Management of Early Progressive Corneal Ectasia

Accelerated Crosslinking Principles

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To Paola, Martina, Giulia and Lucia and to all Authors' Families

Preface

For the last 10 years the advent of Riboflavin-UV-A-induced crosslinking (CXL) has drastically modified the natural history of Keratoconus and secondary corneal ectasia therapy. The number of corneal transplants for Keratoconus has notably reduced over a 30% drop in the last 5 years. Potentially correct and early use of CXL, at an early stage, can lead to its annulment in the next 5–10 years with notable savings for the health care system, of expenses for patients, and above all, radically improving patient quality of life which for years had been the "*sword of Damocles*" of surgery. This ambitious goal is possible if ophthalmologists pay ever more attention to timely diagnosis and early treatment, especially in the paediatric age. Early identification of Keratoconus is the fundamental stage in this "*ideal path*". The Scheimpflug cameras and new partial optical coherence light-based (OCT) tomographers available in the clinical practice allow us to "*surprise*" the illness at its onset. One can, at this point, hypothesize a treatment that immediately stabilizes the pathological process, impeding its evolution, and most importantly stabilizing the cornea when the refractive defect is still modest and correctable, without difficulties with spectacles or contact lenses. Over 10 years have passed since the effective introduction of corneal CXL therapy, and the Dresden protocol remains the most important point of reference because it is the most studied and the most used, as well as the protocol with the most valued clinical results and longest follow-up. The fields of application have rapidly stretched from keratoconus to iatrogenic secondary ectasias, especially post-Lasik, with excellent results and more recently extended to the treatment of therapy-resistant infectious keratitis. Nonetheless, the conventional CXL (C-CXL) protocol has shown some limits: the excessive duration (1 h), the need to remove the epithelium, post-operative pain, risk of infection, haze development (stromal wound healing complications), endothelial risks for thin corneas and long waiting lists. Recently, thanks to the principles articulated in the Bunsen-Roscoe law, accelerated crosslinking treatments (A-CXL) have emerged with the objective of shortening the procedure, maintaining efficiency. Some of these proposals are proving to be valid alternatives of the conventional CXL protocol, while other techniques such as super-fast A-CXL, trans-epithelial and iontophoresis-assisted CXL (I-CXL) have shown notable limits and are thus to be modified and evaluated in the long term, large number of cases and different age groups. Another challenging chapter is the attempt to combine treatments of improving the aberrometric and refractive defect induced by Keratoconus. This is a dream for all patients

who are intolerant of contact lenses: stopping Keratoconus progression and simultaneously improving the visual acuity without the necessity of resorting to corneal transplants. The so-called "*Crosslinking plus*" is already a reality for selected cases, reserved for patients with scarce visual acuity and intolerant to contact lenses, who are usually candidates for lamellar keratoplasty. The existence of clinical and instrumental parameters permits these people to attempt a stabilizing approach associated with contemporary refractive empowerment or postponement before thinking about replacement surgery. The ball is rolling, but adjustments are necessary for patient ease and repetitive, satisfying results. Crosslinking is a therapy that has changed the story of Keratoconus all over the world, and it is in continuous evolution. Accelerated CXL procedures illustrated in the book will be the leading part of the future crosslinking revolution.

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Principles of Accelerated Corneal Collagen Cross-Linking

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1.1 Introduction

Prior to the advent of the corneal collagen cross-linking procedure, no conservative treatment for corneal ectasia existed, with 20% of keratoconus patients progressing to eventually require penetrating keratoplasty [1].

Collagen cross-linking in the cornea as a treatment for ectasia was the breakthrough of Theo Seiler, MD, PhD, a professor of ophthalmology at Dresden Technical University, Germany at the time of the discovery. As he has told the story many times, Professor Seiler had his moment of inspiration in a dental chair, when he learned that UV light is used to harden dental fillings through the induction of cross-links. If the natural increase in non-enzymatic cross-linking that occurred with age led to increased corneal strength, could a physical means of inducing cross-linking be used to stabilize keratoconus? This initiated his first investigation to determine "*whether the elastic modulus of corneal tissue can be increased by cross-links of collagen fibrils induces by UV irradiation of the cornea*" [2].

Cross-linking was developed as a method of stabilizing the structurally weak corneas of patients with keratoconus, and has not only revolutionized the treatment of ectatic disorders, but has also become a platform technology including accelerated crosslinking (ACXL) with numerous additional clinical applications.

