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Biomechanics of Ophthalmic Crosslinking

Brecken J. Blackburn[1], Andrew M. Rollins[1], and William J. Dupps, Jr.[1–4]

[1] Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH, USA
[2] Cole Eye Institute, Cleveland Clinic, Cleveland, OH, USA
[3] Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH, USA
[4] Department of Biomedical Engineering, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

Correspondence: William J. Dupps, Jr., 9500 Euclid Ave/i-32, Cleveland, OH 44195, USA.
e-mail: bjdupps@outlook.com

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Crosslinking involves the formation of bonds between polymer chains, such as proteins. In biological tissues, these bonds tend to stiffen the tissue, making it more resistant to mechanical degradation and deformation. In ophthalmology, the crosslinking phenomenon is being increasingly harnessed and explored as a treatment strategy for treating corneal ectasias, keratitis, degenerative myopia, and glaucoma. This review surveys the multitude of exogenous crosslinking strategies reported in the literature, both "light" (involving light energy) and "dark" (involving non-photic chemical processes), and explores their mechanisms, cytotoxicity, and stage of translational development. The spectrum of ophthalmic applications described in the literature is then discussed, with particular attention to proposed therapeutic mechanisms in the cornea and sclera. The mechanical effects of crosslinking are then discussed in the context of their proposed site and scale of action. Biomechanical characterization of the crosslinking effect is needed to more thoroughly address knowledge gaps in this area, and a review of reported methods for biomechanical characterization is presented with an attempt to assess the sensitivity of each method to crosslinking-mediated changes using data from the experimental and clinical literature. Biomechanical measurement methods differ in spatial resolution, mechanical sensitivity, suitability for detecting crosslinking subtypes, and translational readiness and are central to the effort to understand the mechanistic link between crosslinking methods and clinical outcomes of candidate therapies. Data on differences in the biomechanical effect of different crosslinking protocols and their correspondence to clinical outcomes are reviewed, and strategies for leveraging measurement advances predicting clinical outcomes of crosslinking procedures are discussed.
Advancing the understanding of ophthalmic crosslinking, its biomechanical underpinnings, and its applications supports the development of next-generation crosslinking procedures that optimize therapeutic effect while reducing complications.

Introduction

Crosslinking refers broadly to the formation of bonds between polymer chains, such as proteins. In biological tissues, these bonds, in most cases, tend to stiffen the tissue composed of these proteins, making it more rigid and resistant to deformation. In ophthalmology, the crosslinking phenomenon has been harnessed as a method of treatment to strengthen tissue to resist further biomechanical degradation and morphological changes (as in treating corneal ectasias or degenerative myopia). Crosslinking is often abbreviated as CXL in the case of corneal crosslinking and SXL in the case of scleral crosslinking.

This Review Aims to Summarize:

- The diversity of crosslinking methods applied to the cornea and sclera.
- The variety of analysis methods used to determine biomechanical changes in the corneal and sclera due to crosslinking.
- The biomechanical effects of various methods of crosslinking the cornea and sclera.

Variety of Ophthalmic Crosslinking Methods

Crosslinking can be thought of in two forms, endogenous and exogenous. Endogenous crosslinks are a natural part of tissue structure, maintenance, aging, or disease, and include lysyl oxidase-mediated crosslinking with age and advanced glycation end (AGE) product-mediated crosslinking in diabetes.[1] Endogenous crosslinking will not be discussed in this review. Exogenous crosslinks are intentionally induced in certain ophthalmic treatments, and we will summarize a variety of exogenous crosslinking methods described for ophthalmic applications.

We will consider ophthalmic applications of crosslinking in two groups: First, the "light" methods, which induce crosslinks through irradiation of the target tissue with light (photochemical crosslinking). This irradiation is paired with administration of a photosensitizing agent, which increases the efficiency of the crosslinking process by absorbing photons and indirectly transferring the resulting energy to crosslink formation. The second group includes the "dark" crosslinking methods, which are solely chemically induced and require no photoactivation.

"Light" Methods

"Light" (photochemical) methods of crosslinking rely on energy delivered in the form of light to induce crosslinks between adjacent molecules. The advantage of this method is that light can be focused in a selective manner so that only the region or tissue of interest is crosslinked.

UV-A / Riboflavin

The most commonly used method of ophthalmic crosslinking is UV-A / riboflavin crosslinking. U-VA / riboflavin crosslinking is most commonly used to crosslink the cornea (the application for which it is US Food and Drug Administration [FDA] approved), but may also be used on other ocular tissues. The most widely used protocol for cornea crosslinking, the Dresden protocol,[2] requires removal of the corneal epithelium, instillation of a 0.1% riboflavin / 20% dextran solution onto the surface of the cornea for 30 minutes, followed by 30 minutes of 370 nm UV-A irradiation at 3 mW/cm^2 with continued intermittent administration of riboflavin solution.

