of the eye. It is an avascular connective tissue acting as the refractive surface for the eye to enable vision and providing a transparent protective barrier to the eyeball [13, 14]. The compositions of corneal tissue include cellular and acellular components [15]. The cellular components are the epithelial cells, keratocytes, and endothelial cells. The acellular component is predominantly collagen. There are three major cellular layers of a corneal tissue, from the eye surface to the inner eyeball they are epithelium, stroma and endothelium layers (Figure 1.2). The epithelium layer is around 50 µm thick, providing a smooth and evenly moisturized surface for corneal tissue [14]. Stroma has a thickness of approximately 500 µm. It is the major portion of a corneal tissue composed of collagen fibrils and water, allowing the transparency and surface contour of cornea for the eye to enable vision, while maintaining the cornea tissue biomechanical stiffness [13]. Collagen fibrils in stroma are highly organized, and form lamellar structure. Lamellae are parallel to the surface and almost equal in size, maintaining corneal shape while providing the transparency and refractive properties to the cornea and the whole eye. Endothelium is a single layer of cells that covers the posterior stroma and has a thickness of about 5 µm [14]. Endothelial cells adjust stromal hydration level, and thus maintain
the optical transparency of the corneal tissue [16]. Besides these three major cellular zones of a cornea, there are three thin acellular membrane layers. The tear film is a 3 µm thick fluid layer that covers and lubricates the corneal superficial surface, forming a smooth refractive surface for vision [17]. Bowman’s membrane is a thin layer right underneath the epithelium layer made of fine collagen fibrils, providing a smooth outer surface of epithelium, though the main function remains unclear [18]. Descemet’s membrane is the basement membrane between stroma and endothelium that anchors the endothelium to the cornea, while simultaneously allowing the entrance of nutrients and other molecules into the corneal stroma [19].

Figure 1.2: Schematic diagram of cornea structure.

More than 70% of the dry weight of cornea is counted as COL, and the predominant COL component of cornea COL framework is type I, forming an extracellular matrix containing narrow-diameter (~36 nm) and heterotypic COL fibrils that are spaced and organized uniformly into sheets
of parallel fibrils [5]. Each sheet is arranged orthogonal to its neighbor and to the path of light through the cornea [20]. Other types of COL (type V, VI, XII, XIII, XIV and XXIV) were found with small amounts [5, 21-23]. In corneal heterotypic fibrils, COL type I and V were observed, and type V COL was discovered under corneal superficial surface [5]. In the interfaces with Bowman and Descemet layers COL type XII was founded as the long isoform aligned on type I COL fibril surface, and it is assumed to provide increased stiffness [22, 24]. COL type VI beaded filaments in the stroma stabilize the fibrillar COL array, and it may also be responsible for the extension of the keratocytes lifespan by preventing apoptosis [23]. Type XXIV is responsible for the early regulation of murine corneal fibril diameters in conjunction with type I COL [25]. In posterior stroma collagen type XIII was observed within unknown function. Type XIV is found to be responsible for maintaining the transparency of the avian cornea associated with the surface of type I COL fibrils [26].

1.1.2 Cartilage collagen and structure

Figure 1.3: Schematic diagram of the fibrous architecture of articular cartilage.
Specialized as the connective tissue of synovial joints, articular cartilage is normally 2-4 mm thick, providing a smooth, lubricated low friction surface, and distributing the load applied to the joint to minimize the stress on the subchondral bone [27, 28]. Unlike the other tissues, there are no blood vessels, lymphatics, and nerves within adult articular cartilage, leading to an extremely severe physiological environment [27]. Articular cartilage is composed of COL, proteoglycans (PG), water, a very small amount of other proteins [29, 30], and chondrocytes [31]. Components of articular cartilage combine to form an extracellular matrix (ECM), an environment suitable for a sparse distribution of chondrocytes [32]. More than 80% of the total weight of articular cartilage comes from its water content, most of which is located in the pore space of the matrix, while the rest of water is filling up the intrafibrillar space within the COL [33, 34]. Sodium, calcium, chloride, potassium, and other inorganic elements are dissolved in the water to provide basic nutrients for tissue metabolism [35, 36]. COL is the major provider of tensile and shear strength to ECM in articular cartilage [37]. PGs aggregate along a hyaluronan chain and their electric charge gives rise to Donnan osmotic pressure that helps to resist compressive loads [38]. While the function of quantitatively minor proteins in articular cartilage remains to be fully clarified, they may help with the maintenance and organization of the ECM [39]. Generated by mesenchymal stem cells, chondrocytes occupy about 2%-5% of the entire articular cartilage volume in adult tissue. Chondrocytes have different shapes and sizes in different regions of the articular layer [31]. Their metabolism helps with the development, maintenance, and possibly repair of ECM [31].

There are four zones of articular cartilage, which are distinguished by their COL fibril orientation and microstructure: superficial or tangential zone, middle or transitional zone, deep or radial zone, and calcified zone (Figure 1.3). In addition, there is a several micron deep layer
composed of fine fibrils (4 to 10 nm in diameter) that cover the articular surface called the lamina splendens (Figure 1.3). The superficial zone normally has a thickness around 200 µm in which packed bundles of COL fibrils are thin and oriented primarily parallel to the plane of the articular surface [12]. This major fibrous zone is thinnest at the joint center and slightly thicker at the peripheral zone of the joint. COL fibril diameters were observed to exhibit a greater range in the deeper zones. A significant amount of PGs were observed in middle zone, where COL fibrils are randomly oriented. COL fibrils in the deep zone are also somewhat randomly arranged, however, a preferred orientation is demonstrated to be orthogonal to the superficial surface [40]. COL in the calcified zone mostly contains mature COL oriented perpendicular to the articular surface.

Approximately two-thirds of the dry weight of articular cartilage is counted as COL [41]. COL type II was found to be the predominant COL component in articular cartilage, and the other types (IX, XI, III, VI, XII and XIV) of COL were also reported to a very small level with different functions [41-45], although some of their functions are not specified. COL type IX molecules can be observed on fibril surfaces, particularly those of thin fibrils in the pericellular basket [46]. COL type XI is reported in developing cartilage as a heterotrimeric molecule of two novel COL gene products (α1(XI) and α2(XI)) and a third chain (α3(XI)) identical in primary sequence to α1(II)B, the common form of splicing variant of the type II collagen gene [47]. COL type III was found in normal and osteoarthritic human articular cartilage by immunofluorescence [48]. To maintain its N-telopeptide domain, COL type III was also demonstrated to be spatially overlapped with COL type II in the same fibrils band [42]. COL type VI was studied to be less than 1% of total COL content in articular cartilage and it has been found mostly in fibrocartilages [43]. It forms disulfide-bonded dimers, tetramers and a special filament network, which was discovered to be most concentrated around cells and diffused loosely in spaces throughout the fibrillar matrix [44].
The function of type XII and XIV COL in cartilage was unknown, but they were found to be physically bond to fibrillar COL surfaces instead of covalently bonded in the matrix [45].

1.1.3 Type I and type II collagen

Type I COL assembles into fibers that form structural and mechanical scaffold (matrix) of bone, skin, tendons, cornea, blood vessel walls and other connective tissues [49]. COL type I molecule consists of two αI chains and one α2 chain [αI(1)2–α2(I)], twisted around each other in a characteristic triple helix, although a very small amount of COL type I molecules are characterized by three αI chains [αI(1)3] forming the triple helical structure. Both the αI chain and the α2 chain have a long helical domain preceded by a short N-terminal peptide and followed by a short C-terminal peptide [50].

COL type II is the main COL extracellular matrix (ECM) component of articular cartilage. The degradation of COL type II ECM is corelated with the progression of cartilage osteoarthritis (OA) [51]. It has three identical, same length αI chains composed of around 1060 amino acid residues each, with a long uninterrupted triple helix region and relatively short and nonhelical telopeptides [52, 53]. The three identical αI chains are displaced from one another by one residue in the triple-helix to allow for its proper super coiling.

COL type I and II are closely related, as the structure between those two COL types only differs in the way that three alpha triple helical chains are “phased” with respect to one another. In particular, the difference is the hydrogen bonds that hold the triple helical chains together. In COL type I the NH groups point counter-clockwise when viewed from the carboxyl ends of the chains, whereas in COL type II the opposite situation stands [3]. If an existing set of hydrogen bonds in COL type I is broken, and each polypeptide chain rotated about its own axis by approximately π/3, the NH group would instead be attached to the carbonyl oxygen, forming the hydrogen bond [3].
The work of Rich et al. [3] illustrates the “phase” shift between COL type I and COL type II [3]. Obviously, this structural difference is non-trivial [3]. However, one may employ a model of COL type I to study the properties of COL type II related materials, as the COL type I is much easier to reach.

1.2 Collagen crosslinks

COL crosslinks (CxL) are chemical bonds that link one polypeptide chain to another [1, 2, 6-10]. Properties of COL rich tissues predominantly depend on the formation of CxL, which provide mechanical support and structural stability. Thus, CxL provide structural integrity in tissues, and help establishment of the ECM, as a highly organized network [54]. CxLs can be roughly categorized into intra- and intermolecular COL CxL [55]. Intramolecular CxL in COL are the covalent bonds that connect polypeptide chains within a COL molecule’s triple helical structure [56]. These CxL are responsible for the stability and integrity of COL’s triple helices and partially determine the mechanical properties of COL rich material and tissues. Intermolecular CxL are formed by covalently bonding one COL triple helical molecule to another [57]. Almost all tissues in multicellular systems are COL rich, and the biomechanical properties and structural integrity of collagenous tissues predominantly depend on the level and organization of intermolecular CxL between the COL molecules within the fibers to prevent slippage under load, keep the extracellular space for cells and maintain the tissue shape [57, 58]. The biomechanical properties of tissues are further distinguished by the alignment of the intermolecular crosslinked fibers within the tissues. For example, the parallel alignment of fibers forms the precise layered lamellar structure in corneal tissue may enhance biomechanical strength while facilitating the corneal transparency. On the other hand, the random layered organization in the articular cartilage tissue maximizes compliance,
whereas the parallel alignment of fibrils in different cartilage zones enhances longitudinal and tensile strength.

1.3 Osteoarthritis

Osteoarthritis (OA) is a progressive and complex joint disease that affects millions of Americans [59]. It is the most common form of arthritis worldwide. OA is characterized by breakdown of joint articular cartilage and remodeling of underlying bone, with the most common symptoms being joint pain, stiffness and locomotor restrictions. Other symptoms may include joint deformation or swelling and crepitus. Although any joint in human body could be affected by OA, the disorder is commonly seen in hands, knees, hips and spine. OA of the knee increases in prevalence with age and is more common in women than in men. Approximately 10% of men and 18% of women over 60 years of age are affected by this type of joint disease [60]. The symptoms of OA may develop slowly and worsen over time. Risk factors of OA include obesity, traumatic injury, previous surgery, and occupational bending and lifting [61].

1.3.1 Osteoarthritis formation and progression

While OA is a degenerative joint disorder that may lead to gross cartilage loss and morphological damage to other joint tissues, a lot of subtle biochemical changes occur in the early stages of OA. During onset of degeneration, the collagen (COL) matrix becomes more disorganized and there is a decrease in proteoglycan (PG) content within cartilage [62]. The loss of COL crosslinks (CxL) causes the breakdown of COL fibers, which may result in a net increase in water content due to osmotic swelling caused by remaining PGs [62, 63]. With further loss of COL CxL and the reduced protective effects of PGs, the cartilage matrix can become more susceptible to wear, thus aggravating the degeneration [64].
1.3.2 Current clinical diagnosis of osteoarthritis and standard of care

Diagnosis of OA is typically determined after physical examination triggered by the patient reporting symptoms, with medical imaging and other tests occasionally used to support the diagnosis, or to rule out other problems. Plain film radiography such as X-ray might be necessary in confirming the diagnosis results and ruling out other conditions. OA characteristics include the narrowing of the joint space width, osteophyte formation, and the development of subchondral sclerosis and cysts [65]. If needed, advanced imaging techniques such as magnetic resonance imaging may be used as well for assessment of structural alterations through the progression of the disease [66, 67]. Based on the diagnosis and clinical symptoms, the condition can be classified into different levels, using the WOMAC scale [68] that takes into account pain, stiffness and functional limitation, or other clinical scores such as the Knee injury and Osteoarthritis Outcome Score (KOOS) [69] and the Hip disability and Osteoarthritis Outcome Score (HOOS) [69, 70].

Current clinical options mainly focus on pain management [60], or surgery [71, 72]. Patients may alleviate OA symptoms through muscle-strengthening exercise and weight loss [73]. Pharmacological interventions are playing a major role in symptoms control. Interventions include non-steroidal anti-inflammatory drugs such as acetaminophen and ibuprofen [74], and intra-articular steroid injections [75]. Injection of synovial-like fluid that acts as a lubricant may be used as a treatment, however, it may increase the risk of further pain [76]. Anakinra, a recombinant antagonist of interleukin-1 receptor, also improved symptoms in patients with OA, but the effect was not sustained beyond 4 days after intra-articular injection [77]. If the impact of OA symptoms on quality of life is significant, and conservative treatments ineffective, joint replacement surgery or resurfacing may be recommended. Surgery is the only treatment option for advanced stages of OA [71, 72].
One of major challenges in OA treatment is its long asymptomatic period. Symptoms usually become noticeable when the disease is reaching advanced stages. Although a lot of research [18-20] has been focused on OA treatment, such as surgery to transplant articular cartilage from a non-weight-bearing area to the damaged area [71, 72] and osteotomy [78], currently there is no effective intervention for early stages of OA. In part this is due to lack of a reliable diagnostic modality for detecting early OA.

Fourier-transform infrared spectroscopy (FTIR) has been applied to detect the early changes of structure of OA-afflicted cartilage [79]. This in vitro approach offers a framework to assess complex pathological changes in this heterogeneous tissue. However, since samples have to be fully dehydrated for imaging, this methodology cannot be applied in the clinic [80].

1.3.3 Collagen crosslinks as potential biomarker for osteoarthritis diagnosis and treatment

Hydroxylysine aldehyde-derived crosslinks, both immature and mature, are the predominant common type of CxL in articular cartilage [81]. Two reducible, immature CxL, dihydroxylysinonorleucine and hydroxylysinonorleucine (structures shown in Table 1.1), a senescent CxL, pentosidine (Table 1.1), and two nonreducible, mature CxL, pyridinoline (PYD) and deoxypyridinoline (Table 1.1), were identified in all articular cartilage zones [81, 82]. Dihydroxylysinonorleucine and hydroxylysinonorleucine are divalent immature CxL between COL fibrils. These immature COL CxL in articular cartilage time-dependently turn into trivalent mature CxL, known as PYD and deoxypyridinoline [81, 83]. PYD is the predominant mature crosslinking residue (> 95% PYD, < 5% deoxypyridinoline) in COL type II in an articular layer.
Table 1.1: Cartilage collagen crosslinks

<table>
<thead>
<tr>
<th>Crosslink Type</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature CxL</td>
<td><img src="image" alt="Dihydroxylysinonorleucine" /> <img src="image" alt="Hydroxylsisinonorleucine" /></td>
</tr>
<tr>
<td>Mature and Senescent CxL</td>
<td><img src="image" alt="Pyridinoline" /> <img src="image" alt="Deoxypyridinoline" /> <img src="image" alt="Pentosidine" /></td>
</tr>
</tbody>
</table>

PYD CxL have both inter- and intramolecular forms in articular cartilage COL fibrils, playing the most important role in stabilizing the COL fibril network and ECM in articular cartilage [41]. It is a nonreducible CxL, and its synthesis increases with COL maturation [82]. PYD was used as one of the clinical assays to diagnose the progressive stages of OA [84]. In that study, the quantitative characterization of PYD CxL density was employed to assess the changes that occur within the COL framework in OA progression [85, 86]. A small amount of senescent CxL, pentosidine, relevant to the age of tissue, is also characterized in articular cartilage [87]. It is a condensation product of arginine, lysine, and ribose, and it is an end product of advanced glycation. It is also formed by sequential glycosylation and oxidation reactions [82].
1.4 Raman spectroscopy

1.4.1 Raman spectroscopy

The Raman effect is a non-linear inelastic light-scattering of a photon by molecules which are excited to higher energy levels [88, 89]. When photons from a beam of monochromatic light are scattered by the target material, they produce an oscillating polarization in the molecules, exciting them to a virtual energy state. If the oscillating polarization of the molecules couples with other polarizations, the frequency of photons scattered by the molecules will not change, and the scattered photons maintain the same energy. This type of scattering is known as Rayleigh (elastic) scattering (Figure 1.4a, b). However, if the polarization in the molecules couples to a vibrational state different from the original state, the incident and scattered photons will differ in energy by the amount of vibrationally excited molecules, and the appearance of scattered photons with altered wavenumber and energy is called Raman scattering (Figure 1.4a, b). Raman scattering may lead to two symmetrical outcomes – Stokes and Anti-Stokes Raman scattering (Figure 1.4a, b). When the molecules absorb energy and the emitted photons have a lower energy than the incident photon, the effect is stokes Raman scattering. Stokes Raman scattering refers to Raman bands with exciting wavenumber $\nu' = \nu_0 - \nu_M$. When the molecules loss energy and the emitted photons have a higher energy than the incident photon, the effect is anti-stokes Raman scattering. Anti-stokes Raman scattering refers Raman bands with exciting wavenumber $\nu' = \nu_0 + \nu_M$. The Raman components displayed in the spectrum of the scattered radiation at shifted wavenumbers are termed Raman bands, and collectively they are referred to as the Raman spectrum.

The molecule scattered must have a change in polarizability during the vibration in order to be Raman active. When the distorting electrons change positions from the original state, molecule polarizability changes. Molecule polarizability negatively relates to the change of
electron density or bond strength, and positively relates to the change of bond length. For example, a simple molecular dipole moment model is shown in Figure 1.4c. The symmetric stretch will be Raman active since there is a change in the net dipole, while asymmetric stretch or bending would not be Raman active because the overall molecule polarizability does not change.

When a beam of light is scattered by a target material, within the focal volume the intensity of Raman scattering \( I(v) \) is given by

\[
I(v) = \frac{2^4 \pi^3}{45 \cdot 3^2 \cdot c^4} \cdot \frac{h I_L N(v_0 - v)^4}{\mu v (1 - e^{-h v / kT})} \times [45 (\alpha'_a)^2 + 7 (\gamma'_a)^2]
\]

[90-92], (1); here, \( I(v) \) – the integrated intensity of Raman bands, \( c \) – speed of light, \( h \) – Planck’s constant, \( I_L \) – excitation intensity, \( N \) – number of scattering molecules, \( v \) – molecular vibrational frequency (Hz), \( v_0 \) – laser excitation frequency (Hz), \( \mu \) – reduced mass of the vibrating atoms, \( k \) – Boltzmann constant, \( T \) – absolute temperature, \( \alpha'_a \) – mean value invariant of the polarizability tensor, \( \gamma'_a \) – anisotropy invariant of the polarizability tensor.