1.2 Corneal Structure and Ectasia Pathophysiology

The structure of the cornea, interacting with the forces applied to it (predominantly, intra-ocular pressure and gravity) help define the shape of the cornea. Thus, the cornea can maintain a reasonably constant shape and corneal curvature due to the tensile strength of the stromal collagen fibrils. The orderly structure of the cornea, and particularly the orientation of the stromal collagen fibrils, enables light to pass through it with minimal disruption or scatter [3–6].

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The corneal stroma is composed mainly of collagen, proteoglycans and water. The lamellar organization of the collagen fibrils which make up the corneal stroma is the primary source of the biomechanical strength of the cornea, and is regulated by interaction with proteoglycans [7]. The precise arrangement of the stromal collagen lamellae is critical for maintaining ocular transparency and is thought to be responsible for the corneal shape [7, 8]. Stromal collagen lamellae run in bands across the cornea in a limbus to limbus orientation. Deeper lamellae tend to run parallel to one another, whereas anterior lamellae are more intertwined and insert into the anterior limiting lamina (Bowman's membrane) [7, 9].

Individual stromal collagen fibrils are composed of units of tropocollagen, each containing three protein chains wound together in a helical pattern [10]. Each triple helical tropocollagen molecule is nominally 300 nm long, 1.5 nm in diameter, and held together by interpeptide hydrogen bonds [11–13]. Multiple tropocollagen units are assembled into microfibrils that are natively cross-linked to form the individual collagen fibrils [11, 12]. In the normal eye, the corneal collagen fibrils lie in orthogonal sheets that are parallel to each other and to the plane of the cornea, forming about 200 lamellae and comprising the extracellular matrix of the corneal stroma [14]. The collagen lamellae also exist in two preferred orthogonal orientations, with collagen preferentially aligned uniformly in the vertical and horizontal medians throughout most of the central 7 mm of the cornea [15].

There are regional differences in the arrangement of the collagen lamellae. In the anterior one third of the stroma, collagen lamellae are thin (about 0.2–1.2 μm thick and 0.5–30 μm wide), run obliquely to the corneal surface, and sometimes split into two to three sublayers that branch and become interwoven [16]. In the posterior stroma, collagen lamellae tend to be arranged parallel to the surface and are thicker (about 1.0–2.5 μm thick and 100–250 μm wide) [16]. Additional anchoring collagen lamellae in the periphery also contribute to the increased peripheral thickness. In the periphery, the collagen fibrils weave into the limbal collagen imparting considerable biomechanical strength [15, 17] (Fig. 1.1).

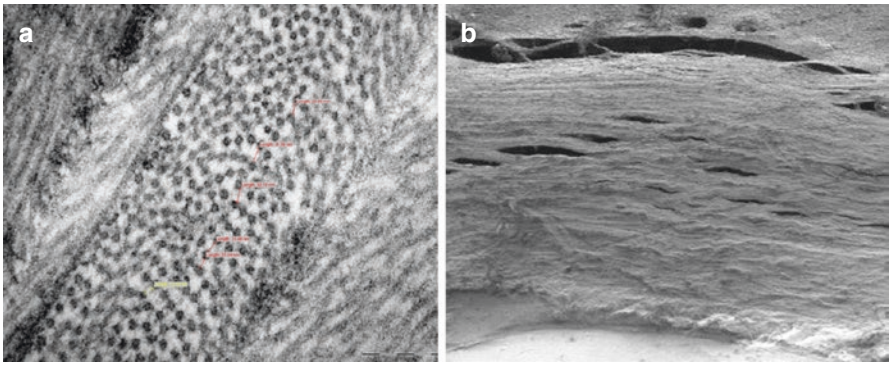

Fig. 1.1 Photomicrograph of collagen fibrils uranyl acetate-lead citrate 89.000X. Orthogonal arrangement of corneal collagen lamellae in the anterior stiff stroma (**a**). Parallel lamellar arrangement of the posterior (weak) stroma (**b**). TEM Philips EM 208S ultra-thin sections photomycrograph