Although it has achieved widespread clinical adoption, modifications to the UV-A / riboflavin crosslinking protocol are under investigation. For instance, there is on-going development of protocols which do not require removal of the corneal epithelium (transepithelial crosslinking) for corneal crosslinking. The Dresden protocol calls for removal of the corneal epithelium prior to instillation of riboflavin. Removing the epithelium allows better penetration of the riboflavin, but it also causes patient discomfort, increases the risk of infection, and causes prolonged changes to function and morphology of the corneal nerves.[3] Less disruption to the corneal nerves is observed in transepithelial CXL procedures[4,5] and the risk of infection is reduced.

The most straightforward transepithelial method is to omit the epithelial debridement from the Dresden protocol and rely on the small amount of diffused riboflavin into the stroma to serve as photoactivator. However, most transepithelial protocols have reduced effectiveness compared to epithelium-off CXL.[6–9] Another method to address the issue is to partially perforate the corneal epithelium to minimize disruption while increasing the ability of riboflavin to cross into the stroma.[10] Other methods of investigation include chemically weakening the tight junctions in the epithelium,[11–14] iontophoresis across the cornea during riboflavin application,[15–17] creation of a intrastromal "pocket" in which to inject the riboflavin,[18] and the addition of solutions beyond riboflavin to enhance the riboflavin penetration.[19]

Adjacent to the matter of transepithelial riboflavin penetration, there has been discussion in the field on the time course of riboflavin application. First, characterizing or reducing duration of instillation needed for the riboflavin to sufficiently penetrate into the desired tissue (commonly, the sclera or corneal stroma) for optimal crosslinking is a question of continued interest.[20–22] Second, if riboflavin is instilled continuously (as called for in the Dresden protocol), a layer of riboflavin is left on top of the tissue surface, and may strongly absorb the UV-A rather than allowing for deeper penetration of the UV-A into the stroma. Washing the surface prior to UV-A exposure may allow for more complete crosslinking.[19]

A third area of investigation for improvement of the riboflavin / UV-A CXL method is reducing the duration of the UV-A irradiation. This is primarily motivated by concerns of patients' comfort and reducing overall procedure time. With 30 minutes of 3 mW/cm^2 370 nm light as a baseline (a total energy delivery of 5.4 J/cm^2), the use of higher intensity light (9–30 mW/cm^2) for shorter periods of time (3 to 10 minutes) has been widely investigated.[23–25] However, the kinetics of the crosslinking reaction are an important (and potentially limiting) factor in high intensity/short duration modifications. The crosslinking reaction

Table 1A. Photochemical Methods for Ophthalmic Crosslinking

Photosensitizer	Light	SXL	CXL	Radicals Generated	Cytotoxicity	Development Stage
Riboflavin	Ultraviolet (365 nm)	X	X	Singlet oxygen	Moderate	Clinically used
	Blue (460 nm)[31]	X		Singlet oxygen	Moderate[32,33]	Animal studies
Eosin Y[34]	Green (525 nm)		X	Singlet oxygen[35]	Low[35]	Ex vivo
Rose Bengal[36]	Green (560 nm)	X[37]	X[38]	Singlet oxygen and electron transfer[39]	Low	Limited human use,[40] Animal studies
Methylene blue[41]	Red (665 nm)	X		Singlet oxygen	Low	Animal studies
Water-soluble-taurine (WST)[42]	Near infrared (NIR) (755 nm)		X	Superoxide and hydroxyl radicals[43]	Low	Animal studies

can be broken down into two types: type 1 (oxygen independent) and type 2 (oxygen dependent). In Dresden UV-A riboflavin CXL, in the first 15 seconds of UV-A irradiation, photochemically generated reactive oxygen species drive the crosslinking process by oxidizing the proteoglycan core proteins and collagen of the stroma.[26] This is representative of a type II crosslinking reaction. After approximately 15 seconds, the endogenous oxygen is depleted and the crosslinking is driven by slower type I mechanisms, in which the energized riboflavin directly interacts with the proteoglycan and collagen molecules in the stroma.

During the 30-minute irradiation of Dresden protocol corneal crosslinking, oxygen continuously diffuses into the stroma (~400 um thick) and resupplies the oxygen within the stroma, allowing some type 2 reaction to continue occurring over 30 minutes. However, in accelerated protocols, the time for this oxygen replenishment is limited. Accordingly, it has generally been found that accelerated protocols (which rely on higher intensity light for shorter durations) result in more superficial stiffening of the stroma. Although short-term outcome studies have shown equal efficacy between Dresden CXL and many accelerated protocols in halting keratoconus, it is not known if the lesser penetration of the stiffening effect elevates the risk of late disease progression.