Within the focal volume, the integrated intensity of Raman scattering \( I(v) \), which could be calculated by the area under the Raman bands, is proportional to the number of molecules being scattered \( N \) [93]. Therefore, the calculation of the integrated area of Raman bands may provide key information for quantitative analysis of targeting materials.
Figure 1.4: Schematic diagram of (a) molecule scattered by light, and (b) energy diagram for Rayleigh and Raman scattering processes. Rayleigh scattering is the most intense scattering process, and Stokes scattering predominates over Anti-Stokes scattering at room temperature. Thickness of the arrow shapes indicates the relative intensity of the three processes (not to scale). (c) Dipole movement molecular model.
1.4.2 Raman spectroscopy biomedical applications

Each Raman band is a characteristic of a specific molecular bond, and may show the detailed information of the bonds associated with protein, DNA, RNA, lipids and other biomolecules present in body tissue or even just a single cell. Collectively, the entire Raman spectrum provides an intrinsic ‘molecular fingerprint’ of the target sample. This fingerprint may demonstrate the identity, composition, structure, and conformation of biological samples, as well as some interactions between macromolecules, such as protein–protein interaction [94]. Based on its ability to accurately detect subtle changes of chemical components within biological tissues non-invasively, several Raman-based techniques [95-98] are currently under development to address the complicated progressive diseases diagnosis needs, providing help to understand changes in COL biochemistry that can be of relevance to the pathologies of disease.

Raman spectroscopy has also been utilized in studies of components of connective tissues [99, 100], as well as OA [101, 102], and cartilage degradation [103]. Frushour and Koenig [104] used Raman scattering to analyze the complexity of COL and elastin networks. Nguyen et al [105] compared Raman spectra of COL type I against COL type IV aiming to develop an easier and non-invasive way to tell the differences between COL types. Zhang et al [106] studied hydration in COL by applying Raman spectroscopy. Jastrzebszka et al [107] utilized Raman spectroscopy to investigate the subtle biomolecular modification of COL structure introduced by glutaraldehyde (GA) fixation. Morris’s group developed a Raman spectroscopy-based method to determine molecular changes associated with OA by identifying signals from subchondral bone and biological fluids [101]. However, this method represents an indirect assessment of biomarkers and is not joint-specific. Lim et al [108] employed polarized Raman spectroscopy to detect biomolecular changes in disrupted porcine cartilage loaded under different stresses; they
demonstrated that Raman spectroscopy has the potential for diagnosing early cartilage damage at the molecular level. Based on this study they were able to differentiate normal and damaged cartilage prior to noticeable histological changes, but the cartilage damage could only be characterized qualitatively. Although these studies have demonstrated the potential of applying Raman for diagnosing cartilage joint disease, the analysis of Raman spectrum obtained from articular cartilage is rather complicated and so far there is no generally accepted quantitative analysis for diagnosing early stages of OA.

**1.4.3 Quantitative Raman spectroscopy analysis for biomedical applications**

Raman spectrum of biochemical materials may consist of a large numbers of Raman bands, in which some of the Raman characteristic peaks may overlap with each other. Traditionally, multivariate analysis, such as principal component analysis and neural networks analysis, are used to analyze Raman spectrum for biological tissues [95, 109]. These methods are capable of analyzing large datasets simultaneously, identifying similar features in the individual spectra, and are especially effective in deducing patterns [110, 111]. However, these analyses often yield binary answers, which are suitable for optical diagnostics and qualitatively determining of the presence of a disease, such as cancer. A more rigorous approach is necessary to detect subtle quantitative changes of molecular compounds in progressive diseases.

The main challenge of applying Raman spectroscopy as a quantitative tool to biomedical fields is the decomposition of a complex biomedical Raman spectrum. Such a decomposition requires an optimization of a lot of different parameters, with multiple Raman bands attributed to individual biochemical compounds, resulting in numerous decomposition solutions for each specific biomedical Raman spectrum [112].
The innovation of the Raman spectrum decomposition method described in this doctoral dissertation is in quantifying the biomolecular targets of interest through constraining the decomposition of Raman spectrum to the well-known biochemical components and their possible vibrational modes and confirmational alterations allowed by the molecular structures. The analytical framework for this method was first studied in prior work on characterization of fused silica [113], and previous research on prostate cancer diagnostics [98, 112]. In both cases it has shown potential as an automated quantitative tool diagnostics. By applying and optimizing this analytical approach, we aim to quantitatively characterize the biomolecular changes of intra- and inter- molecular CxL in COL networks to study CxL kinetics, and potentially correlate changes in CxL with the early subclinical structural changes of OA in the future.

1.5 Refractive errors

Refractive errors occur when an eye is unable to focus light accurately onto the retina due to the shape or other misfunctioning of the eye. The most common types of refractive errors are near-sightedness (myopia), far-sightedness (hyperopia), astigmatism, and presbyopia. Refractive errors have become a growing public health problem worldwide. Its incidence has doubled over the last 50 years in the United States and Europe [114]. It is even more significant issue in some East Asian countries, where its prevalence reaches 70 to 90% [115]. Most affected individuals use spectacles or contact lenses, which generally provide adequate refractive error correction. However, both are subject to limitations. Glasses do not work well in the rain and mist may form on them following changes in temperature or humidity. Contact lenses improve the field of vision and acuity, but many people find their presence on ocular surfaces intolerable [116]. Over the last
two to three decades, refractive surgery for the permanent correction of vision has thus emerged as an attractive choice for many patients [117].

1.5.1 Traditional clinical interventions for vision correction

One of the first reports on laser refractive surgery was by Trokel et al in early 1980s [118]. Utilizing an ArF excimer laser with 193 nm wavelength, authors of the study ablated corneal stroma with accuracy >1 μm without compromising the remaining tissue. The study was performed on \textit{ex vivo} bovine and \textit{in vivo} rabbit models. MacDonald et al [119] first demonstrated the safety and efficacy of the excimer laser vision correction system on human eyes. They also reported that after 6-months follow up, the cornea remained clear and the visual acuity appeared to be stable.

Further, photorefractive keratectomy (PRK) was extensively studied. In PRK surgery, epithelium was first debrided from the center of the cornea and discarded prior to reshaping [120, 121]. An excimer laser was applied to remove the corneal stromal surface [120, 121]. Patients’ refractive power (20/20) and visual function recover after the epithelium layer grows back over the corneal surface. With an acceptable risk to benefit ratio, PRK has been shown to be safe and effective [120, 121]. However, due to the debriding of the epithelium layer, PRK surgery requires a very long wound healing and recovery time [122, 123]. During the recovery, patients may suffer discomfort and an extremely long time to reach stable vision. Typically, vision is about 80% improved at 1 month after surgery and about 95% by 3 months after surgery [124, 125]. The discomfort may be due to the epithelial cells to regeneration, a time consuming process [126]. In addition, the PRK outcomes, are not completely predictable due to the variations in individual corneas and wound healing process. Some patients may still require glasses or additional surgery after the PRK [127].

To minimize the side effects and complications caused by PRK, laser-assisted in situ keratomileusis (LASIK) was proposed in mid 1990s [128, 129]. A mechanical microkeratome was
utilized to create a corneal flap by cutting through the corneal epithelium and Bowman's layer, after a soft corneal suction ring is applied to the eye, holding the eye immobilized in place. An excimer laser was then applied to ablate a thin layer of corneal stroma in a controlled manner without damaging the adjacent tissue [128, 129]. After the reshaping of corneal central stroma, the corneal flap was repositioned over the treated area and checked for presence of air bubbles, debris, and whether the flap fits properly on the eye. The flap remains in position by natural adhesion until healing is completed. LASIK is demonstrated to vaporize the central corneal stroma without affecting the epithelium layer, which will result in a faster recovery time and less pain than PRK [124]. However, the mechanical corneal flap creation could not be accurate with predictable thickness, which may introduce flap complications such as buttonholes, free caps, irregular cuts etc. These could affect the patients’ recovery and wound healing process significantly [117, 124].

To precisely control the flap diameter and thickness, femtosecond laser-assisted LASIK (femto-assisted LASIK) was proposed [130, 131]. In femto-assisted LASIK surgery a flap is created by focusing the femtosecond laser in the interior of the corneal stroma. The laser induces an optical breakdown resulting in formation of a dense plasma, which rapidly expands and forms a gas bubble consisting of the breakdown products. In addition, with the assistance of computer software, one is able to design any pattern to create the corneal flap [132]. By increasing the motorized speed of the laser, one is also capable of placing rows of bubbles closer together to create smooth dissections through applying smaller focal spot. Femto-assisted LASIK greatly improve the predictability and safety compared to traditional LASIK, which creates the flap with a mechanical microkeratome, although the flap creation may take longer. However, the medical complications caused by the flap creation and reposition may still affect patients’ recovery, and may permanently affect patients’
vision and life [133, 134]. In addition, patients’ cornea must have sufficient thickness (thicker than 450 μm) to perform any LASIK surgeries [135].

Another alternative solution to replace PRK is laser-assisted epithelial keratomileusis (LASEK) [136]. A LASEK in a way is a combination of LASIK and PRK. Similarly to PRK surgery, the corneal epithelium is separated from the underlying corneal stroma. However, instead of discarding the removed corneal epithelium layer, an ultra-thin flap that contains only corneal epithelium is created and attached to the cornea by an uncut hinge at the periphery of the epithelium, similar but not the same as LASIK surgery [137]. A high energy excimer laser is then applied to reshape the corneal stroma by vaporizing the stromal tissue. During the last step of LASEK, the epithelium flap is carefully repositioned over the treated area for recovery of the visual function. This flap is much thinner than the corneal flap created in LASIK surgery, which contains both corneal epithelium layer and central stroma [138]. LASEK may be beneficial for patients with naturally thin corneas as less-than-ideal LASIK candidates [138]. The LASEK procedure avoids any corneal flap related LASIK complications, lessens the likelihood of removing too much corneal tissue with the excimer laser and compromising the structural integrity of the eye [138]. By avoiding a deeper flap, there is also a slightly less risk of developing dry eyes. LASEK may also be a better option if a patient has a high degree of myopia, which requires more tissue removal from the central cornea to correct the refractive error. However, LASEK procedure typically involves more discomfort while performing the procedure and a longer recovery time compared with LASIK or PRK surgeries [124].

To improve the efficacy and safety of LASIK or LASEK, research have also been done to develop all femtosecond LASIK or LASEK surgery, which utilizes only femtosecond laser as both flap creating and corneal reshaping resources. Such a surgery, femtosecond lenticule extraction
(FLEEx), was introduced by Carl Zeiss Meditec[139]. In a FLEEx surgery, femtosecond laser creates the back side and then the front side of the refractive lenticule inside the intact cornea. A vertical incision is created by the same femtosecond laser and the lenticule is removed through the incision with minimal disruption of corneal biomechanics. Applying a similar concept as in LASIK or LASEK, the removal of the lenticule reshapes the corneal tissue and thereby achieves the desired refractive correction[140]. An improvement of FLEEx is small incision lenticule extraction (SMILE)[141]. The SMILE procedure involves the same lenticule creating process as it is in FLEEx. However, in a SMILE procedure, a minimum possibility of the corneal incision will be created to remove the corneal lenticule[141]. The SMILE has been shown to be less invasive then LASIK or LASEK with the preservation of a biomechanical properties closer to the cornea’s natural stability[142]. However, a physical removal of partial natural corneal tissue is still applied which may cause several severe post treatment complications[143, 144].

1.5.2 Collagen crosslinking for corneal standard of care

Cross linking as a clinical tool to strengthen corneal tissue was suggested by Wollensak et al and introduced into clinical practice in early 2000s[145, 146]. The concept was created based on the knowledge that the COL in the corneal stroma of some people was abnormally weak naturally or after the eye procedures. This clinical approach is generally employed for the treatment of progressive corneal ectasias with an aim to stiffen the corneal tissue[146]. A conventional crosslinking treatment applies ultraviolet A light onto eyes previously soaked with a riboflavin solution, which acts as a photosensitizer. The procedure creates free radicals, which react with collagen side chains resulting in the increase of intra- or inter-collagen molecule chemical bonds, crosslinks, thus increasing the corneal rigidity[145, 147]. This technique has been demonstrated
to be safe with no severe endothelial or structural damage [146], if corneas are thick enough. However, while the illuminating UVA light can be modified to control the area of tissue that is crosslinked, it has been almost impossible to control the depth of the cross linking process using this photochemical approach [147, 148].

It was noted that the strengthening of the tissue was accompanied by a change in its physical dimension, through the introduction of CxLs. This biophysical alteration may be used to manipulate the shape of the cornea, while stiffening the biomechanical properties [145, 147]. This phenomenon has the potential to be applied to induce refractive power changes thus achieve vision corrections. This idea first comes with the concept of photorefractive intrastromal cross-linking (Pixl) for the treatment of low myopia [149]. Lim et al [150] has shown that Pixl protocol was safe and effective in reducing myopic refractive error with up to 12 months follow-up. However, the involvement of photosensitizer injected into the corneal stromal followed by the UVA excitation may result in tissue damage and cell apoptosis in post treatment, including a significantly reduced number of keratocytes, which play a major role in keeping the corneal transparency, healing its wounds, and synthesizing the components that make up the corneal tissue. This may also induce damages to the corneal endothelial layer if the cornea is thin [151, 152]. In addition, the refractive error correction was relatively small. If the photosensitizer could be absent during the COL crosslinking process, the induced COL CxLs may be applied clinically to change the biophysical dimension of corneal tissue and future manipulate the refractive power of the eye for the purpose of vision correction [153].

1.6 Thesis objectives

The objectives of the first part of this doctoral dissertation are focused on developing a novel, non-invasive, quantitative diagnostic modality, based on Raman spectroscopy that has
potential to detect changes in biochemical composition of COL network in collagenous tissues. Results suggested that the proposed characterization tool may be applied to detect the early changes of extracellular matrix associated with progressive diseases, such as osteoarthritis. Under the second stage of this doctoral dissertation, a novel non-linear ultrafast laser-based COL photochemical crosslinking method is proposed. *Ex vivo* and *in vivo* animal studies suggested that this novel COL crosslinking approach may be utilized as a non-invasive treatment to permanent vision correction through an innovative mode of laser–cornea interaction in which both thermal ablation and optical breakdown are avoided. Furthermore, through the application of the developed methodologies of mechanical and wear properties assessment and homeostatic culture conditions, the effectiveness of this novel COL crosslinking treatment strategy was evaluated, which has shown the potential to apply the proposed intervention to prevent or slow down cartilage osteoarthritis progression.
Chapter 2: Quantitative Raman Characterization of Cross-linked Collagen Thin Films as a Model System for Diagnosing Early Osteoarthritis

2.1 Introduction

Osteoarthritis (OA) is a progressive joint disease that severely affects articular cartilage [64, 154]. OA remains asymptomatic until its late stage, when the treatment options are limited to major interventions such as joint replacement [155]. In adults, articular cartilage is an avascular connective tissue comprised of chondrocytes and collagenous extracellular matrix (ECM). Its main function is ensuring smooth joint movement and shock absorption. The ECM structure consists predominantly of collagens (COL) and proteoglycans (PGs), with the COL matrix providing tensile strength, whereas PGs resist compression. Initiation of OA could be caused by mechanical damage (trauma), proteolytic action secondary to soft-tissue injuries, or slow, age-dependent progression. Regardless of the initiation mechanism, an imbalance in ECM homeostasis is a key pathogenic pathway of OA. This imbalance results in progressive degradation of the ECM cartilage components [59, 156, 157]. To date, however, there is no well-established quantitative diagnostic tool for detecting OA at its early stages, which may be a prerequisite for development of an effective treatment that does not require joint replacement [158].

Collagen is the major structural protein of most connective tissues [159]. It provides the structural support to resident cells in the form of extracellular matrix (ECM) [160]. The structural integrity and mechanical properties of collagen-based joint tissues is significantly affected by collagen cross-links (CxL), chemical compounds that both, connect COL fibrils as well as molecules within the COL [26, 146, 161, 162]. It has been reported that degradation of the COL
CxLs compromises structural integrity of articular cartilage, which may be lead to onset of OA [163]. Asanbaeva et al. [163] developed a model in which β-aminopropionitrile (BAPN) was used to block the formation of pyridinoline (PYD) CxLs in an immature bovine articular cartilage explant model. Further, Albro et al. [164] developed a cartilage explant model in which levels of glucose, cortisol and insulin were controlled in a serum-free, chemically defined media to investigate the evolution of biochemical and mechanical properties of live immature bovine cartilage. In this model cartilage explants lost their structural integrity in the absence of cortisol. It is speculated that loss of structural integrity is a result of degradation of CxL in ECM [165, 166]. Therefore it is of interest to develop a method capable of quantifying CxL concentrations in articular cartilage.

Raman scattering is the process in which a small fraction of incoming photons, provided by the light source, inelastically collide with target molecules. As a result, the optical frequency of the outgoing photons is different from the incident photonic frequency, with the difference being equal to the vibrational energy of the scattering molecule. Raman spectroscopy allows investigation of functional groups and bonding types, providing information about the biochemical composition of tissues on a molecular level [167]. Being a non-destructive and highly versatile analytical technique, Raman micro-spectroscopy exhibits vast potential for in vivo probing and in vitro analysis of biological tissues, especially as it does not require specimen preparation [168]. However, the weakness of the signal, which is often masked by overwhelming fluorescence, has prevented a more widespread use of Raman scattering in the past. The use of infrared lasers as a light source, as well as advances in CCD detectors, have significantly improved the signal-to-noise ratio, enabling the wide use of Raman spectroscopy in probing tissues. This technique has recently emerged as a tool for analysis and evaluation in biomedical applications, such as cancer diagnostics.
Manoharan et al. used Raman spectroscopy to classify normal, benign and malignant breast tissues and demonstrated potential of their technique to diagnose breast cancer [173]. Utzinger et al. applied Raman spectroscopy to characterize cervical cancer through a pilot clinical study that monitored squamous dysplasia, a precursor of this disease [174].