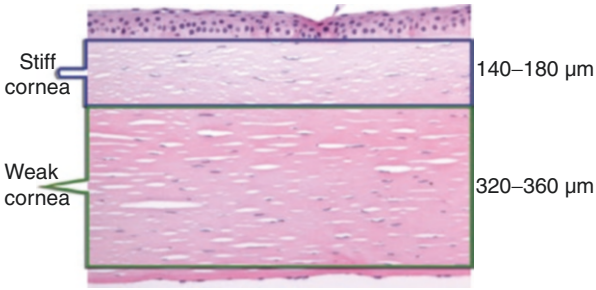

Fig. 1.2 Corneal lamellar organization divide the stroma into a superior biomechanically resistant portion (stiff cornea) and an intermediate-deep weak portion beyond 180 μm

Unlike other collagen connective tissues, the corneal collagen fibrils must maintain uniform spacing to allow light to pass with minimal scattering [3]. The spacing is influenced by proteoglycans, which are protein molecules from which glycosaminoglycan molecules extend in a bristle-like fashion [18].

The cornea's mechanical strength is primarily derived from Bowman's membrane and the anterior third of the corneal stroma because the fibers in these layers are the most interwoven making its biomechanical properties decidedly stiffer [7, 9] (Fig. 1.2).

1.2.1 Keratoconic Microstructure

The term corneal ectasia encompasses a group of disorders that share a common characteristic: progressive stromal thinning and loss of structural integrity resulting in corneal shape change, most typically inferior steepening [19, 20]. This corneal biomechanical weakness may be congenital, as in the case of keratoconus or pellucid marginal degeneration, or iatrogenic, as in the case of post-LASIK ectasia. The corneal thinning that occurs in keratoconic tissue appears to be due to the redistribution of the collagen fibrils [20]. In a normal cornea, approximately two thirds of the lamellae are oriented in a 45° sector around the vertical and horizontal meridian, with the remaining third oriented in the oblique sector between, Fig. 1.1 [21, 22]. This orthogonal arrangement is found to be absent over the area of an apical scar in keratoconus [21]. Meek et al. has demonstrated that a breakdown of cohesive strength of collagen-proteoglycan links leads to shearing between, and possibly within, stromal collagen lamellae in corneas with keratoconus [19]. Shearing between lamellae results in a phenomenon known as "creep," whereby lamellar sliding and collagen reorganization are thought to result in corneal shape change and resulting ectasia [7] (Fig. 1.3).

More recently, Roberts and Dupps [23] proposed that the earliest initiating changes for keratoconus occur in the stroma's biomechanical properties leading to clinical disease progression. They postulated that the initial biomechanical modification is focal in nature, rather than a uniform global weakening, and that the focal reduction in elastic modulus precipitates a cycle of biomechanical decompensation. Asymmetry in these biomechanical properties initiate a repeating cycle of increased strain, stress redistribution, and subsequent focal steepening and

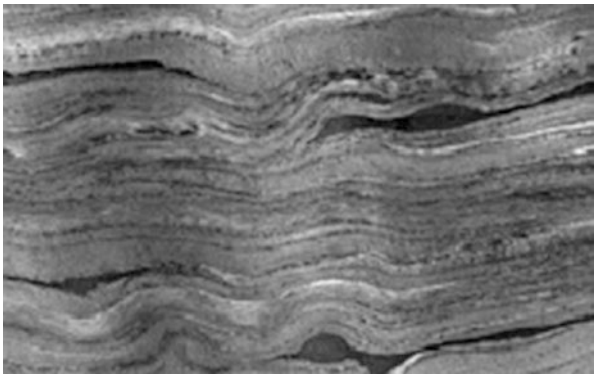

Fig. 1.3 Photomicrograph of collagen fibrils uranyl acetate-lead citrate 89.000X. Shearing between lamellae results in a phenomenon known as "creep," whereby lamellar sliding and collagen reorganization are thought to result in corneal shape change and resulting ectasia