Several methods have been attempted to counteract this oxygen-limited superficial crosslinking result. One method is to perfuse the cornea with oxygen during UV-A irradiation, which has been shown to increase the stiffening effect of accelerated crosslinking.[27] Another method is to use higher intensity light, but pulse the light in a manner which allows oxygen to be replenished in between pulses.[28]

Other Photosensitizer Methods

Whereas generally hailed as a safe and effective treatment, riboflavin / UV-A crosslinking does have some drawbacks. Notably, the high-energy UV-A radiation presents phototoxicity concerns for long-duration tissue exposure, particularly for the cells of the corneal endothelium or retina.[29] For this reason, corneal thickness is an important consideration in riboflavin / UV-A crosslinking, as the bulk of the riboflavin-infused stroma will absorb the UV-A light effectively shielding the endothelial cells. If the cornea is too thin, shielding may not be sufficient and damage may be done to endothelial cells. For this reason, patients with thin corneas are not given Dresden riboflavin / UV-A crosslinking, and instead may be administered a modified CXL protocol.[30] Thus, other pairs of photosensitizers and excitation wavelengths have been explored for ophthalmic crosslinking.

These methods (see Table 1A) operate on a similar theory as riboflavin / UV-A crosslinking, but the photosensitizers may have different absorption wavelengths, different properties with respect to tissue penetration, and different energy dose requirements.

"Dark" Methods

Dark methods of crosslinking rely solely on crosslinking by a chemical agent with no photoactivation. The lack of photoactivation can be an advantage if the tissue targeted for crosslinking is adjacent to photosensitive tissues (for instance, the sclera is adjacent to the photosensitive retina). However, without photoinitiation, spatial selectivity is reliant on the diffusion of the crosslinking agent itself. This diffusion can be difficult to control and cause unintended

Table 1B. Chemical Methods for Ophthalmic Crosslinking

Crosslinker	CXL	SXL	Cytotoxicity	Development Stage	Other Notes
Glyceraldehyde	X[44]	X[45]	Moderately low[46]	Animal studies	
Genipin	X[47]	X[48]	Low	Animal studies	Several proposed crosslinking mechanisms of genipin have been described.[49] However, genipin may discolor tissue.[48]
Methylglyoxal		X[50]	Not studied, potentially high[50]	Ex vivo	
Aliphatic β-nitro alcohols	X[51]	X[52]	Low[53]	Animal studies	Nitrite-related crosslinking is a suspected cause of increased crosslinking with aging
Formaldehyde releasers	X[54]	X[54]	Low[54]	Ex vivo	Related to aliphatic β-nitro alcohols
Decoron	X[55]		Not studied, but likely low	Ex vivo	Decorin is one of the major types of proteoglycans in the corneal stroma, and naturally binds to collagen fibrils. Thus, introduction of additional decoron (the core protein of the decorin proteoglycan chain) to the cornea may serve to stabilize and organize collagen fibrils.[55]
Transglutaminases	X[56]	X[57]	Low[58]	Animal studies	It has been suggested that part of the crosslinking effect of riboflavin / UV-A crosslinking is that the natural lysyl oxidase pathway responsible for age-related corneal crosslinking is activated by the generation of free radicals during photochemical crosslinking. It was found that certain crosslinking enzymes, including transglutaminases, were increased after riboflavin / UV-A CXL, and proposed that these might be responsible for a stiffening effect through crosslinking of glutamine and lysine residues.

effects by crosslinking adjacent tissues. Examples of ophthalmic "dark" crosslinkers are shown in Table 1B.

Ophthalmic Targets for Crosslinking

Ophthalmic crosslinking has found a number of target applications in the eye. The only FDA-approved crosslinking treatment as of this writing is UV-A / riboflavin crosslinking for stabilization of progressive keratoconus or postrefractive surgery ectasia. However, several methods and applications have been trialed in research settings for a variety of ophthalmic conditions.

Cornea

The most common use of corneal crosslinking is to mechanically stabilize ectatic corneas (Fig. 1). Keratoconus is the most common cause of ectasia and many clinical studies have emphasized the effectiveness of corneal CXL to halt the progression of keratoconus.[59] In some cases, the stiffening induced by crosslinking even results in mild flattening of the cornea, effectively reducing the adverse morphological and optical effects of keratoconus to a small degree.[59] In another case of degenerative disease, CXL has been shown to be useful in the treatment of pellucid marginal degeneration.[60] CXL is similarly used to mechanically stabilize corneas in cases of postsurgical ectasia.[61]

Additionally, crosslinking has been explored as a preventative measure in refractive surgery – essentially reinforcing corneas which may otherwise be too thin or weak to safely receive refractive procedures.[62] In addition, CXL itself, if done in a spatially selective manner, can induce subtle and specific changes in cornea geometry, which can provide refractive correction.[63,64] Further, CXL has been shown to be effective in improving corneal clarity and comfort for patients with corneal edema secondary to endothelial dysfunction by imparting some resistance to stromal swelling.[65]

In a different class of procedures, CXL has been explored for its generation of reactive oxygen species, which may inhibit microbial infection. It has been