Raman Infrared spectroscopy has also been used in studies of components of connective tissues [99, 100], as well as OA [101], and cartilage degradation [175]. Frushour and Koenig [176] used Raman scattering to analyze collagen gelatin and elastin. Nguyen et al. [105] compared Raman spectra COL type I against COL type IV. Zhang et al [106] studied hydration in COL. Jastrebszka et al. [107] utilized Raman spectroscopy to investigate glutaraldehyde fixation of COL. Morris’s group developed a Raman spectroscopy-based method to determine molecular changes associated with OA by identifying signals from subchondral bone and biological fluids[29]. However, this method represents an indirect assessment of biomarkers and is not joint-specific. Lim et al. [108] employed polarized Raman spectroscopy to detect biomolecular changes in disrupted porcine cartilage loaded under different stresses; they demonstrated that Raman spectroscopy has the potential for diagnosing early cartilage damage at the molecular level. Based on this study they were able to differentiate normal and damaged cartilage prior to noticeable histological changes, but the cartilage damage could only be detected qualitatively. Although those studies have demonstrated the potential of applying Raman for diagnosing cartilage joint disease, the analysis of Raman spectra obtained from articular cartilage is rather complicated and so far there is no generally accepted quantitative analysis for diagnosing early stages of OA.

In this study, COL thin films are employed as a simplified model of collagenous extracellular matrix found in articular cartilage. Raman spectroscopy is utilized to assess changes of the relative concentration of CxL induced by glutaraldehyde fixation. To achieve this goal, a
quantitative method is developed whereby the relatively complex Raman signal obtained from the collagen is decomposed into known chemical structures and their allowable vibrational modes. Each of the structures is then modeled as a mathematical function and their sum forms an optimization function that produces model of the spectra via curve fitting. Relevant information on the CxL is then extracted from the model. Quantitatively assessing relative CxL concentrations in articular cartilage is proposed as an avenue for diagnosing early OA.

2.2 Materials and Methods

Neutralized collagen solution (PH 7.4) was prepared from 3.1 mg/ml type I collagen from bovine hides (PureCol, Advanced Biomatrix San Diego, CA, USA) in 10× Dulbecco's phosphate buffered saline (DPBS) and 0.1M NaOH. Neutralized COL solutions were applied on alkanethiol treated gold-coated silicon wafers to produce thin films. Before being used to prepare thin films, the substrates were cut into 15 mm × 15 mm pieces and placed in petri dishes. Silicon substrates were coated with a 5 nm layer of chromium and 18 nm layer of gold through thermal deposition (Edwards BOC/Auto 306 Thermal Evaporator) and rinsed with ethanol [177]. Coating was introduced to reduce fluorescence background to the Raman spectra. 1.8 ml of the neutralized COL solution was applied onto a coated silicon wafer in a petri dish and then incubated at 37 °C for 12 h. After incubation, the samples were immersed in 1-hexadecanethiol (0.5 mM; Sigma Aldrich) ethanol solution for 8 h prior to being rinsed with ethanol and dried with filtered N2. Incubated collagen was rinsed with 1× DPBS and deionized H2O 10 times to remove the bulk salt and gels from the surface. After rinsing, specimens were immediately placed in DPBS (1×) and stored at 4 °C. Prior to treatment/characterization, samples were taken out of the DPBS, rinsed with deionized H2O and dried with a filtered nitrogen gun. The degree of fiber polymerization of thin films was assessed via atomic force microscopy (PSIA XE-100 AFM (Park Systems, Santa Clara,
California, USA), whereas thickness and surface uniformity were characterized with optical profilometer (Figure 2.1).

Glutaraldehyde (GA) solution, being a known cross-linker, has been used to introduce various levels of collagen cross-links. Varying exposure and concentration of GA solution controlled CxL concentration levels. Three batches of samples were treated with GA concentrations of 0.05%, 0.1% and 0.2%, respectively. There were seven samples within each batch, fixation times were 1h, 1.5h, 2h, 2.5h, 3h and 4h. 5ml of GA solution was applied onto each sample. After the fixation cross-linked COL films were rinsed with deionized water and desorbed into 20ml PBS (0.2 M, PH 7.4, Sigma Aldrich) for 48-72h. Subsequently, specimens were rinsed with deionized H$_2$O and DPBS (1×) several times, stored in DPBS (1×) at 4 °C and kept sterile. Cross-linked COL thin films were rinsed with deionized H$_2$O prior to Raman characterization.

Raman spectra were acquired with a confocal micro-spectrometer (InVia, Renishaw Wotton-under-Edge, Gloucestershire, UK). Incident laser excitation was provided by helium-neon laser with 632.8 nm wavelength, delivered by 100× objective. Spectral resolution provided by 1800 gr/mm diffraction grating was 1 cm$^{-1}$. Raman signal was acquired with 10 accumulations, each lasting 10 s. Prior to the acquisition of the spectrum at each measurement point; photobleaching was applied for 10 s to reduce the fluorescence background. Spatially resolved characterization was performed to generate spectral maps. A 57 mm × 30 mm window within each sample was assessed and each spectral map included 220 measurement points.
Figure 2.1: Surface morphology of the prepared collagen thin films on a gold coated silicon wafer. (a) COL fiber polymerization level assessed with AFM. (b, c) Optical profiler confirms the surface roughness (c) and thickness (b).

Computational implementation of the proposed model was carried in MatLab (Mathworks, Inc) as a two-step process, each requiring curve fitting of experimental data. First, the signal was pre-processed to remove fluorescence background and then relevant segments of Raman spectra were modeled. Fluorescence is commonly removed by fitting the region of the spectra below distinctive Raman bands with low order polynomial function [178-182]. Fluorescence was iteratively fitted with a fifth order polynomial and subsequently subtracted from the raw signal. In the second step, the vibrational modes of interest were approximated as a damped harmonic oscillator driven by a force whose profile follows a sinusoidal curve [93]. According to the hydrodynamic theory, Raman lines naturally follow a Lorentzian profile [179, 180]. Thus the optimization function is the sum of Lorentzians. The trust region optimization model [183] was employed for curve fitting of the Raman spectra. Conceptually, it is a maximum neighborhood method developed through interpolation between the Taylor series method and the gradient descent method. It sets a problem as the iterative solving of a set of nonlinear algebraic equations. However, if the initial estimate is too far from the optimum, the algorithm will not converge. Therefore the
subset of the region of the objective function is defined and optimized first. The function is approximated with a simpler one which reasonably reflects the behavior of the original function in a neighborhood \( E \) around the point \( x \). This neighborhood is referred to as the trust region. In essence, the trust region represents constraints derived from the underlying biochemistry of the specimen.

### 2.3 Difference in Structure of Type I and Type II Collagen

Collagen in the ECM of articular cartilage is predominantly type II (COLII), whereas due to practical considerations our model utilizes type I (COL I). The major difference between COL I and COL II is a ‘phase shift’ in their chains [3]. In COL I the NH groups point counter-clockwise when viewed from the carboxyl ends of the chains, whereas in COL II the opposite is true [3]. According to Rich and Crick [3], if an existing set of hydrogen bonds in COL I is broken, and each polypeptide chain rotated about its own axis by approximately \( \pi/3 \), the NH group would instead be attached to the carbonyl oxygen, forming the hydrogen bond. Illustration of the ‘phase shift’ between COL I and COL II can be found in [3]. Consequently, Raman spectra of COL I and II are rather similar (Figure 2.2). Further, the proposed cross-link (CxL) concentration assessment relies on quantification of the concentration pyridine aromatic rings, which are centerpieces of both pyridinium CxL induced by GA-fixation in COL I and pyridinoline (PYD) CxL that are present in the COL II of the ECM of articular cartilage. The Raman band associated with in-plane stretching of pyridine ring is observed at the same location in the spectra of COL I and COL II (Figure 2.2c) as well as in articular cartilage explants [165, 166]. The ring is a prominent CxL residue found in fibrillar COLs and most connective tissues, except cornea and skin [107].
2.4 Results and Discussion

Collagen is a large molecule comprised of three polypeptide chains, which form a right-handed triple helix. Each of the chains contains multiple regions of repeating amino acid sequences (Gly-X-Y)$_n$, where Gly is glycine and X and Y are often proline and hydroxyproline. Figure 2.3 depicts typical Raman spectra of collagen type I (COL I) obtained from a thin film sample, superimposed with the spectra of GA-treated COL I. The dominant Raman band at the far right side of the spectrum, centered at 1669 cm$^{-1}$, is assigned to amide I and in COL I is mainly attributed to carbonyl stretching of the peptide bond in the Gly-X-Y tripeptide sequence $[^{105}]$. Two superimposed Raman bands in the center of the signal, with peaks at 1240 cm$^{-1}$ and 1268 cm$^{-1}$, respectively represent coupling of CN stretching with NH in-plane deformation, and are assigned to amide III $[^{107}]$. The peak at 1240 cm$^{-1}$ is associated with proline rich regions, whereas 1268 cm$^{-1}$ corresponds to proline poor regions, respectively $[^{176}]$. Amide I and amide III, together with
C-C stretching of the backbone formed by the Gly-X-Y sequence found at 935 cm\(^{-1}\), suggest an \(\alpha\)-helix conformation [106-108, 184]. Vibrations of the proline ring, specifically C-C stretching, is attributed to Raman bands centered at 853 cm\(^{-1}\) and 918 cm\(^{-1}\) [105], and the hydroxyproline ring gives rise to Raman band with peak at 875 cm\(^{-1}\) [106]. The isolated Raman band located at 1030 cm\(^{-1}\) is associated with C-C stretching of aromatic pyridine ring [185, 186].

![Figure 2.3: Normalized Raman spectra of control and glutaraldehyde-fixed collagen thin films.](image)

GA treatment resulted in a shift of the amide I and amide III bands. The peak of the amide I band shifted to 1674 cm\(^{-1}\), and bands attributed to Amide III became centered at 1235 cm\(^{-1}\) and 1264 cm\(^{-1}\), respectively. In the spectra of GA-fixed samples, a new band appeared at 865 cm\(^{-1}\) [187, 188] which is attributed to COC symmetric stretching. This band is due to ether-type COC C\(\times\)L, which is a result of the reaction of the GA aldehyde group with the carbonyl group of the peptide bond in COL. Newly formed ether-type bond weakens hydrogen bonds, producing conformational modifications of the protein structure, which are seen as Raman shifts of the amide I and amide III bands [107]. Quaternary pyridinium compounds form stable C\(\times\)L in GA-fixed samples [189, 190].
The 1030 cm\textsuperscript{-1} band is due to in-plane deformation of six-membered aromatic ring and is an indicator of the 1,3,5- substituted pyridine ring, similar to the CxL of interest in articular COL [107].

Pyridinium-type CxL in GA-fixed COL samples have a pyridine ring as their central feature whose vibrational modes are similar to monosubstituted six-membered rings [191]. Similarly PYD, which is one of the major COL CxLs and is responsible for the tensile strength and structural integrity of cartilage ECM [192] has a pyridine-like aromatic ring as its central chemical structure (Figure 2.4a). Assessment of relative concentration of pyridine rings in articular cartilage thus can provide information about the concentration of PYD CxLs. Stable vibrational modes of pyridine consist of in-plane ring deformation and symmetric ring breathing modes (Figure 2.4b). The form involves displacing alternate carbon atoms around the ring and is characterized by a rise of the 1030 cm\textsuperscript{-1} band. The latter is attributed to symmetric ring stretching that involves all carbons and nitrogen moving in and out in unison. The Raman band associated with this mode is located at 992 cm\textsuperscript{-1}. The 1030 cm\textsuperscript{-1} band is present in both purified COL (box II in Figure 2.3) and bovine articular cartilage explants [165, 166]. Furthermore, there is no significant overlapping of the Raman band centered at 1030 cm\textsuperscript{-1} and other bands in the COL spectra, which simplifies the analysis to some extent.
Figure 2.4: Similarity between the hydroxylysyl pyridinoline, a major crosslink naturally occurring in articular cartilage and glutaraldehyde induced pyridinium-type crosslink schematic diagrams of hydroxylysyl pyridinoline (a) and pyridinium-type crosslinks (b). Central chemical compound in both crosslinks is pyridine ring. (c) Raman spectra of pyridine.

Quantification of the changes in the relative concentration of pyridinium-type CxL was achieved via modeling of the experimental data, which enabled the analysis of changes in individual Raman bands due to GA-fixation. It is assumed that the relative concentration of the pyridine rings within the focal volume is proportional to the normalized integrated intensity of the Raman band associated with in-plane ring deformation. Modeling of the spectrum was needed to assess contributions of the individual bands to the complex signal that is comprised of a relatively large number of overlapping Raman bands. Subsequently, quantitative information about the particular Raman band of interest could be extracted from the model. By taking advantage of the
additive property of the Raman signal [93], segments of interest in the COL Raman spectra (box I and box II in Figure 2.3) can be seen as the sum of the individual bands. Therefore the optimization function, which is used to model the spectrum, is the sum of the functions describing individual bands [93, 113, 193]. The concentration of CxLs is quantified as the area under the curve of the 1030 cm$^{-1}$ peak in the modeled spectrum, normalized by the area under the hydroxyproline curve assigned to COL. Normalization is necessary to avoid errors which could arise as a result of concentration-dependent changes, as done in FTIR studies [194]. The trust region optimization model [183] used for these analyses, is an adaptive method that utilizes a two-step approach in which the approximation model predicts the improvement of the system being optimized before resorting to a detailed model that confirms the validity of the initial approximation, followed by constrained variation of the trust region. The modeling enforces a locally constrained optimal step in the otherwise unconstrained approximate iteration [195]. The underlying biochemistry, including the location of the peak assignments, helped to introduce appropriate constraints in the optimization model so that a unique solution could be obtained. This approach yields a unique solution when treating large numbers of varying parameters, while being sufficiently sensitive to capture subtle changes in the vibrational spectra that arise from the potentially modest changes in the crosslink density. Two segments of the spectrum have been modeled, and the resulting modeled spectrum closely matches experimental data (Figure 2.5). From the first segment the integrated intensity of the hydroxyproline band has been extracted and used to normalize the integrated intensity of the band centered at 1030 cm$^{-1}$, which is associated with in-plane stretching of pyridine rings.
Figure 2.5: Decomposition and modeling of segments of COL I Raman spectrum (a) corresponds to box I in Figure 2.3; (b) corresponds to box 2 in Figure 2.3.

Spatially resolved characterization of the COL thin films was utilized for generation of spectral maps (Figure 2.6). Each spectrum in the map was modeled as outlined above and the integrated intensity of the 1030 cm\(^{-1}\) band was utilized as a parameter that represents CxL relative concentration. Each map depicted in Figure 2.6 corresponds to a specimen subjected to GA fixation. In these maps it can be seen that an increase in the concentration of GA and prolonged exposure to GA both produce an increase in the relative concentration of pyridinium-type CxLs. Averaged values of CxL concentrations at different exposure times and GA concentrations are summarized in Figure 2.7. COL thin films exposed to 0.05% GA experience a nearly linear increase in the relative CxL concentration,
Figure 2.6: Spatially resolved spectral maps of the normalized integrated intensity (concentration) of the 1030 cm\(^{-1}\) Raman band associated with the pyridinium type cross-linking. Thin films were exposed to (a) 0.05% glutaraldehyde solution, (b) 0.1% glutaraldehyde solution and (c) 0.2% glutaraldehyde solution for different fixation time.
whereas specimens treated with 0.1 % and 0.2% GA solution exhibit a higher rate of increase in the first hour of exposure, after which the increase rate slows down and becomes similar to that seen in samples fixed with 0.05% GA solution.

2.5 Conclusion

A collagen thin film model to simulate changes in relative crosslink concentration in extracellular matrix of an articular cartilage has been introduced. Samples were treated with varying concentrations of glutaraldehyde solutions at different exposure times to induce pyridinium-type crosslinks. These crosslinks are relevant to this model system as their centerpiece is the pyridine ring, which is also the central chemical compound of hydroxylysyl pyridinoline, a crosslink found in the extracellular matrix of articular cartilage and whose degradation is associated with loss of its structural stability. A novel Raman-based method to quantitatively assess the concentration of pyridinium-type crosslinks has been proposed. The spectra of glutaraldehyde treated collagen matrix were acquired and relevant segments of the signal were modeled with an appropriate optimization function comprised of the sum of the Lorentz functions, each of which represents individual bands present in the complex signal obtained from the collagen sample. The known biochemistry of the target material provided appropriate constraints on the optimization
algorithm to yield a unique solution, which was sufficiently sensitive to capture subtle alterations in the vibrational spectra. Normalized integrated intensity of the Raman band associated with the pyridinium-type cross-link was extracted from the resulting curve fit and its fluctuation as a function of exposure to glutaraldehyde was studied. Spatially resolved characterization of collagen thin films yielded spectral maps that showed an increase in the relative concentration of pyridinium-type crosslinks in collagen with prolonged exposure to glutaraldehyde solution. Future work envisions the application of the proposed model to cartilage explants and the validation of this method against available biochemical assays for quantification of crosslink concentrations.
Chapter 3: Use of Raman Spectroscopy to Assess Pyridinium-type Crosslinks and Study Crosslinking Kinetics in Proteoglycan-depleted Immature Bovine Cartilage Explants

3.1 Introduction

Collagen (COL) is one of the most abundant proteins in nature, and it is a prevalent component of extracellular matrix (ECM). COL molecules consist of three parallel polypeptide chains arranged in a right-handed triple helix. Each strand is made of a repeating XaYaGly sequence where Xa and Ya can be any amino acid, but are often proline (Pro) and hydroxyproline (Hyp), and Gly is glycine [196], making ProHypGly the most common COL triplet. Individual COL triple helices assemble hierarchically and form microscopic fibrils and macroscopic fibers and networks. Specifically, type I and II COL form fibrils, with type I being present in all fibrous tissues except cartilage, whereas type II COL is the main component of cartilage extracellular network (ECM). Cartilage fibrils are D-periodic (D ≈ 67 nm), indeterminate in length [196], and have slightly different structures than type I fibrils [197]. They form ECM network characterized by its ability to provide cartilage tensile strength, and withstand compression-relaxation cycles exerted by hydrostatic pressure provided by proteoglycans (PGs), another key component of an articular cartilage.

Crosslinks (CxLs) stabilize the COL fiber network. In cartilage, pyridinoline (PYD) are the major mature CxLs and pentosidine (PSD) are the senescent ones. Lysyl oxidase mediates covalent crosslinking COL fibrils by oxidizing hydroxylysine residues to hydroxylysyl aldehydes which then, through several reactions, lead first to immature CxL, then to stable PYD CxLs [198,
The contribution of crosslinking to tensile strength and stability of cartilage COL matrix has been extensively demonstrated in tissue engineering studies [163].

Stabilizing COL networks by inducing CxLs has been extensively studied [190, 200], and utilized in clinical practice [146]. Glutaraldehyde (GA) is a well-known CxL agent, widely used in stabilization of collagenous biomaterials. It is used in production of bioprostheses, such as heart valves [201], vascular grafts [202], [203] and tendon xenografts [204]. It is also used to immobilize enzymes, and CxL proteins [190]. GA has also found applications in CxL of polysaccharides for controlled drug delivery [205]. The nature of GA CxL process has been studied by many authors [190, 200], due to its importance in diverse fields [6], and yet its chemical nature is not yet fully understood.