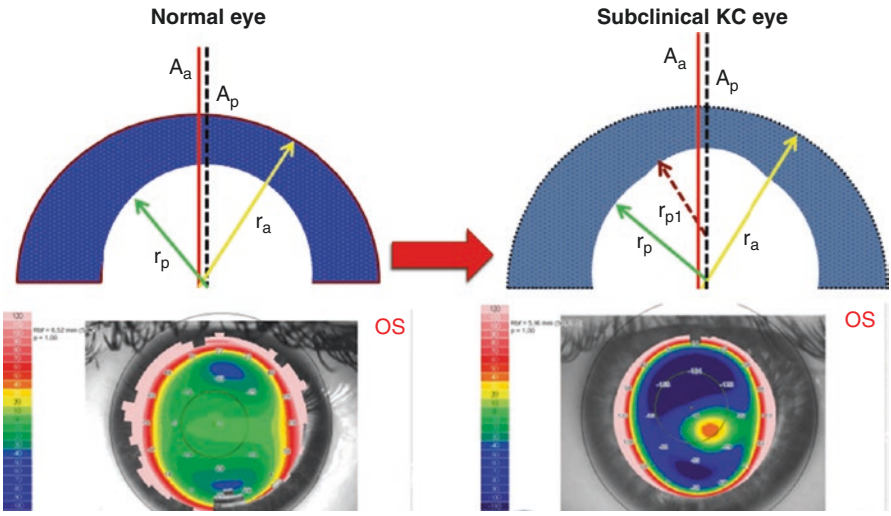

Fig. 1.4 The initial biomechanical modification driving to ectasia is focal in nature, rather than a uniform global weakening, and the focal reduction in elastic modulus generally starts in the infero-temporal region of the posterior corneal surface, precipitating a cycle of biomechanical decompensation

thinning. This is supported by Roy and Dupps [24] who showed through finite element analysis that steepening of Kmax is driven by elastic weakening. Scarcelli et al. utilizing Brillouin Microscopy verified both ex-vivo [25] and in-vivo [26] that the mechanical loss is primarily concentrated within the area of the keratoconic cone and that outside the cone, the mechanical properties of the stroma are comparable with that of healthy corneas. The causes of keratoconus and focal weakening remain unanswered but theories include genetic predisposition and eye-rubbing [23, 27–29] (Fig. 1.4).

1.2.2 Corneal Cross-Links

Keratoconus stabilizes with age [30]. Diagnosis and most rapid progression generally begin in puberty and continue throughout adolescence, arresting in the third or fourth decade of life. The stabilization of keratoconus with age [30], as well as the reduced incidence of post-LASIK ectasia in older patients [31] has provided indirect clinical evidence that the biomechanical properties of the human cornea change over time, which has been supported by laboratory research. Knox Cartwright, et al. describe a twofold increase in corneal stiffness between the ages of 20 and 100 years, demonstrated by a linear decrease in apical cornea displacement in response to change in intraocular pressure equivalent to that which occurs during the cardiac cycle [32].

Age-related increases in corneal stiffness have been attributed to the glycation induced cross-linking that occurs between or within stromal collagen lamellae as a result of the accumulation of non-enzymatic glycation end products over time [33, 34]. Similarly, it has been demonstrated that subjects with diabetes mellitus are significantly less susceptible to the development of keratoconus than non-diabetic subjects [35], attributed to greater corneal cross-linking induced through glycosylation and lysyl oxidase enzymatic activity resulting in greater corneal strength.

Corneal crosslinking using UVA light in combination with riboflavin induces extra covalent bonds. Exactly what molecules and where covalent bond formation is occurring in the stromal collagen microstructure is still not exactly known [19]. A thorough description of possible crosslinking scenarios is discussed by Hayes et al. [19]. The model describes the collagen fibrils, collagen molecules, and the bristle like hairs of the proteoglycan core proteins with their glycoaminoglycans. The possible sites include: (a) Within molecules (intramolecular), (b) Collagen molecular-collagen molecule at fibril surface (intermolecular), (c) Collagen molecule-collagen molecule at fibril surface (intermolecular), (d) Direct collagen fibril-collagen fibril (interfibrillar), (e) Proteoglycan-collagen molecule (fibril surface), (f) Within proteoglycan core proteins (intraproteoglycan), and (g) Proteoglycan core protein-proteoglycan core protein (interproteoglycan) [19]. They suggested that the covalent bonds formed during crosslinking occur predominately at the collagen fibril surface and in the protein network surrounding the collagen thereby stiffening the extracellular matrix. A simple analogy of this process would be likened to sugar coating a floppy piece of string in a glass of sugar water, the sugar coating causing the string to stiffen. They also concluded that "cross-links prevent the usual shrinkage associated with tissue dehydration during electron microscopy processing (which) may misleadingly appear larger in diameter than those in untreated corneas."