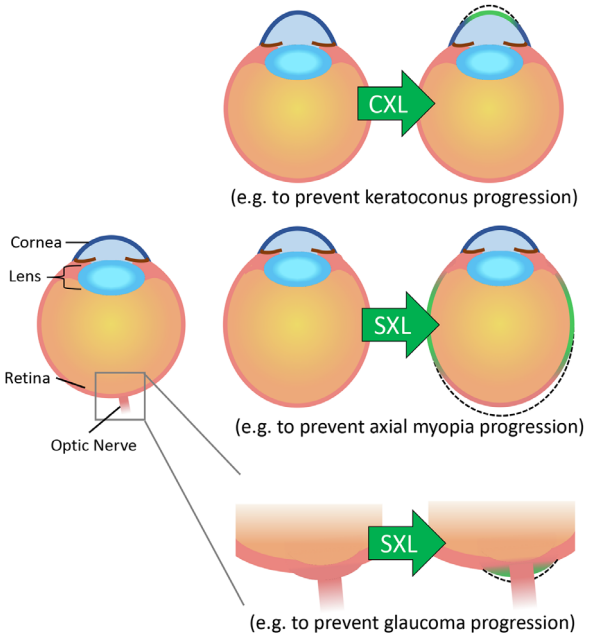

Figure 1. Overview of ophthalmic crosslinking for disease stabilization. Crosslinked regions of tissue are shown in *green*. *Dotted lines* indicate the progression of disease if crosslinking had not been applied. (*Top*) In corneal crosslinking (CXL) for keratoconus stabilization, the stiffening of the cornea prevents the progression of corneal steepening (dotted line). (*Middle*) In scleral crosslinking (SXL) for myopia stabilization, the stiffening of the sclera prevents further axial elongation of the globe (*dotted line*). (*Bottom*) In scleral crosslinking for glaucoma stabilization, the stiffening of the peripapillary sclera reduces strain on the lamina cribrosa and prevents further distention of the lamina cribrosa (*dotted line*).

shown that corneal CXL can be an effective alternative or adjunct to antibiotics in cases of infectious keratitis caused by bacteria, amoeba, and fungi.[66,67]

Sclera

Crosslinking has also been applied as a potential strategy for treating disease that involves the sclera. In the case of progressive myopia,[68] crosslinking of the sclera may halt further axial elongation of the eye and potentially decrease the risk of associated comorbidities, such as retinal tears and choroidal neovascularization related to thinning of the retina, choroid, and Bruch's membrane (Fig. 1).

Although glaucoma is known to be a multifactorial disease, crosslinking has been explored as a strategy to modify glaucoma risk. It is known that, in some fraction of glaucoma cases, there is a weakening of the supportive tissue surrounding the optic nerve head that may contribute to retinal ganglion cell axonal stress when it is excessively deformed at higher intraocular pressures (IOPs) or during wide diurnal fluctuations of IOP.[69,70] Spatially specific crosslinking, which targets this peripapillary scleral tissue, may help relieve strain on the optic nerve head and reduce the effects of IOP and intracranial pressure-related laminar deformations on the retinal ganglion cell axons that traverse this area en route to the brain (Fig. 1).[71] However, crosslinking of the sclera may worsen axonal transport obstruction and exacerbate glaucomatous changes in the optic nerve,[72] so additional work is needed to characterize the mechanical and biological effects of crosslinking in the optic nerve head region.

Measurement of Biomechanical Change Induced by Crosslinking

Many methods have been devised to assess the mechanical properties of ocular tissues. These methods report different mechanical parameters, many of which are changed by crosslink formation. Understanding what these different parameters mean is critical to interpreting the overarching body of work. See Figure 2 for an explanation of various measurement parameters.

Generally, two factors will affect the mechanical property change induced by crosslinking:

1) The total number of crosslinks (titratable through protocol modification).
2) The location of the crosslinks: both distribution throughout the stroma (i.e. anterior more crosslinked than posterior), and location of crosslinks in the collagen hierarchical structure (e.g. within helices, between the proteoglycan coatings, within fibrils).

Evidence from biomolecular studies suggests that not all crosslinking protocols form crosslinks in the same position. Even within photochemical crosslinking techniques, the crosslink position may differ. For example, studies suggest that riboflavin / UV-A and WST / NIR form crosslinks in different locations throughout the collagen hierarchical structure.[42,73] For nonphotochemical crosslinkers, like decoron or transglutaminases, different crosslinking mechanisms are suggested.[55,58]

Crosslinking does not modify all mechanical parameters in the same way. For instance, crosslinking may significantly increase tensile strength, while not increasing viscosity.[74] Further, different types of crosslinking may modify mechanical properties differently.[75] To understand why, it is necessarily to refer to the hierarchical microstructure of collage in the cornea (see Fig. 3). Individual collagen molecules form helical tropocollagen, which in turn make up microfibrils