At the moment there is no available assay that would non-destructively quantify CxL density in collagenous tissues. Biochemical methods, such as high performance liquid chromatography (HPLC), albeit informative, are complex and destructive, which often deems them impractical for use. On the other hand, Raman spectroscopy as a non-destructive, label free method for characterization of fully hydrated tissues ex vivo and in vitro, has in recent years emerged as a viable tool for characterizing ECM network. The Raman effect is the distortion of charge distribution by the primary incident electric field, induced by the monochromatic light source [93]. Incoming photons excite the various chemical species within the focal volume. The charge distortion results in the induction of molecular dipole moments, uniquely determined by the specific chemical bonds in the irradiated material. Each molecule has a specific vibration frequency determined by the geometry of the molecular bonds and the vibration modes of each dipole: an excited molecule can exhibit stretching, bending, twisting and other vibration modes simultaneously. As a result, a molecule can have multiple Raman lines in the overall signal. The
sum of all induced dipole moments acts as a macroscopic polarization, a scattered light of a secondary electric field, determining the intensity of the Raman lines. Therefore, Raman spectra are a signature of the material of interest.

There are a number of approaches to analyze rather complex Raman signal obtained from biological materials. Univariate peak analyses aim to identify single peaks or peak intensity ratios and assign them to bonds in particular molecules [206]. However, such an approach often yields relatively limited specificity due to band overlapping in Raman spectra. Multivariate analyses of Raman spectra, such as principal component analysis (PCA), are commonly used to characterize biological tissues [95, 172]. These methods are capable of analyzing large datasets simultaneously, identifying similar features in the individual spectra, and are effective in deducing patterns. Multivariate analyses do not require prior information to perform the analysis, and can determine whether there is significant enough difference between the individual spectra in a dataset, and identify the features of the spectra that are changing by clustering them [99, 207]. Algorithms used can be unsupervised, seen in PCA [208], Multivariate Curve Resolution (MCR) [209] and Clustering Analysis (CA) [210], or supervised, such as Partial Least Squares (PLS), Non-negativity Least Squares (NNLS) and Spectral Unmixing [206]. However, despite variety of analysis techniques applied to a wide range of tissues, quantification of data obtained with Raman spectroscopy has thus far not been quantitative in nature [206]. Efforts have been made to quantify the interpretation of Raman data utilizing multivariate curve resolution [206]. Such an approach utilizes biochemical variance via alternating least square algorithm to deconvolute the spectra, and has achieved to measure the concentration distribution of cartilage ECM constituents [211].

The aim of this investigation is two-fold, to introduce quantitative analysis of Raman spectra via selective decomposition of a segment of Raman spectra to detect subtle changes in
molecular composition of target tissue, and to study the kinetics of the GA induced crosslinking process. We hypothesize that GA-induced reactions continue in the target tissue after the crosslinking solution has been removed from it, and aim to study the kinetics of such an interaction. Rather than deconvoluting the entire Raman spectra and using statistical analysis to assess changes, we target specific molecules, and decompose segment of the Raman spectra containing bands associated with them. To achieve this goal, we combine the understanding of the underlying biochemistry of the said process with the utilization of optimization algorithms, which enable us to extract the individual Raman bands from the overall signal and quantify changes induced by biochemical reactions. This approach has been used in relatively simple materials but is rarely attempted in the analysis of complex biologic tissues [212, 213]. We have previously used constraints based on the biochemistry of the target material to decompose Raman spectra of COL type I thin films subject to GA treatment (please see Chapter 2), which served as a simplified two-dimensional model for the proposed analysis of Raman signal [102, 165]. In this study, by identifying Raman bands that originate in vibrational modes of pyridine, we aim to quantify the concentration of key COL CxLs induced by GA in the COL type II network in ECM obtained by digesting of the proteoglycans in native articular cartilage, which represents the next, more complex, level of approximation. Validation of the proposed approach is achieved by comparing the results obtained from analysis of Raman data against the mechanical testing, as it is known that increase of CxL density correlates with the increase in the stiffness.
3.2 Materials and methods

3.2.1 Sample preparation and proteoglycans depletion

Cylindrical cartilage explants were harvested from the condyles of immature bovine knee joints (Green Village Packing, NJ) at the day of slaughter. Cylindrical biopsy punches with $\varnothing 5$ mm in diameter were extracted from fresh bovine articular cartilages, after which the middle and deep zones were microtomed, resulting in a thickness of $1.96 \pm 0.2$ mm. PG Depletion: Explants were digested of their proteoglycan content using the protocol of Schmidt et al. [214]. Briefly, proteoglycan of articular cartilage was digested out through sequential exposure and incubation to the following three enzymes: chondroitinase ABC (Sigma Aldrich), trypsin (Sigma Aldrich) and Streptomyces hyaluronidase (Sigma Aldrich) (Figure 3.2a).

3.2.2 Glutaraldehyde treatment

0.2% glutaraldehyde was prepared in phosphate buffer saline (PBS, Sigma Aldrich) at pH 7.36 from 25% glutaraldehyde solution (Sigma Aldrich). Samples ($n=6$ for each group) were individually exposed to 25 mL of 0.2% glutaraldehyde for 30 sec (Group 1) or 2 min (Group 2) at 25 °C, then washed in 50 mL of PBS for 2 min (Group 1) or 5 min (Group 2). Samples in the control group were exposed to PBS as a sham treatment, but otherwise followed the same protocol as the treated group. Another group of samples (Group 3) was prepared and exposed to GA solution for 5 minutes and desorbed in PBS for 10 min, however, the results showed that longer exposure to GA degraded Raman spectra due to excessive background fluorescence from induced CxLs. Thus, results from Group 3 were not included here.

3.2.3 Mechanical testing

Explants were tested in unconfined compression, by stress-relaxation test. Equilibrium compressive modulus ($E_Y$) was calculated from equilibrium load, displacement and sample
geometry, following the protocol described in detail elsewhere [215]. Briefly, cartilage explants were tested in a custom device under unconfined compression [215], using a creep tare load (0.1 N, 400 s) followed by stress-relaxation to 10% strain (0.5 μm/s ramp, 1800 s). The equilibrium compressive modulus (EY) was calculated from the explant cross-sectional area, the equilibrium load and the displacement.

3.2.4 Raman spectroscopy

Raman spectra was acquired at four locations per sample 2 hours before, then again immediately after the GA treatment, with a Raman micro-spectrometer (InVia, Renishaw Wotton-under-Edge). Incident laser excitation was provided by helium-neon laser with 632.8 nm wavelength, delivered by 50× objective. Each Raman signal was collected with 10 second acquisition time and 30 accumulations. Prior to each acquisition, photobleaching was applied for 10 seconds to reduce the fluorescence background.

3.2.5 High performance liquid chromatography

HPLC experiments were performed on a Shimadzu HPLC equipped with SIL-20A autosampler, LC-6AD pumps, a DGU-20A5 degasser, a FRC-10A fraction collector, CBM-20A controller, SPD-20A UV-vis detector, and an Agilent Poroshell 120 EC-C18 (3.0 x 150 mm, 2.7 μm pore size) column. The mobile phase consisted of ultrapure deionized water and acetonitrile (HPLC grade) with 0.1% trifluoroacetic acid (v/v) at a flow rate of 0.5 mL min⁻¹ with 4:1 H₂O/CH₃CN.

3.2.6 Confocal fluorescence imaging

Ø5mm diameter bovine cartilage explants (Green Village Packing, NJ) were cut vertically to form ~1mm thick slices of tissue. 0.2% glutaraldehyde was prepared in phosphate buffer saline (PBS, Sigma Aldrich) at pH 7.36 from 25% glutaraldehyde solution (Sigma Aldrich). The tissue
was exposed to 25 mL of 0.2% glutaraldehyde for 2 minutes, then washed in 50 mL of PBS with proclin 950 and EDTA for 5 minutes. The sample was then placed in a petri dish, submerged in PBS bath, and imaged under confocal microscope (Olympus Fluoview FV1000 Confocal Microscope) periodically over 4 hours at 10X, with a 473 nm laser at 25% power, 20.0 us/pixel, 1024 × 1024 pixels, with an detector sensitivity of 855.

3.2.7 Raman spectra processing

Raman signal was pre-processed to remove fluorescence background [102, 216] and then relevant segments of Raman spectra were modeled. The hydrodynamic theory and trust region optimization were applied for the modeling and decomposing of the fluorescence removed signal [102]. The concentration of crosslinks (CxLs) at the focal spot is quantified as the area under the curve of corresponding Raman peaks in the modeled spectrum, and normalized by the area under proline curve assigned to COL. Normalization of Raman spectra is rather common, and in COL in particular has been used in FTIR studies to avoid errors as a result of concentration-dependent changes [194]. The additive property of Raman spectra allows for establishing correlation between the area under the curve (integrated intensity) and the concentration of the particular chemical compound [93, 217]. The molar ratio of CxLs to COL directly corresponds to the ratio of Raman Areas: they represent ratios of number of molecules and ratios of number of molecular bonds, respectively. We have verified this point by measuring the PYD standard (Quidel, San Diego, CA) at different concentrations and compared it against the high performance liquid chromatography (HPLC) measurements of different concentrations of PYD standard (Figure 3.1).
Figure 3.1: (a) Raman spectroscopic characterized PYD relative concentration. PYD CxL concentration is characterized by the integrated intensity of the pyridine ring; (b) HPLC characterized PYD relative concentration.
Figure 3.2: (a) Photographs of native and PG-Depleted explants; (b) representative Raman spectra of native and PG-Depleted explants. Decomposition and modeling of segments of PG depleted cartilage explants: (c) boxed region I and (d) boxed region II.

Two segments of Raman signal (boxed regions I and II in Figure 3.2b) were decomposed into three Raman bands each, with individual Raman bands following a Lorentzian profile. In the first segment, 853 cm$^{-1}$, 865 cm$^{-1}$ and 874 cm$^{-1}$ Raman bands represent proline [105, 206], COC symmetric stretching [107] and hydroxyproline/tryptanthrin [107], respectively. In the second
segment, 1030 cm$^{-1}$, 1042 cm$^{-1}$ and 1063 cm$^{-1}$ Raman bands are pyridine rings associated with PYD and pyridinium-type CxLs [107], pyranose ring stretching [108], and glycosaminoglycans (GAG) [206], respectively. Concentration of PYD/ pyridinium-type CxLs was quantified as the area under the 1030 cm$^{-1}$ peak in the modeled spectrum, normalized by the area under the proline curve (853 cm$^{-1}$) assigned to collagen [102]. Two-way ANOVA was performed with factors of treatment (GA+PBS and PBS Control) and timepoint (before, after), with repeated measures on the latter, for $E_Y$ and each Raman band (1030 cm$^{-1}$, 1042 cm$^{-1}$ and 1063 cm$^{-1}$). Tukey’s post hoc testing of the means ($\alpha = 0.05$) was performed.

### 3.3 Results

A new CxL formation of pyridinium-type crosslinks due to GA treatment, measured as the increase pyridine rings at the focal point of the incident laser light, has been observed. Samples were subjected to PBS only for control group, or 30 second or 2 minute exposure to GA for treatment groups. Longer exposures produced overwhelming fluorescence in the spectra, due to which Raman bands could not be extracted. The relative CxL concentration in the samples significantly increased after treatment with GA, whereas in controls, subjected to PBS only, the concentration remained unchanged (Figure 3.3a). The increase in CxL density was much more pronounced with longer exposure to GA. When exposed to GA for 30 seconds, the normalized integrated intensity of the Raman band associated with the pyridinium CxLs increased about 0.5 A.U. (A.U. = arbitrary units), compared against the pre-treatment values. In contrast, 2 minute exposure resulted in CxL density doubled. The other two Raman bands, centered at 1042 cm$^{-1}$ and 1063 cm$^{-1}$ and associated with pyranose and GAGs, respectively, were also decomposed in the modeling of the analyzed segment of the spectrum. The integrated intensity of these two Raman
bands has not significantly changed by GA treatment (Figure 3.3b, c), suggesting that the proposed analysis indeed captured changes in CxL density rather than random change that affect all Raman bands in the spectra.

Mechanical testing (Figure 3.4) has shown trend that corresponds to Raman results. 30 seconds exposure to GA yields about $31 \pm 9\%$ increase in Young’s modulus ($E_Y$), whereas extended GA exposure to 2 minutes produced an average percent change in modulus of $115 \pm 20\%$. Both are significantly higher than the average $4 \pm 4\%$ change for control samples, ($p < 0.0001$). The linear correlation between the change in PYD concentration and % change in $E_Y$ had an $R^2$ value of 65% (Figure 3.4a).
Figure 3.3: (a) Pyridinium CxLs concentrations of PG depleted cartilage explants. Integrated intensity represents CxL density and is assessed before and after the treatment by either PBS for control group, or glutaraldehyde solution for 30 s and 2 min for the treatment groups, *p < 0.05. Integrated intensities for Raman bands located at (b) 1042 cm$^{-1}$ and (c) 1063 cm$^{-1}$, *p > 0.3 for both.
Figure 3.4: (a) % Change of $E_Y$ due to the treatment * $p < 0.05$; (b) linear fit between change in CxL concentration increased by exposure to GA solution and change in $E_Y$.

Confocal imaging has captured increase in the fluorescence of samples after the exposure GA solution, and its subsequent removal (Figure 3.5). The calculated fluorescence intensity continues to increase throughout the 20 hours of imaging, indicating the glutaraldehyde is continuing to react and form new crosslinks or bonds within the tissue. The rate of fluorescence intensity increase is relatively high within the first 4 hours post-treatment, and then it slows down from 4 hours to 15 hours after the exposure to the GA solution (Figure 3.6). 15 hours after the GA
treatment the fluorescence intensity is starting to reach plateau, however, up to this time point the reaction appears to continue, albeit at a rather slow rate (Figure 3.6).

Figure 3.5: Fluorescence confocal images of cross-sections of PG depleted cartilage explants before and after the treatment. The images are acquired at constant time intervals ranging from 3 min to 20 hours after the treatment. Increase in fluorescence intensity indicates that crosslinking continues even though the GA solution has been washed out from the sample.
The proposed method to analyze the Raman spectra relies on quantifying the molecular targets of interest by constraining the decomposition of selected segment of Raman spectra to known chemical bonds and their modes of vibration allowed by the molecular structures. The main challenge is a large number of optimization parameters, with multiple peaks from individual chemical compounds, resulting in non-unique decomposition solutions. Restricting the analysis to a segment of the spectra somewhat alleviates the problem. Further, understanding of the underlying biochemistry to introduce initial conditions for the modeling allows us to obtain unique and meaningful solution to the optimization problem. Once the segment of the spectra is properly
modeled one can extract quantitative information out of it. Detailed description of the trust region optimization model can be found in Chapter 2, where it was applied on a simplified 2D model of a COL type I thin film.

![Pyridinoline cross-links](image)

![Pyridinium cross-links](image)

Figure 3.7: Schematic diagrams of (a) pyridinoline (PYD), a major crosslink naturally occurring in articular cartilage and (b) glutaraldehyde induced pyridinium-type crosslink. Pyridine ring prominently features in both CxLs; (c) Representative Raman spectrum of pyridine.

The GA-induced COL CxLs have been extensively studied [189, 190, 218-220]. A number of factors influence GA reactions with proteins including pH, concentration and temperature [219]. Further, Raman spectroscopy has been previously used to identify structural differences between COL types, and chemical bonds formed in GA-fixed porcine pericardium and isolated bovine COL
The reaction of the GA aldehyde group with the carbonyl group of the peptide bond in COL results in ether-type COC bond between GA and COL, weakening hydrogen bonds within the secondary polypeptide chain of COL and altering its conformation. In the GA treated samples, a new band due to COC symmetric stretching vibration appears at 865 cm$^{-1}$, along with shifts in amide peaks (Figure 3.2c) [107, 221], similarly to changes seen in COL type I thin films (please see Chapter 2). In addition, quaternary pyridinium compounds form stable CxLs in GA treated articular cartilage explants [189]. In plane deformation of 1,3,5- substituted pyridine ring associated with both PYD and Pyridinium-type CxLs, prominently features in the Raman spectrum at 1030 cm$^{-1}$ (Figure 3.7). It appears in both PG depleted and native cartilage explants (Figure 3.2b), however, depletion of PGs simplifies the region of the Raman spectrum by reducing it to only 3 Raman bands, which makes the modeling relatively straightforward. Further, we hypothesize that the integrated intensity of the in-plane stretching of pyridine ring can be used as the biomarker for the pyridinium-type CxLs, which we believe dominate the crosslinking process in presented experiments, because the increase in pyridinium-type CxL concentration correlates well with the increase of E$\gamma$ (Figure 3.4b). However, it should be mentioned that in-plane deformation of pyridine structures can be associated with both, naturally occurring PYD CxLs, and GA treatment induced pyridinium-type CxLs. Althoguh PYD CxL density should hypothetically be a function of bovine joint age, its concentration is relatively stable, as seen in Raman spectra of control samples. On the other hand, the Pyridinium-type CxLs are influenced by exposure time and concertation of GA solution (Figure 3.3a). By varying GA exposure time we initially induced different levels of pyridinium-type CxLs in PG digested articular cartilage explants. After the treatment, only the integrated intensity of the Raman band selected as a biomarker associated with Pyridinium-type CxLS density increased within the modeled segment
of the spectra, which provides us with confidence that the proposed method for the spectra analysis can indeed provide quantitative information about the CxL concentration. Correlation between the described CxL density and $E_Y$ (Figure 3.4) indicate that produced pyridinium structures indeed dominate the crosslinking process.

Confocal imaging has shown us a very interesting finding, GA induced crosslinking process continues long after the glutaraldehyde solution has been washed out of the specimen. To the best of our knowledge the time-dependent nature of GA crosslinking kinetics has not been observed before. The observed phenomenon requires further investigation and analysis.