Exactly which molecules which are creating covalent crosslink bonds is still being investigated. McCall et al. [36] showed that carbonyl-based reactions dominated with minimal role of free amino groups for RFUVA cross-linking in the corneal stroma and suggested that these reactions may involve "histidine, hydroxyproline, hydroxylysine, tyrosine, and threonine." Brummer [37] discussed

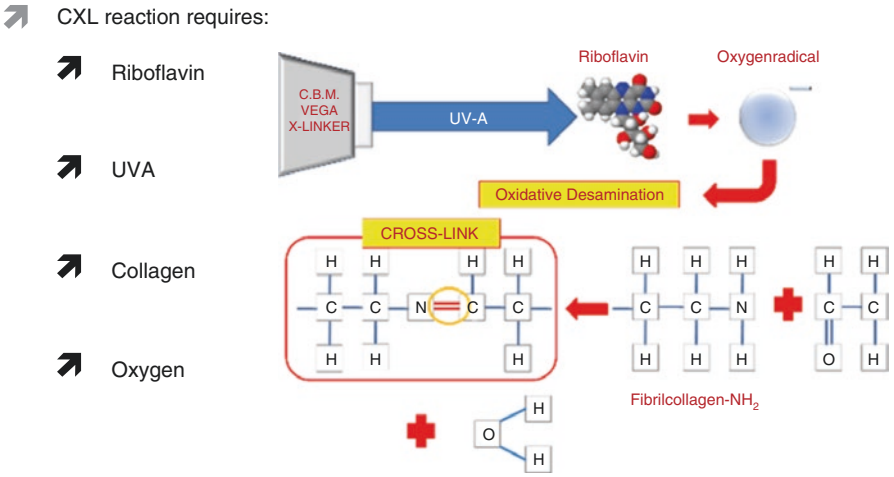

Fig. 1.5 Corneal crosslinking using UVA light in combination with riboflavin induces extra covalent bonds between and within collagen fibres and between collagen and proteoglycans

how these carbonyl-based reactions and advance glycation end products (AGEs) are involved in crosslinking formation and are similar to glycation in aging, diabetes, and cigarette smoking. Additionally, Zhang et al. [38] showed RFUVA causes crosslinking of collagen molecules among themselves and proteoglycan core proteins among themselves, together with limited linkages between collagen and keratocan, lumican, mimecan, and decorin. A table summarizing Zhang's results may be found in Meek and Hayes [39] (Fig. 1.5).

1.2.3 Standard Crosslinking and the "Dresden Protocol"

"We would emphasize that the in vitro-experiments reported here are intended to the first step in the direction of a conservative treatment of keratectasia" [1]. In this seminal paper, Spoerl, Huhle and Seiler performed the first series of experiments to determine whether the induction of crosslinks stiffened the cornea with riboflavin. They compared photochemical crosslinking with riboflavin using 365 and 436 nm light, 254 nm UV light only, sunlight and compared these with known chemical crosslinkers of glutaraldehyde and Karnovsky's solution utilizing uniaxial extensiometry. Their results demonstrated that corneal crosslinking and stiffening of the cornea was achievable with riboflavin in the presence of UVA light.

The group then investigated and developed many aspects of the science underlying corneal crosslinking. They began the search by measuring the dose response to various stiffening techniques of the cornea [40]. This was followed by a series of other studies which looked at keratocyte cytotoxicity [41], endothelial cytotoxicity in-vitro [42], endothelial cell damage in-vivo [43], stress-strain measurements

of human and porcine corneas [44], collagen fiber diameter [45], thermomechanical Behavior [46], and resistance to enzymatic digestion [47] fully characterizing the safety and developing many of the methods for measuring crosslinking utilized today.

In 2003, Wollensak et al. [48] presented the results of the first clinical study of the use of riboflavin-5-phosphate and UVA light cross-linking as a treatment for progressive keratoconus. Twenty-three eyes of twenty-two patients with confirmed progressive keratoconus were treated in a prospective, non-randomized pilot study, with follow-up ranging from 3 to 47 months. The epithelium was mechanically debrided from the central 7 mm of the cornea of treated eyes. A solution of 0.1% riboflavin-5-phosphate and 20% dextran T-500 was applied 5 min before irradiation with 370 nm UV light, and every 5 min during irradiation. Irradiation was delivered using two UV diodes calibrated to an irradiance of 3 mW/cm^2 for 30 min, corresponding to a dose of 5.4 J/cm^2. In all cases, progression was stopped, as measured by change in maximum keratometry value (Kmax). In 16 of 22 eyes, reduction of Kmax was observed, with a mean flattening of 2.01 and of the refractive error of 1.14 diopters. In this first study, transient stromal edema was noted until re-epithelialization. No other complications were reported, and endothelial cell density remained unchanged from baseline.