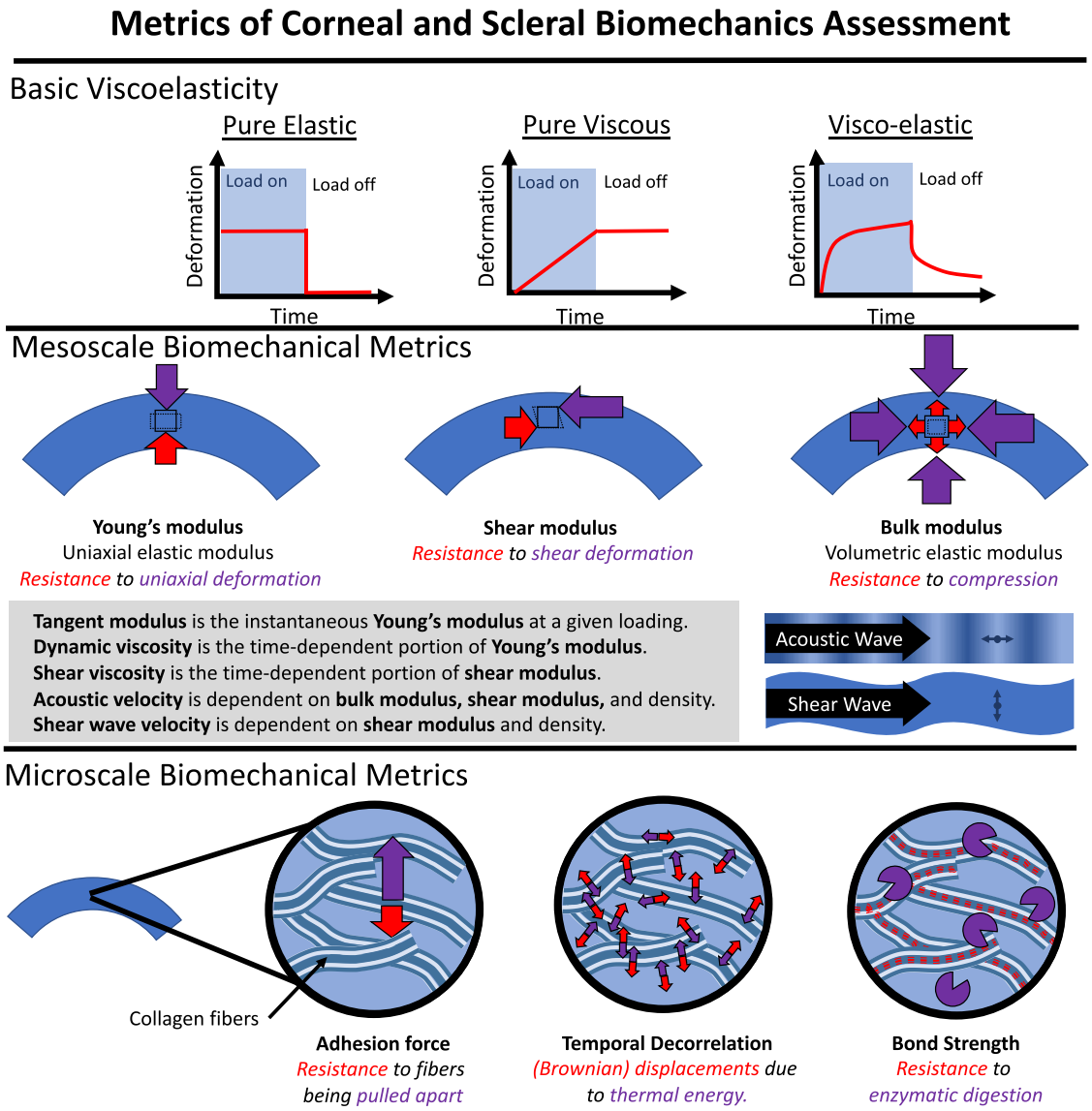

Figure 2. Overview of metrics used to quantify mechanical changes due to crosslinking the cornea or sclera. (*Top*) A sample which is wholly elastic will immediately deform when a load is added or removed. A sample which is viscous will continue to deform over time if a load is present. A sample which is viscoelastic, such as the cornea, will have both a viscous and elastic component in its deformation response to load. (*Middle*) Many different mechanical moduli are reported in ocular biomechanics literature. Young's modulus, also known as the uniaxial elastic modulus, is the resistance to deformation from a uniaxial load. Shear modulus is resistance to deformation due to a shear load. Bulk modulus, also known as the volumetric elastic modulus, is the resistance to deformation given a volumetric compression. The tangent modulus is the instantaneous slope of the stress-strain curve at a given load (stress) when the curve is no longer linear (if the stress-strain curve is linear, tangent modulus is the same as Young's modulus). Dynamic viscosity and shear viscosity are the time-dependent (rate-dependent) equivalents of Young's modulus and shear modulus, respectively. Acoustic velocity is the propagation speed of a pressure wave and is dependent on the material's bulk modulus, shear modulus, and density. Shear wave velocity is the propagation speed of a shear wave, and is dependent on shear modulus and density. (*Bottom*) Methods of assessing even smaller-scale mechanics include: adhesion force, which is the force required to retract an atomic force microscopy cantilever embedded in the sample; temporal decorrelation, as measured by DLS or OCT, is a measure of the quasi-Brownian displacements which result from random thermal energy within the sample; bond strength can be measured by the time required for the sample to be digested by enzymes.