3.5 Conclusion

In this study a novel method to analyze Raman spectra of biological media, and articular cartilage in particular, has been proposed. The proposed modality allows for detection and quantification of subtle changes in chemical composition of the tissue, and it is achieved by decomposition and modeling of particular segments of the Raman spectra. Obtaining unique and meaningful optimization models relies on deep understanding of the underlying chemistry of the target tissue, which provides initial and boundary conditions for the optimization model. The analysis has been performed on the PG depleted immature bovine cartilage explants; increase in concentration of Pyridinium-type CxLs due to exposure to glutaraldehyde solution have been assessed, and results corroborated with changes in the mechanical properties. Raman band associated with in-plane deformation of 1,3,5- substituted pyridine ring has been proposed as a biomarker for measurement of PYD CxL density in cartilage. The post-treatment fluorescence imaging revealed that the crosslinking process continues long after the GA has been washed out of the system. Future studies will include detailed investigation of crosslinking kinetics, as well as
application of the proposed method onto a native bovine tissue. Subsequent work will likely include analysis of Raman spectra from human cadaver joints.
Chapter 4: Femtosecond Laser Crosslinking of the Cornea for Noninvasive Vision Correction

4.1 Introduction

The prevalence of myopia has increased worldwide over the last 50 years. Its incidence in the United States and Europe is now almost twice that 50 years ago, and it is even more prevalent in East Asia. Spectacles and contact lenses remain the most common means of vision correction, but the permanent correction of refractive errors, by refractive surgery, has emerged as an attractive alternative. However, such surgery is an invasive procedure that may compromise corneal structure, and postsurgical complications have been reported. We propose a novel, non-invasive approach to permanent vision correction based on a different mode of laser-cornea interaction. Our approach induces the formation of a low-density plasma that produces reactive oxygen species, which react with the surrounding proteins, forming cross-links and triggering spatially resolved changes in mechanical properties. We show that the proposed method changes the refractive power of the eye, and confirm its safety and stability.

Myopia, or near-sightedness, is an eye condition in which incoming light is focused in front of, rather than on the retina. It is the most common refractive error and constitutes a growing public health problem worldwide. Its incidence has doubled over the last 50 years in the United States and Europe [222]. Myopia has also become an important issue in some East Asian countries, where its prevalence reaches 70 to 90% [223]. Most affected individuals use spectacles or contact lenses, which generally provide adequate refractive error correction. However, both are subject to limitations. Glasses do not work well in the rain and mist may form on them following changes in temperature or humidity. Contact lenses improve the field of vision and acuity, but many people find their presence on ocular surfaces intolerable [116]. Over the last 25 years, refractive surgery
for the permanent correction of vision has, thus, emerged as an attractive choice for many patients [117]. Vision correction surgery, such as laser-assisted *in situ* keratomileusis (LASIK) and photorefractive keratectomy (PRK), reshapes the corneal curvature via laser-assisted ablation of a portion of the corneal tissue. These procedures are invasive and may weaken the cornea, leading to postsurgical complications. Furthermore, such procedures cannot be performed in a significant fraction of the population, due to patient’s thin cornea, insufficient tear production or other abnormalities [117]. Efforts have been made to use riboflavin/ultraviolet (UV) light to correct myopia [224]. In such procedures, which were initially introduced for the treatment of keratoconus, corneas are soaked with riboflavin and then exposed to UV light, to induce corneal cross-linking. However, this approach is suitable only in cases of weak myopia. We propose a novel, non-invasive approach to permanent vision correction through a novel mode of laser-cornea interaction in which both thermal ablation [225] and optical breakdown [226] are avoided. Our approach is based on the induction of a low-density plasma (LDP) [227], to generate an ionisation field within biological media without the production of damaging thermoacoustic [228] and shock waves [229]. When applied to collagenous tissues, LDPs induce the production of reactive oxygen species, which react with the surrounding proteins to form cross-links and trigger spatially resolved changes in mechanical properties. We show here, with pig eyes *ex vivo*, that the proposed method effectively changes the refractive power of the eye. Follow-up experiments on rabbits *in vivo* confirmed its safety and stability. This method could expand the pool of patients eligible for permanent vision correction, while simultaneously eliminating the adverse effects associated with current forms of surgery. Furthermore, the approach described is also suitable for the treatment of keratoconus [230], and may have possible applications in other collagen-rich tissues, such as articular cartilage, in which it has been used to slow the progression of early osteoarthritis [231].
4.2 Results and Discussion

Laser-induced cross-link (CxL) formation alters the overall mechanical properties of the cornea, increasing its rigidity [230]. The concentration of laser energy achieved with this technique results in spatially resolved changes in mechanical properties, which are, thus, controllable. This technique may be particularly useful for slowing or reversing keratoconus and post-LASIK ectasia. Furthermore, the treatment of specific volumetric regions of the corneal stroma leads to macroscopic changes in overall corneal curvature (Figure 4.1), which could be useful for the treatment of myopia, hyperopia, astigmatism and irregular astigmatism. We present here 1) an *ex vivo* study on pig eyes, providing proof-of-principle for this technique, and 2) an *in vivo* study on rabbits, demonstrating the stability and safety of the induced changes. Pig eyes are frequently used in ophthalmic *ex vivo* research [145], and rabbits are widely used as a model for the correction of refractive errors [232].

![Figure 4.1: Step-by-step illustration of the use of the proposed laser-assisted process for the non-invasive correction of refractive errors.](image)
Figure 4.2: (a), (b) and (c) Time course of the change in normalized effective refractive power (EFR) after the laser treatment of pig eyes ex vivo; (a) Flattening (treatment of myopia); (b) Steepening (treatment of hyperopia) (c) Control study analyzing the effects of the treatment protocol. The treatment involves applying laser pulses such that the path of the laser follows a zigzag trajectory, thus treating a planar area at a specific depth. The treatment is repeated at different depths, effectively inducing ‘treatment layers’. Multiple treatment layers parallel to the surface were created, with a distance of 50 μm between consecutive planes. The y axis corresponds to effective refractive power normalized against diopter (D) values before treatment. Changes in the refractive power of the eye relative to the measurement performed immediately before treatment are shown. The error bars indicate the standard deviation.
4.2.1 Amending the effective refractive power of pig eyes *ex vivo*

We present two examples in the *ex vivo* study: corneal flattening, which is used to correct refractive errors due to myopia, and corneal steepening, which is used to treat hyperopia. We used a total of 60 fresh pig eyes for the study. Fifteen of these eyes underwent corneal flattening, and the treated eyes were paired with 10 control eyes. Thirteen eyes underwent laser irradiation to induce post-treatment steepening; these eyes were also paired with 10 control eyes. The remaining 12 eyes were used for a separate control study, to evaluate the effects of the experimental setup.

For the flattening treatment (Figure 4.2a), we treated a square in the middle of the eye. A strong change in corneal curvature, corresponding to a change in effective refractive power (ERP) of about 12% (a mean of about 5.11 dioptres), was initially observed, followed by partial recovery. Most of the change in curvature occurred within eight hours of treatment, after which, the cornea stabilised at a refractive power about 92% the initial level (about 3.45 dioptres, on average). This significant change became evident when corneal topography before and after treatment was paired with the corresponding virtual vision findings, demonstrating the effects of the refractive error correction applied (topography and virtual vision are illustrated in the Chapter 5). The initially large change in refractive power is due to a combination of the effects of the treatment itself and experimental conditions, including the temporary flattening of the cornea with a coverslip to ensure even volumetric exposure of the stroma to laser irradiation. The coverslip has an effect analogous to that of orthokeratology (ortho-K) [233], a temporary reshaping of the cornea used to reduce refractive errors, and the duration of the effect is similar to that of an ortho-K procedure. Once the effect of the coverslip wears off, the adjusted curvature remains stable throughout the rest of the 24-hour period. By contrast, laser treatment of the peripheral zone of the cornea leads to its steepening (Figure 4.2b). The effective refractive power (ERP) of pig eyes is significantly

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increased by treatment of a ring-shaped region. In the case of corneal steepening, the effective power of the eye increases gradually over a 12-hour period, after which it stabilises at a new value higher than that before treatment. This indicates that the induction of new CxLs counteracts the influence of the coverslip. For confirmation that the induced changes were photochemical in nature, and not due to the thermal denaturation of collagen fibrils, we measured the laser-induced changes in corneal temperature. The relative change in temperature at the focal volume and in its immediate vicinity was less than 7°C (results shown in Chapter 5). The heating induced by the treatment was, therefore, well below the threshold for the thermal denaturation of collagen. Furthermore, examination under a light microscope equipped with Nomarski interference contrast optics revealed that there was no difference in refractive index between the treated and untreated parts of the cornea, consistent with an absence of corneal hazing (see Chapter 5 for further details).

Figure 4.3: Two-photon fluorescence (TPF) images of cross-sections of (a) control and (b) laser-treated pig eyes. Three regions are imaged in the treated eye: an untreated region (left), a transitional region (middle) and the central region (right). Crosslink density is similar in the control sample and the untreated region of the laser-irradiated specimen. Scale bar: 60 μm.
The depth of the cross-linked region in the cornea was assessed by two-photon fluorescence (TPF) imaging. TPF imaging can be used to visualise the changes induced in the cornea [234], because collagen is a primary extracellular source of non-linear emissions [235]. The difference in structure between the control and treated regions of the cornea is shown in Figure 4.3. This difference is due to the excitation of tyrosine and dityrosine oxidation products [235] and pyridinium-type fluorophores[236], strongly indicating that femtosecond laser treatment results in a significant increase in CxL density in the treated region [236, 237].

These findings are consistent with reported results for the TPF imaging of glutaraldehyde-cross-linked collagen hydrogels [236], and riboflavin/UV light-cross-linked rabbit corneas [237]. The depth of the treated region was about 220 µm, consistent with the projected depth of treatment, suggesting that cross-linking does not decrease in efficiency as the focus of the laser pulses shifts toward the posterior stroma. Furthermore, cross-link density appeared to be fairly uniform throughout the treated volume, even though consecutive treatment planes were 50 µm apart.

In addition to evaluating changes in ERP and cross-link density, we also performed standard histological examinations of the pig corneas. No laser-induced damage was observed on haematoxylin- and eosin (H&E)-stained histological sections of corneas (results shown in Chapter 5). The pig corneas were also cultured for one week after treatment, to determine whether cross-link density remained stable, and to check for degradation in the cross-linked layers of the stromal matrix. Treatment delayed the injury-induced apoptosis of stromal keratocytes (for results and further discussion, see Chapter 5).
Figure 4.4: Time course of treatment-induced changes in the corneal effective refractive power (EPR) of live rabbits: group 1 (a), group 2 (b), and group 3 (c). ERP did not change in control eyes, whereas it decreased following laser treatment, subsequently remaining stable for three months after the procedure. *p<0.05: statistically significant change in refractive power.
4.2.2 In vivo studies in the rabbit model

Having obtained proof-of-principle in these initial ex vivo experiments on pig eyes, we then used the rabbit model to assess the stability of the changes in corneal curvature induced by laser treatment, and the safety of the procedure. A protocol almost identical to that used on pig eyes was applied to rabbit eyes in vivo, with a view to assessing changes in ERP 24 hours, seven days, and then weekly up to three months after treatment. Three groups of animals were assessed (see Methods). In Group 1, the mean change in ERP 48 hours after treatment was 1.74 dioptres. (Figure 4.4a) relative to the pretreatment value. In Group 2, the change in ERP seven days after treatment was 1.64 dioptres (Figure 4.4b). No significant change in ERP was observed in the control eyes of the rabbits of these two groups over these periods (Figure 4.4a, b). The stability of the ERP changes induced by the treatment was monitored in the third group of animals (Figure 4.4c). These changes remained stable for three months after treatment, with a relative change in ERP of about 1.94 dioptres for treated eyes.

The structure of haematoxylin- and eosin (H&E)-stained histological sections of corneas obtained 48 hours, one week and three months after treatment was similar to that of the control samples (Figure 4.5). Specifically, no wound or wound-healing response resembling that observed after refractive surgery[232] was detected, and no collagen disorganisation, epithelial cell and stromal oedema, intrastromal vacuole formation or endothelial cell detachment was observed; all these features are associated with thermal damage to stromal tissue, and we can, therefore, conclude that no such damage occurred [238].
Figure 4.5: Histological sections of hematoxylin-eosin (H&E)-stained rabbit corneas: (a) two days after treatment; (b) seven days after treatment; (c) three months after treatment; (d), (e) and (f) corresponding untreated controls. Scale bar 100 μm.
Confocal laser scanning microscopy (CLSM) images of rabbit eyes were obtained immediately after the rabbits had been killed (Groups 1 and 2) and in vivo (Group 3). The distance between two consecutive image planes was 2 μm for the epithelium and the stromal keratocyte network. The monolayer of endothelial cells was imaged separately. A comparison of CLSM images for intact rabbit eyes and laser-treated rabbit eyes revealed no significant difference in cellular structure and density (the CLSM images are presented in Chapter 5). Our CLSM images of the epithelial layer, stromal keratocyte network, and endothelium are similar to those obtained in other studies [239-241]. Our CLSM images of the endothelium (see in Chapter 5) showed that cell shape and density were similar for treated eyes and their paired controls.

4.2.3 Low-density plasma produces reactive oxygen species

The formation, by reactive oxygen species (ROS), of intra- and intermolecular covalent bonds between collagen fibrils has been observed following the exposure of riboflavin-soaked eyes to UVA light to initiate the cross-linking of corneal collagen for corneal stabilisation in patients diagnosed with keratoconus [242]. However, ROS generation as a by-product of plasma generation in aqueous media has, itself, been the focus of considerable interest [243]. Free radical formation through two-photon ionisation and the dissociation of water molecules was initially achieved by irradiation with high-power UV picosecond lasers [244]. Advances in femtosecond laser technology subsequently made phasing to multiphoton ionisation (MPI) possible [243]. In aqueous environments, laser-induced ionisation and dissociation occur as a cascade of reactions that can be classified as primary, secondary or tertiary. The primary reactions include the formation of solvated electrons and of the water cation radical, H$_2$O$^+$. This ROS is unstable and reacts with a water molecule to generate the H$_3$O$^+$ ion and the hydroxyl radical OH'. The excited water molecule simultaneously dissociates, H$_2$O$^+$→H + OH', generating another OH'. The primary reactions are
followed by secondary and tertiary reactions leading to the formation of H, O$_2^-$, OH$^-$, H$_2$, H$_2$O$_2$, HO$_2$ and other species [244].

The laser intensities used in studies focusing on ROS generation [243, 244] were well above the irradiance threshold for femtosecond breakdown in aqueous and ocular media ($\sim 10^{13}$ W/cm$^2$) [227], a level at which the density of free electrons released by photoionisation reaches a critical value, resulting in the formation of a dense, optically opaque plasma [229]. However, as the number of free electrons produced during a single pulse is a function of irradiance, pulses generated by a femtosecond oscillator can be focused on aqueous or ocular media such that the density of laser-generated free electrons is well below the critical value for the formation of a dense plasma, but high enough for the generation of a LDP [227]. In this situation, femtosecond irradiation is below the energy level required for optical breakdown, but atoms within the focal volume can be ionised, because the ionisation probability has a number of resonance maxima due to the intermediate transition of the atom to an excited state [245]. In the vicinity of such maxima, the ionisation cross-section increases by several orders of magnitude, making it possible for ionisation to occur, even if the frequency of the incoming electromagnetic wave is below the ionisation potential [245]. Multiple photons interact simultaneously with bound electrons to overcome the band gap, producing an electron-hole pair [229, 246]. Under these conditions, the ionisation of aqueous media is considered possible, and our hypothesis that the LDP produces ROS in aqueous solutions was confirmed by the use of electron paramagnetic resonance spectroscopy to capture OH$^-$ in an aqueous solution irradiated with a femtosecond oscillator (Figure 4.6a).
a

Intensity (a.u. x 10^3)

B0 (G)

3,430 3,450 3,470 3,490 3,510

b

Tyrosine Oxidation
Tyrosil radical Recombination

Enolization

Dityrosine crosslinks

<table>
<thead>
<tr>
<th>Emission at 400 nm (a.u. x 100)</th>
<th>Control</th>
<th>Treated</th>
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<td></td>
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<td><strong>50</strong></td>
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* Indicates significant difference.
Figure 4.6: Mechanism of curvature adjustment: (a) Electron paramagnetic resonance (EPR) spectrum demonstrated the OH• and O2• generated by ionization of the solution with a femtosecond oscillator; (b) Fluorescence spectrum of the laser-treated and control samples, accompanied by a schematic diagram of the oxidative modification of tyrosine. Oxidative modifications of amino acids generated the tyrosyl radicals. Two tyrosyl radicals combine to form 1,3-dityrosine; (c) Differential scanning calorimetry: The thermal denaturation temperature of the treated corneal samples is about 2°C higher than that for untreated samples; (d) Second harmonic generation (SHG) images of the anterior stroma of the control (I) and laser-treated (II) pig eyes. The bar chart (e) shows the number of peaks above the cutoff line on the optical density plots for the control and laser-treated corneas, used to quantify relative changes in the density of collagen structures in the corneal stroma23. *p<0.05: statistically significant difference.
4.2.4 Cross-link Formation

ROS initiate the photo-oxidation of proteins, leading to the formation of chemical CxLs[247]. All amino acids are susceptible to modification by OH$^{-}$ and OH$^{-}$ + O$_{2}^{-}$ ($^{+}$O$_{2}$) radicals, but tryptophan, tyrosine, histidine, and cysteine are particularly sensitive [248]. The amino acids involved in CxL formation in corneal tissue include histidine, hydroxylysine and tyrosine [249]. The oxidative modification of tyrosine is characterised by the abstraction of phenolic hydrogen atoms from tyrosine residues. The tyrosyl radical is relatively long-lived and can react with another tyrosyl radical or with tyrosine to form a stable, covalent carbon-carbon bond, resulting in the creation of 1,3-dityrosine. The formation of this specific product of protein oxidation leads to intra- or intermolecular cross-linking [250] (Figure 4.6b). This reaction primes pathways leading to cross-linking of the corneal stroma upon irradiation with a femtosecond oscillator (Figure 4.6c, 3). Furthermore, preliminary quantitative analyses of second-harmonic generation (SHG) images [251] (Figure 4.6d) showed that CxLs locally increase the density of collagen structures in the anterior layers of the stroma, resulting in a homogeneous, signal more regular than that of the untreated cornea. These changes directly influence overall corneal curvature, leading to changes in the refractive power of the eye. We performed a more detailed analysis of SHG images in a follow-up study [252].