The Dresden group achieved their 1998 goal as there have been numerous clinical studies utilizing the Dresden protocol (*See* Sect. 2.1) demonstrating stabilization of the cornea or continued flattening of Kmax with little to no long term side effects for the "conservative treatment of keratectasia". For over a decade it has been considered the standard of care for keratoconus and post Lasik ectasia across the world and was recently approved in the United States for both procedures [49].

The now more standardize "Dresden Protocol" has the main elements of de-epithelization of the corneal epithelium, a 30 min of pre-soak with 0.1% riboflavin-5-phosphate and 20% dextran T-500, followed by 30 min of 365 nm UVA light treatment at 3 mW/cm^2 for a total dose of 5.4 J/cm^2.

1.3 Accelerated Crosslinking, Photochemical Kinetic Principles

Although very effective, the Dresden protocol is a lengthy procedure and requires removal of the epithelium. In an effort to improve upon the Dresden protocol, newer technologies have emerged addressing these issues making the procedure more comfortable for patients and cost effective for hospitals [50]. The first of these technologies is accelerated crosslinking.

Accelerated cross-linking protocols follow one of the fundamental laws of photochemistry called the Bunsen-Roscoe Law of Reciprocity [51]. This law states that the photochemical biological effect is proportional to the total energy dose delivered regardless of the applied irradiance and time. Irradiance refers to the power per area delivered to the surface of the cornea. It is given in units of W/cm^2. The energy

per area delivered to the surface of the cornea is the dose. Dose is given in units of J/cm^2. Irradiance and dose are related by the following equation:

$$Irradiance\left(Watts/cm^2\right)\times Time\left(s\right)=Dose\left(J/cm^2\right)$$

The Dresden protocol uses 3 mW/cm^2 irradiation over 30 min, which delivers a 5.4 J/cm^2 dose.

$$\left(0.003W/cm^2\right)\times\left(1800\,s\right)=5.4\,J/cm^2$$

To deliver an equivalent energy dose, an accelerated protocol using 30 mW/cm^2 irradiance requires only 3 min to achieve the same dose of 5.4 J/cm^2 obtained in 30 min of irradiation with 3 mW/cm^2 irradiance.

$$\left(0.030W/cm^2\right)\times\left(180\,s\right)=5.4\,J/cm^2$$

Other accelerated protocols may be derived using this equation, and have been employed clinically up to 45 mW/cm^2 irradiation.

The Bunsen-Roscoe reciprocity law may hold if all other parameters are controlled. However, in the case of corneal cross-linking, there are many other factors beyond the UVA dose that contribute to the total amount and 3-dimensional distribution of cross-linking obtained in the cornea. Factors related to the clinical procedure include the beam profile and illumination pattern of the UVA delivery device, the concentration and diffusion rate of the formulation of the riboflavin used, the length of the riboflavin presoak time, the viscosity of the riboflavin film, as well as the presence and concentration of oxygen in the stromal tissue. Individual patient variability, including corneal structure and baseline corneal biomechanics may also influence the outcome of the procedure.

The first reported laboratory investigation and clinical use of accelerated crosslinking for bullous keratopathy was by Krueger [52] utilizing 15 mW/cm^2 for 7 min and a staged intrastromal delivery riboflavin. Krueger also retrospectively reported the first laboratory investigation of accelerated crosslinking [53] using a 370 nm UVA source they crosslinked porcine globes with irradiances of 2, 3, 9, and 15 mW/cm^2 continuously and 15 mW/cm^2 fractionated (with alternate cycles of 30 s "ON" and 30 s "OFF" exposure) for a total dose of 5.4 mJ/cm^2 and performed extensiometry to measure increases in stiffness. Their results showed no statistically significant differences between standard and higher irradiances.

Since that initial study, accelerated crosslinking has been extensively studied utilizing various methods for understanding and quantifying corneal crosslinking.