(coated in proteoglycans), which make up fibers, which make up lamella, which interweave through the bulk of the corneal stroma. The location of crosslinks within this hierarchy is structurally important. For instance, if crosslinks are only formed within corneal collagen microfibrils (as may be the case in enzymatic crosslinking), it could be surmised that the cornea will better withstand tensile stresses. However, if no additional

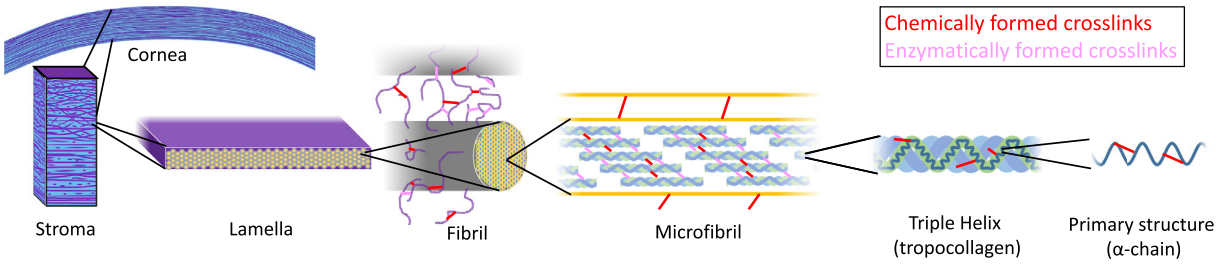

Figure 3. Crosslinks within the collagen hierarchical microstructure (Referencing figures and text[1,79–81]) From *right* to *left*: Chemical crosslinks can be formed between residues in the primary collagen molecule, between collagen molecules in the tropocollagen triple helix, between tropocollagen in the microfibril, and within or between components of the ECM which surround the collagen fibrils (which are mostly proteoglycans[82,83]). Interlamellar crosslinks are not formed by chemical crosslinking.[76] Enzymatic crosslinks are generally formed between tropocollagen in the microfibril or can be formed among the proteoglycans surrounding the fibrils.

crosslinks have been formed between collagen fibers, they may slide over each other as easily as before crosslinking, resulting in a relatively unchanged shear modulus, as has been shown to be the case in CXL for keraotoconus.[76] Conversely, if chemical crosslinking increased binding between proteoglycans surrounding the collagen fibers, essentially increasing gelation, shear modulus may increase significantly but tensile strength may be relatively unchanged. Understanding and modeling the mechanical effects of various crosslinks in a hierarchical collagen structure has remained challenging.[75]

Thus, when choosing a biomechanical measurement to assess crosslinking efficiency, consideration should be given both to the anticipated mechanical change and desired outcome. For instance, if the desired clinical outcome is inhibiting keratoconus progression, enzymatic digestion may be the most appropriate metric. However, if the desired outcome is reducing the risk of postsurgical ectasia, measuring the tensile strength of the cornea may be the best indicator. Similarly, if the crosslink formation is anticipated to occur between proteoglycans, a shear modulus measurement may be most sensitive to crosslink formation. If the crosslink formation is anticipated to occur within microfibrils, a tensile strength measurement may again be best.

The crimping and tensioning of collagen fibrils is also an important consideration. The tension on collagen fibrils during crosslinking may affect the number and type of crosslinks formed.[77,78] This is an important consideration if crosslinking is being induced in tissues ex vivo where IOP is exogenously maintained or tissue which is dissected and therefore has no tension. Collagen molecules are still slightly "crimped" in their natural state. Under tension, they straighten. Different crosslink formation may differently affect this uncrimping process and other deformation mechanics.[78]

Finally, biological tissues, and particularly the cornea, exhibit mechanical properties which are highly dependent on environmental factors, such as hydration state of the tissue, tissue boundary conditions, prestress, and other pretreatment effects. Thus, even for studies using the same protocol and reporting the same mechanical parameter, results may not be comparable.

Overview of Methods

A variety of methods have been devised to measure the mechanical properties of the cornea and sclera. A brief description of each major method of assessing ocular biomechanics is given in Table 2. (With no significance of order listed.) They are listed below for cornea (Table 3) and sclera (Table 4) and organized by modulus reported. Some methods report multiple moduli. The t-value expresses the magnitude of the crosslink-induced change relative to the variation in the data (the difference in units of standard error, where higher values indicate a larger difference). This gives a sense of how sensitive each method is specifically to the change induced by crosslinking. Although individual experimental setups may impact the t-value, overall, examining t-value across methods and moduli may yield insight into which characteristics of the cornea fundamentally change as a result of crosslinking.