4.2.5 Safety of the proposed procedure

The corneal stroma was cross-linked by the formation of free radicals, which have the potential to cause cell damage. For example, both in vitro [253] and in vivo [254] studies of the cytotoxicity of riboflavin/UVA procedures have shown that UV light-induced collagen cross-linking in the presence of riboflavin leads to an immediate loss of stromal keratocytes throughout the entire volume of the affected stroma. It can take up to six months to repopulate the corneal
stroma with activated keratocytes [255], but this treatment is considered safe for humans unless the cornea is less than 400 μm thick. In such cases, the main safety concern is the potential for endothelial damage reported in both \textit{in vivo} and \textit{in vitro} studies [256, 257]. The endothelium of the healthy cornea plays a key role in maintaining corneal hydration and transparency via active sodium–potassium adenosine triphosphatase (ATPase) and bicarbonate-dependent magnesium ATPase ion pumps. The corneal endothelial cells cannot replicate, and compensation can be achieved only by sliding [258]. If cell density decreases to levels below the critical limit, endothelial barrier dysfunction may occur, resulting in vision loss. Clinical studies have shown that endothelia less than 400 μm thick in humans are susceptible to riboflavin/UVA treatment-related toxicity [152, 259], and this has led to restrictions on the use of this technique. Our procedure is wavelength-independent, so an ultrafast laser operating in the infrared frequency domain can be used, avoiding the damaging effects of UV radiation. A quantitative analysis of keratocytes and endothelial cells with FIJI imaging software [260] showed that cell counts were similar in treated and control eyes. Keratocyte density 48 hours after treatment (39464.29 ± 2288.57 cells/mm$^3$) was similar to that in untreated controls (39523.82 ± 5868.68 cells/mm$^3$). Keratocyte density remained stable for three months, in both treated and control eyes. Endothelial cell counts 48 hours after treatment were 2925.00 ± 64.14 cells/mm$^2$ for laser-irradiated corneas and 2908.33 ± 101.04 cells/mm$^2$ for untreated controls. No change in endothelial cell density was observed seven days, one, two and three months after treatment (the quantitative analysis is summarised in Figure 5.5 in Chapter 5). The observed keratocyte and endothelial cell counts were within the normal range [261, 262]. Confocal microscopy can reveal the presence of inflammatory cells in the cornea at an early stage of disease, before the appearance of external signs [263-265].
but CLSM imaging showed that there was no inflammation-driven immune cell migration to the corneal stroma following laser treatment.

4.3 Conclusion

In this study, we used the LDP generated by a loosely focused femtosecond oscillator, a side effect of multiphoton imaging, to develop a method for treating ocular media. The proposed method, which exploits the nature of the interaction between ultrafast laser-generated LDP and collagenous tissue, can be used to induce CxLs in collagen-rich tissues without the use of photosensitizers, to modify the properties and geometry of these tissues. The novel laser-tissue interaction model described here was tested on pig eyes ex vivo and in rabbit models in vivo. It amended the refractive power of these eyes, suggesting that it could potentially be developed as a method for non-invasive vision correction. Selective alterations to the biochemical properties of pig and rabbit corneas improved refractive power. These changes in ERP remained stable for three months after treatment. Furthermore, histological and confocal imaging studies of the eyes revealed no signs of laser-induced damage, suggesting that the procedure is safe. Future studies will include the establishment of a mechanical model of the relationship between CxL density and the full-field deformation response of the corneal stroma, as an understanding of the underlying corneal biomechanics is a prerequisite for the development of a clinically relevant technique. Finally, the laser-tissue model was applied exclusively to ocular media in this study, but the approach proposed could be extended to serve as a robust platform for use with other collagen-rich tissues requiring remodelling or an enhancement of mechanical properties.

4.4 Methods

*Ex vivo Eye treatment.* Pairs of fresh pig eyes were recovered from an abattoir (Green Village Packing, Green Village, NJ), less than two hours after the animals had been killed. They
were placed on ice and immediately transported to the laboratory. We used 60 pig eyes in total. Fifteen eyes were subjected to flattening treatment. The treated eyes were paired with 10 control eyes. Thirteen eyes underwent laser irradiation to demonstrate post-treatment steepening. These eyes were also paired with 10 control eyes. The remaining 12 eyes were used for a separate control study, to evaluate the effects of the experimental set-up. Excess tissue was removed from the isolated eyes, which were then rinsed with Dulbecco’s phosphate-buffered saline (DPBS, 1x, Sigma-Aldrich) and brought to room temperature in a damp chamber. The eyes were examined and any defective samples were discarded. The epithelial layer of the retained specimens was removed by gentle scraping with a 10 mm scalpel blade [266] and the eye globes were mounted on a custom-built eye holder (see Chapter 5 for a schematic diagram). The epithelial layer was removed to ensure consistency between the specimens, as most had experienced superficial damage during handling at the abattoir. Intraocular pressure was maintained (~16 mm Hg) by connecting an intravenous (IV) system filled with 0.9% sodium chloride solution (Hospira Inc, Lake Forest, IL) to the eyeball via a 22G needle (BD, Franklin Lakes, NJ). A customised digital pressure gauge with an Omega PX154 low-differential pressure transmitter was used to adjust the pressure. Immediately before treatment, the corneal surface was covered with a microscope coverslip (#1 Microscope Cover Glasses, VWR International, PA) to ensure homogeneous volumetric irradiation of the cornea. An Nd:Glass femtosecond oscillator (High Q Laser, Austria) was used to generate laser pulses with a temporal pulse width of 99 fs and a repetition rate of 52.06 MHz, at a wavelength of 1059.2 nm. A Zeiss Plan Neofluar 40x objective lens with a numerical aperture (NA) of 0.6 was used to focus the beam, and the mean power of the laser system after the objective lens was 60 mW. The samples were mounted on a three-axis motorised PT1 translational stage powered by Z825B motorised actuators (Thorlabs, Newton, NJ). The treatment consisted of
laser pulses applied by moving the stage in an $x$-$y$ plane, such that the laser path followed a zig-zag trajectory at a feed rate of 2.2 mm/s, resulting in the treatment of a planar area at a specific depth. The objective focused the laser on a spot of about 2 μm in diameter. There was, therefore, a spot overlap of about 90%. The treatment was repeated at different depths, resulting in ‘treatment layers’. Multiple treatment layers were created parallel to the surface, with a distance of 50 μm between consecutive planes, giving an effective depth of treatment of about 200 μm. The laser beam was focused on the anterior cornea, from the corneal surface to a depth of up to 200 μm. With every ‘treatment layer’, the laser was focused exclusively on the cornea, ensuring that the lens was not damaged. The flattening treatment took about 37 minutes, whereas corneal steepening took about 90 minutes to complete under the conditions described. The experimental set-up is shown in Chapter 5, and schematic diagrams of the treatment paths are provided in the main text (Figure 4.2). A paired control eye was subjected to a protocol identical to that used for the treated eyes, and was mounted on the same stage whilst the other eye was being treated, but was not itself exposed to the laser. The coverslip was carefully removed immediately after treatment. During the 24-hour characterisation period, we applied 3 ml of phosphate-buffered saline (PBS) to each eye at 20-minute intervals, to stop the eyes drying out.

*Animals.* In vivo experiments were performed on young adult Dutch Belted rabbits, each weighing 1.8-2.0 kg. Rabbits are commonly used as models for the correction of refractive errors, despite anatomic differences between human and rabbit eyes, such as the higher regenerative capacity of the endothelial layer and doubts about the possible presence of a Bowman’s layer in rabbits [232]. The animals were delivered to the Columbia University Institute of Comparative Medicine (ICM) animal house one week before laser treatment, to allow the animals time to get used to their new environment. The experimental protocol, and pre- and post-treatment handling
procedures, were reviewed and approved by the Institutional Animal Care and Use Committee of Columbia University. The 12 animals used in the study were assigned to three groups. The animals in the first group \( (n = 3) \) were killed and their eyes were removed 48 hours after laser treatment, to investigate the acute effects of laser irradiation. The animals in the second group \( (n = 3) \) were killed after one week, to allow the eyes to undergo at least partial healing if the laser had damaged the tissue. In this group, the refractive power of the eye was also determined 48 hours after treatment. The last group of animals \( (n = 6) \) was monitored to investigate the long-term stability of the induced changes in refractive power. Half of the animals in the last group were killed after three months, to determine whether there was any treatment induced damage or other morphological changes. The animals were killed 48 hours (Group 1), seven days (Group 2), and three months (half the animals in Group 3) after treatment, by the intravenous injection of pentobarbital \( (100 \text{ mg/kg}) \) into the marginal ear vein. The cornea, retina, and lens were isolated, fixed by incubation with 10% formalin overnight, desorbed by incubation with 70% alcohol for 24 hours, and sent to Columbia Medical Center Histology Service for histological staining. The samples were embedded in paraffin wax and cut into 5 μm-thick cross-sections, which were then stained with haematoxylin and eosin. These sections were observed with a VHX 5000 digital microscope (Keyence Corporation, NJ) and the acquired images were processed with ImageJ software [267, 268].

In vivo eyes treatment. Before treatment, the animal corneas were subjected to a macroscopic examination and slit-lamp evaluation by a veterinary surgeon, to ensure that there were no abnormalities or eye injuries. The rabbits were anaesthetized with an intramuscular injection of ketamine \( (3.5 \text{ mg/kg}) \) and xylazine \( (5 \text{ mg/kg}) \), placed on a warm heating pad, and monitored until fully unconscious. The depth of anaesthesia was confirmed by the absence of pedal
and ear-pinch reflexes [269]. Anaesthetized rabbits were gently placed on their sides, in a custom-built, heavily padded holder for immobilisation (see the figure in the Chapter 5). The eye facing upwards was treated, and the eye facing downwards was used as the untreated control. Proparacaine (0.5% ophthalmic solution) drops were applied to the treated eye for local anaesthesia, followed by GenTeal water-based gel (Novartis, Alcon, Inc, TX, USA) to prevent corneal dehydration. GenTeal gel was applied to both eyes and replenished during the procedure, as required. The treated eye was gently pressed with a coverslip, to ensure the homogeneous volumetric application of laser pulses. The laser treatment protocol was based on the procedure developed on porcine eyes ex vivo, as described above. However, the laser beam (Nd:Glass ultrafast laser, Hi-Q Laser, Austria) focused, via an objective with a high numerical aperture (Zeiss, Plan Neofluar 40x/0.6 NA), on the desired volumetric zone of the cornea, was delivered to the rabbit eye by mounting the objective on a custom-built three-axis motion system with three translational stages (PT1, Thorlabs, Newton, NJ) coupled to motorised actuators (Z825B, Thorlabs, Newton, NJ). A number of optical components, including mirrors and lenses, were mounted on the motion system, to steer the laser beam into the back aperture of the objective. As in the ex vivo study, laser pulses were rasterised by moving the objective in an x-y plane such that the laser beam followed a zig-zag trajectory, resulting in the treatment of a circular planar area (∅ 5 mm) at a specific depth (Figure 5.2c in Chapter 5). Again, as in the ex vivo study, the treatment was repeated at different depths, to generate ‘treatment layers’. Five treatment layers parallel to the surface were created, with a distance of 50 μm between consecutive layers. This treatment resulted in corneal flattening (Figure 4.4). The rabbits were returned to the ICM animal facility immediately after treatment.
Ex vivo corneal topography characterisation. We used an EyeSys Vision clinical eye topography characterisation instrument (EyeSys System 2000, EyeSys Vision Inc, TX) with version 1.50 software to capture corneal topography and calculate effective refractive power immediately before treatment, and after laser irradiation. Measurements were made at regular intervals over a 24-hour period after the treatment of the eyes. During this characterisation, the corneal surface was moistened with Systane Ultra Lubricant Eye Drops (Novartis). Following the application of an eye drop, the eye holder was moved gently in a circular motion, to distribute the lubricant evenly over the ocular surface and allow any excess lubricant to slide off the eye. The topography of a pig eye before and after treatment is shown in Chapter 5. Topographic results are paired with virtual vision results to demonstrate the effects of the laser treatment.

In vivo topographic measurements. Topographic measurements were performed with an Eyesys Vision non-contact eye-topographer (EyeSys Vision Inc, Houston, TX) [127, 270]. Topographic measurements of the entire corneal area were performed before treatment, 48 hours after treatment (Groups 1, 2 and 3), seven days after treatment (Groups 2 and 3), and then once weekly (Group 3), to assess the effects of laser light-induced corneal cross-linking on potential changes in the refractive power of the eye. Corneal topography measurements were also made immediately after treatment. Highly irregular patterns and abnormal results were observed in some cases, due to an anaesthesia-driven drop in intraocular pressure, and these results were excluded from the analysis.

TPF microscopy. In total, eight freshly harvested corneal samples, four from control eyes and four from treated eyes, were cut into 2 mm² blocks with a custom-built slicer, mounted in a 3 mm Petri dish and processed for TPF microscopy. We used a two-photon microscope (Bruker, MA, USA), with a Mai Tai Deep See Ti:Sapphire laser (Spectra Physics, Santa Clara, CA) as the
excitation source. A 40x/0.8 NA water immersion objective (Olympus, Japan) was used to acquire
the fluorescence signals, which were registered with two different photomultiplier tubes, one in
the red (580-620 nm) and the other in the green (480-570 nm) wavelengths of visible light. The
excitation wavelength was 826 nm. The green channel was used for data acquisition.
Measurements were processed with ImageJ software.

Confocal laser scanning microscopy (CLSM). Cellular evaluations of corneal tissues were
performed by CLSM imaging with the HRT3-RCM laser scanning system (670 nm laser beam,
Heidelberg Engineering, Dossenheim, Germany) equipped with a 63x/0.95 NA water immersion
objective (Zeiss, Germany). Imaging was performed immediately after death for the rabbits of
Group 1 (48 hours after treatment) and Group 2 (7 days after treatment), and in vivo for Group 3
(one, two, and three months after treatment). A disposable sterile plastic cap was placed on the
objective, to maintain the distance between the corneal surface and the objective. The animals were
placed in a custom-built holder during the process, with the eyelids of the imaged eye gently pulled
open by hand. GenTeal water-based gel was applied as a coupling medium. The entire corneal
volume was scanned and recorded, with optical sections through the epithelium, stroma, and
endothelium [265, 271].

Electron paramagnetic resonance (EPR) spectroscopy. EPR spectroscopy was performed
on laser-irradiated Dulbecco’s phosphate-buffered saline (DPBS). The radicals produced by the
laser irradiation of DPBS were captured with the spin-trapping reagent 5,5-dimethyl-1-pyrroline-
N-oxide (DMPO, Cayman Chemicals, USA). DMPO was dissolved in DPBS, at a final
concentration of 100 mM. We added 170 μL of the sample to a shallow dish (2x8 mm) covered
with a microscope coverslip. The dish was then placed on a three-axis motorised stage for laser
treatment. A control sample was prepared in parallel, by the same procedures. Immediately after
treatment, as described in the Eye Treatment section, both treated and control solutions were collected into 0.5 mL tubes, which were then placed in a canister of liquid nitrogen for transport to the EPR spectrometer (Bruker BioSpin EMX Electron Paramagnetic Resonance Spectrometer, Bruker BioSpin GmbH, Germany) for characterisation. The total time from sample treatment to EPR analysis was less than 15 minutes in all cases.

Fluorospectrometric detection of dityrosine. We prepared 5 mM L-tyrosine (Sigma-Aldrich) in Tris buffer pH 10. Samples were placed in shallow 170 μl dishes, covered with a microscope coverslip, and treated with a femtosecond oscillator, as described in the Eye Treatment section. After laser irradiation, the samples were transferred to a black 96-well plate (Thermo Fisher Scientific) and their fluorescence was measured with excitation at 325 nm and emission at 400 nm [272], on a Gen5 Microplate Reader (BioTeK Instruments, Inc.). Untreated L-tyrosine solution was subjected to the same procedure, under identical conditions, as a control.

Differential scanning calorimetry (DSC). Corneal punches of 5 mm in diameter were extracted from laser-treated and untreated corneal samples, sealed in plastic film to stop the tissues from drying out and stored frozen at -20°C. The punch samples were thawed immediately before characterisation, rinsed in DPBS, blotted on a paper towel folded in half to remove excess solution, and samples weighing about 26 mg were sealed in 50 μL aluminium pans (Perkin-Elmer Universal Crimper Press; Perkin-Elmer, Waltham, MA) and loaded into the DSC autosampler (Perkin-Elmer DSC 6000 autosampler) for the measurement of denaturation temperature. Samples were characterised as described elsewhere [273]. Briefly, they were heated to temperatures of 40°C to 70°C, at a scanning speed of 18°C/min. Denaturation curves representing differential heat flow over time were generated (see the figure in Chapter 5) and analysed with Pyris (version 11.0).
**SHG microscopy.** Corneas were harvested immediately after laser irradiation of the eyes. They were fixed by incubation overnight in 4% paraformaldehyde at 5°C. After fixation, 2 mm² blocks were dissected from the central region, washed in PBS, mounted in 50% glycerol in PBS on microscope coverslips and imaged. The sample preparation process has been described in detail elsewhere [274]. The second harmonic signal was generated with a laser (Chameleon Vision II, Coherent, Santa Clara, CA) tuned to 850 nm, on an A1RMP laser scanning system mounted on an Eclipse TiE microscope stand (Nikon Instruments, Melville, NY) equipped with a 25x/1.1 NA ApoLWD water-immersion objective. The back-scatter configuration was used to acquire the SHG signal, with the non-descanned detector (Nikon, Japan) and a 400-450 nm bandpass filter. The microscope was controlled with NIS Elements software (Nikon, Japan). The images acquired were analysed as described in a previous study [251]. The images were preprocessed with the spatial frequency filter. We used set-ups with 100 and 20 pixels, to analyse large and small features, respectively. After initial processing, the images were converted to binary signals, and plotted. The optical density of the black and white areas on the binary images was assessed by determining the number of peaks crossing the median cut-off intensity. The crossing densities measured at the vertical midline are considered to represent the complexity (irregularity) of collagen structure patterns: the higher the crossing density, the more complex the collagen bundle pattern. Crossing density is therefore used here as a surrogate for pattern irregularity [251]. SHG imaging and analysis were performed on the central regions of anterior corneas. Groups of laser-treated and control samples were prepared for SHG imaging, with each group containing four corneas.

**Temperature measurements.** For temperature measurements, fresh *ex vivo* pig eyes were placed in a customised holder under the three-axis motorised femtosecond laser head. The conditions for laser treatment were as described in the Eye Treatment section. The corneal tissue
was moistened with PBS before measurement. The needle-like tip of a customised thermocouple (Omega Single Strand, Insulated Thermocouple Wire, Φ0.07 mm, temperature measurement range 0-100°C, Stamford, CT) was inserted into the middle of the cornea, parallel to the surface. Real-time temperature readings were displayed on a computer equipped with a LabView temperature input module (National Instruments Cooperation LabView Student Edition 2016). The focal point was carefully aligned with the tip of the thermocouple and temperature distribution was measured as the focal volume was moved laterally away from the tip of the thermocouple (see the schematic diagram of the procedure in Chapter 5). Three eyes were used for characterisation, and 14 measurements were performed per eye. Each consecutive temperature measurement was made along a testing line 18° away from the previous measurement, in the same plane.

Refractive index measurements. Nomarski interference contrast characterisation was performed on corneas, with an Olympus BX 60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a 20x/0.45 PLAN APO objective (Olympus, Tokyo, Japan), 24 hours after treatment. A Nomarski interference contrast (refractive index sensitivity of about 0.08) prism was used to enhance the contrast between regions of the cornea with potentially different refractive indices. Before the examination, corneas were fixed by incubation in 10% formalin overnight, followed by desorption in 70% alcohol for 24 hours.