1.3.1 Laboratory Methods for Quantifying Crosslinking

While there is currently no direct method for measuring the exact amount and distribution of cross-links that occur in tissue, there are a number of methods available to indicate the relative efficacy of cross-linking procedures. These measures include theoretical modeling of the cross-linking process, mechanical techniques, such as

extensiometry or inflation testing commonly used as measures of the change in stress-strain behavior of the cornea, Interferometry, OCT, Brillouin microscopy, the measure of oxygen consumption, as well chemical assessment through enzymatic digestion techniques. This information has been used to indirectly describe changes in the biomechanical properties of the cornea.

1.3.1.1 Interferometry

Interferometry is a family of optical techniques which uses the addition and subtraction of combined light waves to assess the properties of an object. It has been used to measure the biomechanical properties of the cornea using electronic speckle interferometry (ESPI) [32, 54] and low coherence optical tomography [55].

1.3.1.2 Stress-Strain Techniques: Extensiometry and Inflation Testing

Young's modulus, measured in Pascal, describes the resistance of a material to change in length, and is defined as the ratio of stress to strain. Stress refers to the force applied to a material per unit area, and strain refers to the change in length per unit of the original length. The Young's modulus of a material is a constant to a point of breakdown, after which the force applied exceeds the limit of proportionality and the material will no longer rebound from deformation.

The Young's modulus of a material may be obtained through tensile testing of the material, by applying a known force and measuring change using extensiometry techniques. In corneal tissue, extensiometry may be performed using a uniaxial method with a long strip of tissue, or by using corneal flaps [2, 32, 56]. Since corneal tissue is radially oriented, uniaxial strip extensiometry is a less accurate measure than a biaxial extensiometry due to lamellar disruption in one axis, leaving half the collagen fibers in an unknown state. Biaxial extensiometry has the benefit of measuring the collagen fibers in a manner more closely related to the forces they see in-vivo [57].

Corneal tissue used in bi-axial extensiometry may be full thickness, or separated into flaps of known thicknesses. The anterior and posterior cornea have different biomechanical behaviors, and different cross-linking techniques may create deeper or shallower cross-linking, it is therefore important that the thickness of the flap used in extensiometry be known. Commercially available biaxial extensometers use biorake attachments that allow for minimal and repeatable handling of the tissue. Each sample is lowered into a temperature controlled bath and stretched at a constant rate (μm/s) until sample failure.

Inflation testing is another method of obtaining the biomechanical properties of the cornea. An advantage to inflation testing is that the force is applied radially, which more closely mimics physiological conditions [58]. Inflation methods using whole globes were first used to test for ocular rigidity [59–64]. In other inflation studies, only the cornea with varying amounts of scleral ring are mounted to pressure chambers in a variety of configurations to allow their measurement [32, 54, 58]. As with extensiometry, it is critical that temperature and hydration are controlled as these affect the tissue biomechanics and the reproducibility of the results.

1.3.1.3 Tissue Preparation

In-vitro cross-linking is usually performed using human or various animal whole globes. Preconditioning of the globes is a first and necessary step in obtaining repeatable laboratory so tissue hydration is stabilized. Additionally, as corneal crosslinking is a photochemical process, variations in temperature can change the dynamics of the reaction and impacts the amount of cross-linking and the subsequent mechanical response of the tissue [65]. Therefore maintenance of physiologic temperature and hydration of the globes during experimental cross-linking is recommended in addition to maintaining the globes intraocular pressure. This helps to ensure the precision and accuracy of the experimental measurement.

1.3.1.4 Oxygen Monitoring

The use of oxygen monitoring may also function as a proxy for understanding the chemical kinetic mechanisms of corneal cross-linking [66]. In this method, oxygen levels are measured using an O_2 sensor. Drops of riboflavin solution are instilled onto corneas which are then exposed to 365 nm UVA under varying irradiance and temperature. Oxygen concentration in the cornea at a known depth is monitored during UVA illumination. The oxygen dynamics are then used to gather insight into the mechanisms of corneal cross-linking.

1.3.1.5 Chemical Digestion

The use of chemical digestion with enzymes for measuring corneal cross-linking was first performed by Spoerl et al. [47]. In this experiment, porcine corneas were irradiated at three different doses. The corneas were then trephined and allowed to digest in different enzymes. The resistance to enzymatic degradation was measured as a function of time, demonstrating that increased corneal cross-linking caused increased resistance to digestion. Enzymatic digestion has also been utilized to study corneal crosslinking several others [18, 67, 68].