However, not all methods are equally sensitive to crosslink-induced changes in the ocular tissue. In Tables 3 and 4, methods are grouped by the modulus they report. Both tables are limited to riboflavin crosslinking (either UV-A or blue light) to avoid a confounding comparison of various crosslinking methods. For each, the t-value is shown for differentiating untreated samples from crosslinked samples. When choosing a mechanical assessment method to determine the efficacy of crosslinking treatment, a method which has previously shown high t-values

Table 2. Methods of Assessing Ocular Biomechanics

Method Name	*In Vivo*?	Brief Description
Enzymatic digestion[84]	No	Samples incubated with enzyme, degradation rate measured
Atomic force microscopy (AFM)[85]	No	Micro-cantilever tip impacts sample repeatedly, recording forces during contact and withdrawal.
Brillouin microspectroscopy[86]	Yes	Optically detects acoustic velocity within a sample using a precise spectrometer.
Acoustic microscopy[87]	No	Acoustic waves are focused and transmitted through coupling fluid to the sectioned sample, and reflected back from both the sample surface and substrate behind the sample, allowing the acoustic velocity within the sample to be measured.
Phase-decorrelation OCT[88]	Yes	Using optical coherence tomography (OCT), measures random displacements of scatterers due to thermal energy fluctuations.
Ultrasound acoustic velocity[89]	Yes	Using ultrasound, measures the acoustic velocity across a sample.
Ultrasound shear velocity (supersonic shear imaging)[90]	No	Using ultrasound, measures the shear velocity across a sample.
Optical coherence elastography (quasi-static)[91]	Yes	Using OCT, observes internal deformations of tissue as compressional loading (from ocular pulse, compression lens, etc.) is applied. Internal deformations are related to local mechanical properties.
Shear wave optical coherence elastography[92]	Yes	Using OCT, observes shear wave propagation (induced by air puff, ultrasound, ocular pulse, etc.) through tissue. Wave velocity is related to local mechanical properties.
Dynamic shear rheology[93]	No	A piece of tissue is removed, usually with a biopsy punch, and subjected to shear forces at different frequencies. The resistance to shear at each frequency is recorded, characterizing the viscoelasticity of the sample.
Strip extensiometry[94]	No	A strip of tissue is fixtured at the edges or ends, and resistance to mechanical loading is recorded, yielding stress-strain curves. The curves typically provide Young's modulus, creep, and hysteresis.
Inflation testing[95]	No	An *ex vivo* globe is mounted and the relationship between globe expansion and change in intraocular pressure is observed, over short[95] or long[96] periods of time.
Thermal shrinkage[51]	No	As collagenous tissue is heated, tropocollagen denatures, resulting in significant tissue shrinkage. The threshold temperature of this denaturation indicated the stability (and crosslinking) of the collagen structure.

Table 3. Corneal Crosslinking (CXL) Mechanical Properties Sensitivity to Riboflavin / UV-A Crosslinking

Property Measured	Method	In Vivo?	*t*-Value, Untreated or Pretreated to CXL Corneas
Young's modulus	Atomic force microscopy	No	17.89[97]
			2.43[98]
			5.3[99]
	Shear wave optical coherence elastography	Yes	7.29[100]
	Inflation testing	No	6.3[96]
	Supersonic shear imaging	No	83.4[90]
Tangent modulus	Strip extensiometry	No	2.98[101]
Shear modulus	Shear rheometry	No	2.79[102]
Shear viscosity	Shear wave optical coherence elastography	Yes	5.37[100]
Acoustic velocity	Brillouin microspectroscopy	Yes	0.38[103]
	Ultrasound	No	3.64[104]
	Acoustic microscopy	No	4.38[87]
Brownian dynamics	Phase-decorrelation OCT	No	18.38[105]
Molecular bond strength	Enzymatic digestion time (collagenase, pepsin, matrix metalloproteinases, or trypsin)	No	29.79[106]
Adhesion force	Atomic force microscopy	No	3.99[99]
	Strip cleavage	No	Not significant[76]
Parameter Measured	Method	In Vivo?	*t*-Value, Untreated or Pretreated to CXL Corneas
Corneal resistance factor (CRF)	Ocular response analyzer	Yes	1.65[107]
			2.27[108]
			No change[109]
			No change[110]
			1.06[111]
			*2.09[112]
			*0.82[113]
			Not significant[114]
P2area		Yes	3.25[109]
Lateral to imposed axial displacement ratio (posterior central)	Optical coherence elastography (quasi-static)	No	0.75[115]
L2	Corvis ST*For many more parameters, see references*	Yes	3.31[112]
SP-A1		Yes	Significant[116]
Integrated concave radius		Yes	Significant[114]

*Indicates that the change detected was the opposite of the direction the other listed studies detected.