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Chapter 5: Safety and Continued Studies on Femtosecond Laser Crosslinking of the Cornea for Noninvasive Vision Correction

5.1 Experimental setup of ex vivo and in vivo laser treatment

Figure 5.1: Experimental set-up and laser treatment of ex vivo porcine eyes. (a) Experimental set-up; (b) schematic diagram depicting laser beam delivered to the eye ex vivo. The eye is placed in a custom-built eye chamber.
Figure 5.2: *In vivo* experimental set-up and laser treatment pattern. Experimental set-up for live animal treatment (a) and (b). Rabbits were deeply anesthetized and placed in a customized holder. The treatment (c) involved applying laser pulses such that the laser followed a zigzag trajectory over a circle of Ø 5 mm, resulting in the treatment of a planar area at a specific depth. The treatment was performed at five different depths, effectively inducing ‘treatment layers’. Multiple treatment layers parallel to the surface were created, with a distance of 50 μm between consecutive planes.
5.2 Ex vivo topographic measurements and virtual vision simulation

Figure 5.3: Ex vivo topographic measurements and virtual vision simulation. Corneal topography of isolated pig eyes (a) before and (b) after laser treatment; Results are shown together with virtual vision, (c) and (d), to demonstrate the effects of the refractive error correction applied. The corneal elevation maps show effective refractive powers of 45 diopters before and 43.5 diopters after treatment. The virtual vision for the corneal effective refractive powers shown corresponds to (c) 45 diopters and (d) 43.5 diopters, assuming that 43.5 diopters corresponds to a visual acuity of 20/20 (normal vision).
5.3 In vivo confocal laser scanning microscopy

Day 2 Post Treatment
(I) Treated
(a) (g) (m)

(II) Control
(b) (h) (n)

Day 7 Post Treatment
(III) Treated
(c) (i) (o)

(IV) Control
(d) (j) (p)

One Month Post Treatment
(V) Treated
(e) (o) (y)

(VI) Control
(f) (p) (z)
Figure 5.4: Representative in vivo confocal microscopy images of laser-treated (rows I, III, V, VII and IX) and control (rows II, IV, VI, VIII and X) rabbit eyes obtained 48 hours (rows I and II), 7 days (rows III and IV), one month (rows V and VI), two months (rows VII and VIII) and three months (rows IX and X) after treatment. (a-j) – corneal epithelium; (k-t) – keratocyte network; (u-z, aa-dd) – corneal endothelium (scale bar = 50 μm).
Figure 5.5: In vivo keratocytes and endothelial cells counting. Rabbit corneal keratocyte (a) and endothelial (b) cell densities in vivo, on days 2 and 7, one month, two months and three months after laser.
5.4 Differential scanning calorimetry of laser treated pig corneas

Figure 5.6: DSC thermograms of untreated (left) and laser-treated (right) samples of pig cornea. The thermal denaturation temperature of the treated samples is about 2°C higher than that for untreated samples. Comparison of the averaged data for the control and treated samples confirms the higher denaturation temperature for the laser-irradiated samples (right).

The results obtained were similar to those for previous studies on pig corneas. For example, Kampmeier et al. [275] obtained a DSC thermogram for pig corneas with a maximum temperature peak at about 63°C, a temperature similar to that obtained here. The slight difference between our results and those of Kampmeier and coworkers [275] probably reflects differences in experimental conditions. We also re-examined the data published by Spoerl et al. [152] for the thermomechanical behavior of riboflavin/UV-crosslinked pig corneas. Spoerl et al. [152] assessed the hydrothermal shrinkage of the corneal strips. The hydrothermal shrinkage temperature was higher for crosslinked corneal samples than for the untreated control.
5.5 Temperature and refractive index measurement under experimental conditions

Figure 5.7: Temperature measurement of femtosecond irradiated corneal tissue. (a) Experimental setup and (b) the lines along which the measurements were taken; (c) Temperature distribution as a function of distance from the focal volume.

Figure 5.8: Refractive index characterization of ex vivo pig eyes. Nomarski images of control (a) and laser-irradiated (b) corneal tissue 24 hours after laser treatment. The treated region is boxed. The images demonstrate the absence of hazing after the laser treatment. Scale bar: 100 μm.
5.6 Studies on Cultured Pig Corneas

Pig corneas were cultured after treatment, to assess the effects of femtosecond oscillator irradiation. We wished to determine whether any degradation occurred in the crosslinked layers of the stromal matrix and whether there were any adverse effects on cellular components. Cultured corneas were evaluated on days 1 and 7, consistent with earlier studies in which riboflavin-soaked corneas were crosslinked with UVA light [276].

5.6.1 Methods

Freshly harvested pig eyes were treated with the femtosecond oscillator, according to the procedure outlined in the methods section. We used a total of 40 eyes in the study: 20 of these eyes were treated, and the other 20 were paired controls. After laser treatment, the corneal topography of the eyes was monitored for 24 hours under controlled conditions. After the topography observation period, eyes were removed from the custom-built eye holders, rinsed three times with 20 ml of sterile phosphate-buffered saline (PBS) per rinse, immersed in 20 ml of 3% polyvinylpyrrolidone-iodine (PVP-I) solution for about 1 minute and rinsed again three times with sterile PBS. After the second rinse, corneas were dissected from the eye together with about 1 mm (thickness) of the scleral rim. Half of each sample was immediately examined (24 h time point), and the other half was placed in a sterile culture vessel (Fisher Scientific, CAT#08722E). The vessel was filled with 8 ml of a customized incubation medium consisting of low-glucose Dulbecco’s Modified Eagle Medium (Thermo Fisher) supplemented with 8% fetal bovine serum and the appropriate antibody. Corneas were cultured at 37°C in a tissue culture incubator (Thermo Scientific Series 8000 DH, Waltham, MA). Cell viability (Live/Dead Assay Kit, Invitrogen) in the corneal stroma was assessed for both the 24-hour and one-week after treatment time points[277]. We punched out 5 mm-diameter cylinders from the central part of with the cornea for confocal
microscopy assessment (Olympus Fluoview FV1000, Waltham, MA). The images were taken from the anterior side of the corneal samples, in the middle part of the sample thickness. In addition to cell viability tests, we performed standard histological characterization on corneas 24 h and one week after treatment. Corneas were fixed by incubation with 10% formalin overnight, desorbed by incubation with 70% alcohol for 24 hours and sent to Columbia Medical Center Histology Service for histological staining. There, samples were embedded in paraffin wax, and cut into 5 μm sections, which were stained with hematoxylin and eosin (H&E).

![Figure 5.9: Live/Dead staining of corneal punch specimens: (a) control and (b) laser-treated at 24 h; (c) control and (d) laser-treated at 1 week. Live cells are labeled green and dead cells are red. Scale bar: 200 μm.](image)

5.6.2 Results and discussions

Confocal images of samples stained with Live/Dead assay kit provided no evidence of a loss of viability 24 hours after treatment (Extended Data Fig. 9a,b). Similarly, no loss of viability
relative to the untreated control was observed for the specimens cultured for one week after treatment (Extended Data Fig. 9c,d). These results demonstrate that the laser treatment does not affect cell viability for at least week, as we observed no significant post-treatment keratocyte depopulation in the corneal stroma of treated samples. However, qualitative observations of the control and post-treatment confocal micrographs (Extended Data Fig. 9) revealed that cell density was higher in treated than in control specimens. This observation was consistent with the findings for H&E-stained slides of tissue (Extended Data Figs. 10, 11).

**Figure 5.10: Hematoxylin and eosin (H&E)-stained histological cross-sections of (a) untreated control after 1 week of culture; femtosecond laser-irradiated pig corneas after (b) 24 hour and (c) 1 week of in situ incubation. Blue dots represent keratocytes [278, 279]. Scale bar: 100 µm.**

H&E-stained corneal sections of the femtosecond laser-treated eyes displayed no significant change in stromal structure and endothelium integrity relative to the untreated control (Extended Data Fig. 10), but the treated anterior segments of the corneas remain populated with keratocytes 24 hours after treatment, and after culture in tissue culture medium for 7 days at 37°C (Extended Data Fig. 11). After one week of culture, keratocytes were still present, throughout the
entire cross-section, in the treated samples (Extended Data Fig. 10c), whereas they were present only in the posterior regions of cultured control corneas (Extended Data Figs. 10a, 11).

Figure 5.11: Hematoxylin and eosin (H&E)-stained histological cross-sections of the anterior portion of (a) femtosecond laser-irradiated pig corneas and (b) untreated controls. Both after one week of in situ incubation. Blue dots represent keratocytes [278, 279]. Scale bar: 100 µm.

The finding was unexpected, as debriding of the corneal epithelium results in apoptosis-driven keratocyte depopulation[280]. In this study, we removed the corneal epithelium because most samples suffered superficial damage due to handling at the abattoir. We therefore expected to find an absence of keratocytes in the anterior stroma. Previous reports suggested that epithelial-stromal apoptosis serves as an antiviral response mechanism, limiting the proliferation of pathogens, such as herpes simplex virus, from the injured corneal epithelium to the stroma[281]. A similar disappearance of keratocytes from the anterior stroma has been observed after photorefractive keratectomy (PRK) [282], in which scraping of the epithelium is followed by excimer laser-assisted photoablation of the corneal stroma to correct refractive errors. It has been suggested that damaged epithelial cells release interleukin-1 (IL-1) into the corneal stroma, regulating keratocyte apoptosis [283, 284]. In normal homeostasis, IL-1[285] maintains tissue organization through apoptotic and, possibly, negative chemotactic effects on adjacent keratocytes[284]. However, its effect on individual keratocytes depends on its local concentration and, if it remains below lethal concentrations, the keratocyte will respond by negative chemotaxis rather than apoptosis[284]. In
the experiments presented, the epithelium was scraped off the pig eyes before laser treatment, which should have triggered the release of IL-1 into the corneal stroma. However, no major keratocyte apoptosis was observed in the anterior stroma. We suggest that either propagation of the IL-1 signal was retarded by the treatment or that cell-cell interactions were altered. Further studies will be required to determine which of these possibilities applies. Follow-up studies could potentially pave the way to a better understanding of the pathophysiological mechanisms of keratoconus development.

As for the characterization of crosslink density described in the main body of the text (please see the Methods section of the manuscript for two-photon fluorescence), two-photon fluorescence (TPF) imaging was used to visualize laser-induced changes in crosslink density in the treated parts of cultured pig corneas (Extended Data Fig. 12). The structural difference between the control and treated regions of the cultured corneas was similar to that observed in the ex vivo study (see the main text), indicating that the induced crosslinks remained stable one week after treatment. The affected region was larger in cultured samples, which we attributed to the swelling of the corneal tissue during culture for seven days.

Histological examination of the treated cornea showed that the proposed laser treatment had no adverse effects on the stromal tissue. No collagen disorganization, stromal edema, intrastromal vacuole formation or endothelium detachment was observed. The density of induced cross-links appeared to remain stable during culture of the cornea for one week. Furthermore, the treatment appeared to retard the injury-induced apoptosis of stromal keratocytes, potentially shedding new light on the development of keratoconus, a corneal disease characterized by a loss of stromal structural integrity modifying the mechanical properties of the cornea and resulting in myopia, irregular astigmatism and a loss of visual acuity[286]. Crosslinking is known to strengthen
the mechanical properties of cornea and, thus, to stabilize keratoconic eyes, but the pathophysiology of keratoconus is not fully understood[286]. However, corneal fibroblasts in keratoconic eyes have four times as many IL-1 receptors than those of normal eyes[284]. In normal homeostasis, IL-1 balances keratocyte proliferation and apoptosis. It is possible that this balance is disrupted in keratoconic eyes, which lose keratocytes over long periods of time[284]. Our findings suggest that crosslinking may be beneficial in keratoconic eyes through cellular effects, in addition to the stabilization of mechanical properties. However, further studies on live animals are required to confirm and extend these findings.

Figure 5.12: Two-photon fluorescence (TPF) images of cross-sections of (a) control and (b) laser-treated pig eyes. Three regions are imaged in the treated eye: the untreated region (left), the transitional region (middle) and the central region (right), as in the procedure performed on eyes ex vivo (shown in the main body of the study). The control sample and the untreated region of the laser-irradiated specimen after one week in culture ex vivo had a similar crosslink density to the 24 h sample in the ex vivo study. Samples cultured for one week had a larger laser-irradiated region, due to swelling of the corneal tissue during culture. Scale bar: 100 µm.
Chapter 6: Femtosecond Laser Crosslinking of Articular Cartilage as a Treatment Modality for Early Osteoarthritis

6.1 Introduction

In the previous chapter it has been shown that one can utilize a femtosecond oscillator to introduce photochemical reaction that leads to crosslinking of the target tissue by restricting the lasing parameters such that an optical breakdown is never achieved. In this chapter we work with collaborators to expand proposed treatment modality onto an articular cartilage. As discussed in detail in 4.2.3, when a femtosecond oscillator irradiation is restricted to a regime below the optical breakdown, free electron cloud formed by the impact and multiphoton ionization does not have sufficient density to form a dense plasma. However, under proper conditions, a low-density plasma is formed within the focal volume. The low-density plasma does not inflict shock waves or significant thermal effects often seen more traditional use of femtosecond lasers [228, 229], but is energetic enough to produce localized ionization field, which can ionize and dissociate interstitial water in the target tissue. Such a scenario results in a formation of a short-lived burst of reactive oxygen species [244]. We hypothesize that the proposed treatment modality relies on a photochemical effect triggered by ultrafast laser irradiation, because newly formed free radicals oxidize amino acids in collagen triple helix to produce covalent bonds.

Laser intensities employed in studies focused on ROS generation [243, 244] have utilized intensities well above the irradiance threshold for a femtosecond laser induced of an optical breakdown in aqueous and ocular media (~$10^{13}$ W/cm$^2$) [229]. At those irradiances the density of photoionization-formed free electrons reaches a critical value, resulting in formation of a dense, optically opaque plasma [245]. This is a well-known, a widely studied phenomenon. However,
free electrons density generated by the ultrashort laser pulse is a function of irradiance, and therefore it can be controlled to restrict its value below the optical breakdown threshold. Low electron density will usually yield no changes in the biogical media, as seen in multiphoton imaging, however, if the lasing regime can be tailored such that the density of the laser-generated free electrons is significant enough for the generation of low-density-plasma (LDP) [246]. In that case, although the optical breakdown is avoided, ionization of atoms within the focal volume is possible because the ionization probability has a number of resonance maxima due to intermediate transition of the atom to an excited state (Please see Chapter 4 for more details). In our previous work, we have shown that LDP produced ROS, and specifically we captured OH* in aqueous solution irradiated by a femtosecond oscillator with post-treatment of electron paramagnetic resonance spectroscopy (Figure 4.6).

![Image](image_url)

**Figure 6.1: Step-by-step illustration of the proposed use of the proposed laser-assisted process for the treatment of early OA.**

As stated in Chapter 4, it has been widely reported that free radicals induce intra- and inter-molecular CxLs in collagen fibrils. ROS can be introduced by activating riboflavin with UVA light, which is now a clinical application to treat patients diagnosed with keratoconus [242].
Independently, the production of ROS as a byproduct of plasma generation in aqueous media has studied as well [244]. Water molecules have initially been ionized and dissociated to form ROS via exposure to high-power UV picosecond lasers [244]. Advances in femtosecond lasers have enabled phasing to multiphoton ionization (MPI) [287]. In aqueous environments, laser-induced ionization and dissociation occur as a cascade of reactions, and has been described in detail in Chapter 4. Briefly, the process is broken down into primary, secondary and tertiary reactions. In primary reactions solvated electrons are formed together with the cation radical of water H$_2$O$^+$. This is followed by H$_2$O$^+$ reacting with a water molecule, and producing a hydrogen ion H$_3$O$^+$, and hydroxyl radical OH$^-$. Concurrently, dissociation of the excited water molecule occurs, H$_2$O$^*$→H$^+$ OH$^*$, providing another OH$^-$. Primary reactions are followed by secondary and tertiary reactions [244]. We are particularly interested in OH$^*$ radicals because it is believed that they, together with singlet oxygen, are at least in part responsible for CxL formation [288]. Although exact pathways in which oxidative modification of amino acids results in covalent bonds in collagen network is not well understood, role of ROS in CxL formation in collagen type I is well documented [254]. We hypothesize that collagen type II that forms extracellular matrix in articular cartilage can undergo similar modification, and that we can utilize laser-induced free radicals to strengthen articular cartilage and improve its wear resistance via increase of CxL density.

6.1.1 Background

Articular cartilage is a specialized tissue that minimizes wear and friction, while simultaneously transmitting load to adjacent joints. About 9 percent of Americans suffer from degenerative diseases of articular cartilage, such as osteoarthritis (OA) [60]. OA is a complex and progressive joint disease characterized by asymptomatic early stages, followed by debilitating late development [60]. Articular cartilage has limited ability for self-repair, and over the lifespan of an
individual the damage accumulates, resulting in painful loss of function. One of the main challenges of OA is lack of symptoms in its early stages [60]. Late stages of the disease are characterized by incapacitating pain, and after pharmacological remedies are exhausted, the treatment is usually reduced to a partial or full joint replacement [60]. At the moment there are no conclusive treatments for early stages of OA.

6.1.2 Disruption of Crosslinks in extracellular matrix one of major factor in OA progression

Two main pathways lead to OA, acute mechanical trauma and/or gradual development due to joint aging [60]. The common denominator of these two pathways is homeostatic imbalance of the extracellular matrix (ECM). Primary components of ECM are collagen (COL) and proteoglycans (PG), each of which has a distinct function within the articular cartilage. Former provide tensile strength and integrity of the ECM structure [37], whereas latter is responsible for the hydrostatic pressure [38]. COL in the articular cartilage forms a fibrillar network, which is bound together by CxLs. Role of CxLs in establishing structural integrity and material properties of COL matrix has been broadly studied [6-8, 10, 54]. Degradation of the CxLs in the network results in inability of the tissue to counter the PG induced pressure, leading to diminishing of its function in the joint. Animal and clinical models have shown that loss of CxLs result in degradation of ECM, regardless of the status of PGs. [51, 86, 289].

6.1.3 Current OA treatment options

Clinical options for addressing OA are mostly reduced to pain management. Patients suffering from OA are encouraged to undergo muscle-strengthening exercises [60] to relieve pressure on the joint. In addition, doctors usually address symptoms by recommending pharmacological remedies for pain relief [74]. These include both, anti-inflammatory drugs [75], and steroid injections [76], sometimes administered directly to the joint. Current laser treatment
options include smoothing of fibrillated articular cartilage [290, 291]. In theory, smoothing of the cartilage surface should reduce friction and wear. However, such an approach relies on thermal ablation of the tissue surface, and due to the use of excimer and solid-state lasers in these procedures [290, 291] has potential for significant side effects, thermal injury in particular, while offering temporary pain relief and no lasting improvement to the cartilage tissue.