Another enzymatic digestion method utilizes the enzyme papain in combination with spectrofluorometer analysis as a means to quantify the amount of cross-linking in porcine corneal flaps that have undergone various UVA-riboflavin based corneal cross-linking protocols [69]. This method ensures that the same amount of tissue is utilized minimizing corneal thickness variation often seen in uniaxial testing.

In this method, porcine globes are preconditioned as previously described and treated under various cross-linking protocols. Using a femtosecond laser, corneal flaps of various thicknesses are excised after cross-linking. The thicknesses of the corneal flaps are confirmed using ultrasonic pachymetry. To prepare for digestion, the corneal flaps are washed with distilled water 15 times to remove residual riboflavin, and dried in a vacuum until the weight change becomes less than 10%. Each flap is digested for 2.5 h at 65 °C with 2.5 units/mL of papain (from Papaya latex, Sigma) in 1 mL of papain buffer [1× PBS (pH 7.4), 2 mM L-cysteine and 2 mM EDTA]. Papain digests are centrifuged for 20 s at 2200 × G (Mini centrifuge 05-090-100, Fisher Scientific) and diluted 0.5 times with 1× PBS solution.

Fluorescence of the of the corneal flap digested solutions is measured with excitation of $\lambda ex = 360$ nm in a QM-40 Spectrofluorometer (Photon Technology Int., London, Ontario, Canada) with the emission fluorescence measured between 375

and 650 nm. Fluorescence of remaining riboflavin is subtracted out of the recorded fluorescence and normalized to untreated controls. The fluorescence at 450 nm is indicative of the amount of cross-linking in the corneal flap [70]. Using this technique, statistically significant results ($P < .05$) are observable for small variations in protocol. One significant advantage of this method is that multiple flaps can be excised from one cornea to analyze the amount of cross-linking at different depths.

1.3.2 Photochemical Kinetics of Corneal Crosslinking with Riboflavin and UVA Light

There is a large body of literature starting in the 1950s and 1960s, but especially from the 1990s and early 2000s with regard to its photochemical kinetic mechanisms of riboflavin chemistry. The initial hypothesis of the mechanism of UVA and riboflavin for mediated corneal cross-linking was that exposure of riboflavin to UVA light in an oxygenated environment causes the formation of singlet oxygen, which then acts on tissue to produce additional cross linked bonds [35, 71]. Current understanding suggests that the photochemical kinetic mechanisms involve both Type I and Type II photochemical kinetic mechanisms.

Much of this literature through analysis of the photochemical kinetics chemistry, observed tissue oxygen concentration and resultant cross-linking under varied treatment conditions suggests that ROS and especially the type I electron transfer kinetic mechanism are mostly responsible for creating crosslinks within the cornea and that reactive oxygen species (ROS) act as the predominant agent [66, 72, 73].

Several families of reactions underlie riboflavin cross-linking chemistry. Some of these reactions are UV mediated while others occur without UV light. Among these reaction paths, some mechanisms enhance the production of radical species that promote cross-linking, while others degrade riboflavin without creating crosslinks. To optimize the clinical cross-linking process, it is important to understand these reactions, how they relate to one another and to environmental conditions.

Some of the major kinetic reactions involved in Type I and Type II mechanisms derived from the literature are shown in Fig. 1.6 [66, 72–85]. In the presence of light, riboflavin can exhibit photosensitizing properties reacting with a wide range of electron donating tissue sites (such as amines or amino acids) [35, 70, 76, 85, 86] through mixed Type I – Type II photochemical mechanisms [81].

The reaction diagram in Fig. 1.7 generally illustrates these major reactions and their interactions with each other. In the Type I mechanism, the sensitizer excited state generates radicals or radical ions, predominately ROS, which react with the tissue through hydrogen atoms or electron transfer. For the Type I crosslinking mechanism the first pathway in which radical Rf on its own creates crosslinks is a very minor contributor amount of crosslinks [72, 73]. The second pathway in which ROS and predominately hydroxyl radical create the majority of crosslinks is the major contributor to the process [72, 73]. In the Type II mechanism, the excited sensitizer reacts with oxygen to form singlet molecular oxygen. This energy transfer process although present has only a minor impact on the total amount of crosslinking produced during UV treatment [72, 73].