6.2 Methods

6.2.1 Femtosecond oscillator treatment of immature bovine cartilage explants

Immature (2-3 months old) joints were obtained from a local abattoir (Green Village Packing, NJ) at the day of slaughter. In the first study, cylindrical plugs with 3 mm in diameter and height of 1 mm were extracted from fresh bovine articular cartilages using a 3D printed slicer, and devitalized by freezing at -30°C. A total of 36 explants was used. Samples were divided into three groups: 1) control group, 2) laser treatment of the superficial zone at depths 0-200 um and 3) samples laser treated at depths 100-300 µm from the articular surface. The experimental setup was designed to address two questions, effectiveness of the treatment at given lasing conditions, and the depth dependent efficacy measured from the surface of the cartilage explant. Thus, all samples were divided in 3 × 2 matrix with 6 samples in each cell.

In the second study, live immature bovine cartilage explants were treated. A total of 24 samples were collected, and divided into treated specimens and paired controls. Cell viability was assessed using Live/Dead Assay Kit (Molecular Probes), 24 hours and two weeks after the treatment. After the laser treatment explants were cultured in a chemically defined media, a modified typical cartilage culture media, described in detail in [164]. Modification of a typical
cartilage culture media was introduced to maintain homeostatic conditions. The compressive stiffness (please see 2.3.1.) was assessed as well.

In the third, preliminary, study, two larger (Ø10 mm × 1.2 mm) sample pairs were obtained from immature bovine joints, to test whether the lasing induced matrix heterogeneity. These cartilage discs were subjected to the same laser treatment (please see below) as the rest of the batch. However, due to their size, only central region has been treated. The treated area was a 3 mm × 3mm square.

Samples were treated with a femtosecond oscillator (High-Q Laser, Austria), which provided 99 femtosecond long pulses with wavelength centered at 1059 nm at 52 MHz repetition rate. The laser was coupled with a custom built beam delivery setup (Figure 6.2) centered around 3-axis motorized stage (Thorlabs Inc, NJ). The laser beam was focused onto the cartilage sample via microscope objective (Zeiss, plan neofluar, 40x/0.6). Samples were placed in a petri dish and immobilized with a custom made ring that formed an interference fit with the sample. The petri dish was filled with PBS to ensure that the sample remains sufficiently hydrated during the experiment. The samples were covered with a cover slip and subjected to laser irradiation similar to that described in chapter 4.4. Briefly, the laser beam was focused in the interior of the cartilage explant and moved along zig-zag pattern in x-y plane parallel to the surface of the explant. The treatment occurred at pre-determined depth, again measured from the articular surface. After covering a 3 × 3 mm area the focal volume was moved along the z-axis, and zig-zag rasterizing of the laser irradiation was repeated, resulting in a multi-layered treatment.
6.2.2 Femtosecond oscillator treatment of human articular cartilage

Fresh-frozen devitalized fibrillated human articular cartilage sample was obtained from human cadaver joints. The joints were obtained from a tissue bank (Platinum Training, LLC, Dallas, TX). A total of 3 joints were used (1 male and 2 female; donor ages were between 73 and 95 years). OA was diagnosed in all three joints and had the overall Outerbridge score (OS) from OS1 to OS3 [292] (Figure 6.3, 6.4). Paired cartilage explants were extracted from joints. One explant was subsequently laser treated and the paired sample served as a sham control. The samples were similar in size to immature bovine plugs, and also extracted with a 3D printed slicer. 7 paired specimen were harvested from the region with local OS1, and another 5 pairs obtained from areas scored with local OS2. Regions of the joints with localized OS3 were too soft for successful sample
extraction. The laser treatment followed the same protocol as the one applied to immature bovine cartilage plugs treated from articular surface to 200 µm (in 6.2.1).

Figure 6.3: Human OA distal femur with overall Outerbridge score OS1 to OS3. The samples were harvested in pairs (boxed circles) to ensure similar conditions between treated samples and paired controls. The circles represent approximate location of the samples prior to extraction, all of which on lateral condyle. Harvested sites are shown in the inset. Histology was performed as well and the location of the histology section (Please see Fig. 6.4b) is shown with the white dashed line.
Figure 6.4: (a) Example of delamination wear in a human femoral condyle with OS3 score. The delamination damage is highlighted with a white arrow. (b) Another example of human femoral condyle with OS3; Advanced OA shows a missing surface zone again highlighted with a white arrow as well as typical fibrillation. (c) Fibrillated surface in delaminated bovine cartilage sample (d) Representative sample, after being harvested from human lateral condyle (Fig. 6.3). The specimen got OS2 as score.

6.2.3 Testing of Mechanical Properties

6.2.3.1 Measurement of the compressive stiffness

Stiffness of the samples was assessed by comparing the equilibrium compressive stiffness \( E_Y \) between treated samples and paired controls, as well as comparing \( E_Y \) of the specimen before and after the treatment. \( E_Y \) was measured across the full thickness of an explant, following the previously published protocols [215, 293]. Briefly, cartilage explants were tested in a custom device under unconfined compression [215], using a creep tare load (0.1 N, 400 s) followed by stress-relaxation to 10% strain (0.5 μm/s ramp, 1800 s). The equilibrium compressive modulus
(Ey) was calculated from the explant cross-sectional area, the equilibrium load and the displacement.

6.2.3.2 Wear testing

Friction and wear testing was performed using custom, 2-axis loading device. The device consists of a 2-axis translational stage (JMAR) coupled with a linear encoder (RSF Electronics, MSA 65x series), which has 5-10 um resolution and can be operated at speeds up to 10 mm/s. It also has 6-degree-of-freedom load cells (JR3 Inc.) and is located in a clean environment. The setup is controlled by custom LabVIEW software (National Instruments Corp.). Subset of laser treated samples (in 6.2.1) were subjected to constant stress of 0.18 MPa and reciprocal sliding. The conditions were realized by placing the articular cartilage sample into shallow recess within a dish filled with PBS. The dish was sitting at the translational stage. The samples were immobilized by ensuring that the recess that the sample have interference fit. The load was applied either via a flat glass plate with a diameter order of magnitude larger than the specimen. This was a stationary contact area (SCA) configuration, and the method was described in detail in [294-296]. The plate was carefully lowered until a contact with the explant was established and appropriate load applied. Subsequently the translational stage started moving back at forward +/- 5mm at 1 millimeter per second, resulting in reciprocal sliding. The test was performed for 4 hours and had a total of 1440 cycles.

Two larger cartilage plug pairs, were subjected to the wear test that utilized a spherical glass lens with radius of curvature R = 12.7mm, and applied load of 4.45 N. The wear test consisted of sliding cycles with 4 mm travel at 1 mm/s for 12 hours. This test represents a more physiological, mitigating contact area (MCA), configuration, which sustains interstitial fluid pressurization [297] and low friction.
6.2.4 Histology and Cell Viability assessment

Cell viability was examined by live/dead staining [277] and subsequent confocal imaging. Histology preparation included fixation, sectioning and fixation with picrosirius-red [298] and safranin-o [299].

6.3 Results

6.3.1 Laser treatment of immature bovine cartilage plugs

Results of the laser treatment of immature bovine cartilage are depicted in Figure 6.5. The bar chart shows that the primary outcome of the study, the compressive equilibrium unconfined compression modulus, $E_Y$, has nearly doubled in laser treated samples, when compared against untreated controls ($p < 0.03$ for all). Further, moving the treatment deeper into the tissue (from 100 µm to 300 µm) has produced similar results as when the top 200 µm of the cartilage ($p = 0.47$, Figure 6.5), measuring from the articular surface, were treated. Live explants from the second study have exhibited similarly significant ($p< 0.03$) increase of $E_Y$ (0.58 ± 0.21 MPa) after being subjected to the femtosecond laser irradiation when compared against paired untreated controls (0.36 ± 0.21 MPa).
Figure 6.5: Equilibrium compressive stiffness of immature bovine cartilage samples. Bar charts shows controls (blue), laser irradiated specimens in which the treatment covered volume from 0 to 200 µm (orange), and samples in which the laser treatment encompassed volume from 100 to 300 µm (yellow), all measured from the articular surface. Error bar indicates the standard deviation. *p < 0.03.

The wear tests (Figure 6.6) have shown significantly improved wear resistance of laser treated samples, when compared against untreated controls. In addition to lack of morphological changes that would suggest damage from the wear testing in treated samples, there was no substantial difference (p = 0.13) in $E_Y$ before (0.50 +/- 0.03 MPa) and after (0.49 +/- 0.03 MPa) the wear test. On the other hand, control samples were visibly damaged, and $E_Y$ dropped significantly (p < 0.03) from 0.38 +/- 0.01 MPa prior to wear testing to 0.32 +/- 0.02 MPa after it.
Figure 6.6: Side view of bovine cartilage samples before and after the Wear test. Controls (top row) were visibly damaged after the test, as highlighted by arrows, whereas laser-treated explants show no significant morphological changes (bottom row).

In the third, preliminary study, in which the central region of larger bovine cartilage discs ($\varnothing 10 \text{ mm} \times 1.2 \text{ mm}$) was laser treated, wear testing resulted in visual delamination for untreated control samples prior to completion of 12 hours of sliding against a glass lens. In contrast, laser treated samples have shown no signs of wear or visual delamination after 12 hours (Figure 6.7). Delamination in paired controls occurred close to the center of the disc, and is characterized by formation of a blister, which can be observed in both, photographs and 3D scans (Figure 6.7). In addition, the friction coefficient remains similar in magnitude in both, laser irradiated, and untreated control samples (Figure 6.8). The same similarity in friction coefficient magnitude is observed for both testing configurations (SCA and MCA).
Figure 6.7: Bovine cartilage discs after wear test. Top row (a) shows photographs of discs, whereas the bottom row (b) represents laser surface scans. Delamination wear appeared on the control sample in a form of blister (left), accentuated with white arrows; Laser-treated bovine disc had no damage.

Figure 6.8: Friction coefficient $\mu$ obtained during wear tests. $\mu$ remains similar in magnitude in both, controls and treated samples. under MCA configuration, 12-hours wear test yields: $\mu_{\text{final}} =$ 0.005 undamaged laser treated, $\mu_{\text{final}} =$ 0.008 damaged untreated control. Representative friction coefficient obtained from SCA configuration is much higher when compared against MCA for both, treated discs and paired controls. It is also time dependent.
Confocal imaging of laser treated and control live explants of immature bovine cartilage (Figure 6.9) shows similar cell viability 24 hours and two weeks after the laser irradiation. The results suggest that lasing of the cartilage has limited adverse effects on the chondrocytes.

![Image of live/dead staining](image)

**Figure 6.9:** Live/Dead staining of immature bovine cartilage plugs. Top row shows representative results 24 hours after the treatment; Top left image shows (a) control whereas top right (b) shows laser treated sample; Bottom row shows cell viability after two weeks in a culture; bottom left (c) untreated control, and bottom right (d) laser treated specimen.

6.3.2 Laser treatment of human OA cartilage samples

The equilibrium compressive modulus (\(E_Y\)) was tested for both, control and laser treated samples before and after the treatment. Paired controls were subject to sham treatment, i.e. they were exposed to identical conditions as the lased samples, except the exposure to the laser beam. Findings of the testing are depicted in Figure 6.10. \(E_Y\) in controls remains the same before and after the sham treatment (\(p = 0.34\)) for both, OS1 (0.63 +/- 0.43 MPa) and OS2 (0.14 +/- 0.05 MPa). On the other hand, The compressive modulus of the laser treated groups of both OS1 (\(p = 0.04\)) and OS2 (\(p = 0.004\)) has significantly increased after the treatment, compared against the before treatment values (Figure 6.10).
The results presented in the previous section demonstrate that the treatment modality proposed for addressing keratoconus and the correction of refractive errors has potential to be extended to crosslinking of collagen type II in articular cartilage towards slowing down or retarding progression of early osteoarthritis. Increasing crosslink density via ultrafast laser irradiation of the cartilage superficial zone enhances its mechanical properties and increases wear resistance. The wear testing under SCA configuration has shown a striking visual contrast between controls and treated cartilage plugs. After being subjected to reciprocal sliding against a glass plate, controls exhibited fatigue failure characterized by swelling and softening, whereas laser treated samples retained original shape and stiffness. Large cartilage discs were also subjected to wear test, but in this case MCA configuration has been applied. Controls have exhibited delamination
characterized by formation and rupture of a blister in the central zone, whereas laser treated samples had no noticeable damage after 12 hours of cyclic loading. This finding alleviated concerns that the treatment focused on a small region of the cartilage surface could result in unfavorable matrix heterogeneity. Difference in localized stiffness of the articular surface has not shown to be detrimental to ability of cartilage disc to sustain wear resistance.

Proposed ultrashort pulsed laser-based modality enhanced main cartilage characteristics, resilience to wear and sustaining compressive loads, while simultaneously retaining low friction coefficient. Further, the viability studies have showed that subjecting live bovine cartilage explants to femtosecond oscillator to induce crosslinks has very limited detrimental effects on the cell viability within the extracellular matrix. Time-constrained post-treatment culturing has therefore produced promising results that should be validated in animal models in the future.

CxL stabilize collagen fibers in extracellular network [12, 86, 162, 192, 300]. In the cartilage Lysyl oxidase oxidizes hydroxylsine residues leading to a cascade of reactions to initially form immature pentosidine CxLs, and subsequently mature hydrosylsyl pyridinoline CxLs [192, 301-303]. In the immature bovine cartilage this process has obviously not been finalized, which likely leaves an abundance of vacant crosslinking sites. On the other hand, in human cartilage samples OA fibrillates the articular surface. The fibrillation may be increasing the availability of sites that can be crosslinked, which makes the OA cartilage susceptible for the proposed treatment. Interestingly, the achieved values of E\text{Y} were the same for OS1 and OS2 (Figure 6.10), which may suggest that the induced cross-link density, reaches the same plateau regardless the starting number of available CxL sites. The latter, on the other hand, we hypothesize to be a function of fibrillation, and therefore it is unlikely that over-treatment will result in excessive stiffening, which is indirectly corroborated by the treatment of the small region of the
cartilage disc. However, the potential over-treatment poses a risk of inducing too many free radicals in the cartilage.

Avascular nature, low oxygen levels and limited glycolysis prompt free radicals to play a significant physiological roles in cartilage [304-306]. In response to partial oxygen pressure variations, mechanical loading and inflammatory mediators, chondrocytes produce elevated levels of ROS [307]. These are mainly nitric oxide (\(^{\text{•}}\text{NO}\)) and superoxide anion (\(\text{O}_2^{\text{•}}\)), which are responsible for generation of other radicals such as peroxynitrite (\(\text{ONOO}^{\text{•}}\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)) [308-310]. Cellular response to ROS is a function of its redox status, and as long as the reducing capacity of the cell matches its oxidant level, ROS remain within their physiological concentrations. When ROS production exceeds the antioxidizing potential of the cell, an ‘oxidative stress’ is generated which contributes to cartilage degradation [307]. The overproduction of ROS in pathological cartilage has been associated with the accumulation of lipid peroxidation products and nitrotyrosine \(\text{in situ}\) [305, 311, 312]. Although we hypothesize, that the laser treatment induces short-lived bursts of non-nitric ROS, their excess amount could be detrimental to the target tissue. One approach to mitigate adverse effects of excess free radicals is application of ascorbic acid phosphate (AAP), after the laser treatment, following the evidence that antioxidant supplementation slows OA progression [313, 314]. In a preliminary investigation (Figure 6.11) we observed that lasing bovine cartilage bathed in PBS + 1M AAP produced \(E_Y = 0.37 \pm 0.19\) MPa, comparable to untreated cartilage (Figure 6.5) and significantly lower (\(p = 0.04\)) than samples treated in PBS bath only, \(E_Y = 0.64 \pm 0.20\) MPa (\(n = 4\) per group). Therefore, AAP appears to be capable of inhibiting ROS activity even during laser treatment, and may be applied to the future work to prevent any possible damaged by over-induced ROS.
Currently there are no available treatments that would effectively slow down or stop progression of OA. Existing options are limited to pain management or, at the late stage of the disease, highly invasive partial or full joint replacement. Therefore there is a need for a treatment option that would slow down or in best case scenario stop progression of OA. The proposed modality offers promise that such a therapy can be achieved with laser induced crosslinking, which this study has shown to improve mechanical properties and wear resistance of OA afflicted
articular cartilage. If successful, the proposed treatment could be used together with other options to either delay or entirely eliminate need for a joint replacement.
Chapter 7: Conclusion and Future Work

In conclusion, a novel, non-invasive, quantitative modality based on Raman spectroscopy that has potential to quantify changes in biochemical composition of collagen network in collagenous tissue was studied. Initially a two-dimensional collagen type I model was developed and the proposed characterization applied onto it to assess changes in crosslink density as a function of dosage and time exposure to glutaraldehyde, a known crosslinker. In the next level of approximation, a more complex model, proteoglycan depleted immature bovine articular cartilage, was employed to see whether changes in crosslink density in collagen type II fiber matrix can be characterized. The study also included preliminary findings on the kinetics of the glutaraldehyde crosslinking process. The future work will include transfer of the proposed model onto native bovine and human tissue, as well as to investigate whether the modality can assess changes of extracellular matrix associated with progressive diseases, such as osteoarthritis. Finally, the kinetics of the crosslinking process will be studied in detail.

A novel, femtosecond laser-based collagen crosslinking method was proposed and applied on corneal tissue reshape the corneal curvature. The treatment modality was then extended to articular cartilage tissue for the treatment of early osteoarthritis.

The *ex vivo* and *in vivo* live animal studies suggested that ultrafast laser-based crosslinking of corneas has a potential to be utilized as a non-invasive treatment for the permanent vision correction. The proposed modality relies on a innovative mode of laser–cornea interaction in which both, thermal ablation and optical breakdown are avoided. Further, treatment modality has been also applied on devitalized and *in vitro* live cultured immature bovine cartilage explants, as well as human cadaver cartilage. The mechanical properties and wear resistance of the articular cartilage were significantly improved, with minimal adverse effects. Although more research is
needed to fully understand the nature of the laser-tissue interaction, and the extent of different parameters in the treatment envelope, the results obtained thus far suggest that the proposed treatment, in tandem with other options, could potentially delay or, hopefully eliminate need for a joint replacement of progressive osteoarthritis.


98. Wander, J. and S. Vukelic. *Influence of tissue treatment onto the Raman spectra obtained from prostate histopathological slides for diagnostic purposes.* in Photonic Therapeutics and Diagnostics IX. 2013. International Society for Optics and Photonics.


Appendix: List of Publications under Candidature

Full-length publications


Conference podium presentations


**Conference posters**