Note that for studies where the data provided was not sufficient to calculate the t-value, but the change was significant ($P < 0.05$), "significant" maybe listed instead. "No change" indicates that the *t*-value was <0.1.

Table 4. Scleral Crosslinking (SXL) Mechanical Property Sensitivity to Riboflavin Crosslinking

Property Measured	Method	In Vivo?	*t*-Value, Untreated or Pretreated to SXL Sclera
Young's modulus	Inflation testing	No	~19.2[117]
	Strip extensiometry	No	16.9[118]
			~8.3[119]
Shear viscosity	Dynamic shear rheology	No	11.52[120]

may be preferred. However, several caveats are noted. (1) Methods which report changes relative to a paired control or as a percent change to a pre-test value are likely to report higher t-values than studies which report on unpaired samples without a self-control. (2) Studies which observe crosslinking treatment on diseased tissue may report a larger difference than studies which report the effect of crosslinking treatment of healthy tissue. (3) Although these studies all nominally use riboflavin / UV-A, small differences in protocol, such as hydration and pre-tensioning, may affect the amount of crosslinking. (4) Crosslinking may have a greater stiffening effect on human corneas than porcine corneas, although porcine corneas are commonly used in CXL research, and are included in Table 3 below. With

Table 5. Methods of Spatial Resolution of Mechanical Changes in the Cornea or Sclera

Method Name	Comments on Ability to Spatially Resolve Mechanics	Spatial Dimensions	Mechanical Components
Acoustic microscopy	Can laterally resolve down to 1 um, however, this requires good mechanical coupling and a smooth sample surface.[87,126]	2	1
Atomic force microscopy (AFM)	May resolve with high precision (down to 10 nm, depending on tip size) in a prepared, ex vivo lateral (2D) cross-section[127]	2	1
Brillouin microspectroscopy	Can resolve, non-contact and without perturbation in vivo, across three dimensions with approximately 2 um resolution. The resolution is dependent on the optical system and sample properties.[128] Note that Brillouin spectroscopy is noisy in turbid media, making scleral measurements challenging.[129]	3	1+
Phase-decorrelation OCT	Can resolve, non-contact and without perturbation in vivo, across three dimensions with approximately 40 um resolution.[88]	2+	1
Ultrasound (supersonic shear wave imaging)	Can resolve, across the lateral (2D) extent of the cornea in vivo, acoustic properties with a resolution of 400 um.[117]	2	1+
Optical coherence elastography (OCE) – quasi-static	Resolution is highly dependent on sample contrast, method of perturbation, scan pattern, and processing. Capable of resolution across three dimensions in vivo, ranging between 10 and 200 um.[130]	2+	2+
shear wave optical coherence elastography (SW-OCE)	More capable of resolving in the plane of wave propagation, as opposed to the transverse direction. Similar to OCE, resolution is highly dependent on sample contrast, method of perturbation, scan pattern, and processing. Given an appropriate setup, SW-OCE is capable of resolving over a volume, in vivo, approximately 400 um resolution.[131,132]	2+	2
Inflation strain mapping	While this method may be possible in vivo,[133] current ex vivo results from cornea show a resolution of 26 um axial and 112 um lateral.[134] Could theoretically be applied over a volume.	2+	2+

A "+" indicates that higher dimensionality is not typically reported, but may result from a natural extension of the technique (e.g. adjusting scan pattern or collection angle).

these caveats in mind, it appears that enzymatic digestion, phase-decorrelation optical coherence tomography (OCT), supersonic shear imaging, and shear wave optical coherence elastography are the three most sensitive methods to crosslinking-induced change in corneal mechanical properties (given the experimental conditions and regions of interest specified by their accompanying studies). Note that the bottom half of the table summarizes methods which report parameters rather than properties. These parameters do not have an inherent physical meaning in the same manner as, for instance, Young's modulus. Additionally, a large family of similar yet different parameters may be synthesized from the same original set of measurements (as in waveform analysis for the ocular response analyzer [ORA]). For these reasons, only selected parameters are reported in the table below. More parameters can be found in individual articles.

Similarly, Table 4 reviews methods of SXL assessment, which has considerable overlap with CXL assessment techniques. Fewer studies overall have been reported for scleral crosslinking mechanics, both because SXL is less-studied and because dark crosslinkers are more popular in SXL studies. Fewer methods are represented here because fewer studies have been conducted using riboflavin crosslinking.

Resolving Mechanical Changes After Crosslinking

Choosing methods with appropriate resolve mechanical properties is important to detecting the mechanical changes induced by crosslinking. Because the cornea is biomechanically complex, exhibiting nonlinear viscoelastic behavior, which also varies over spatial and temporal scales, it is not straightforward to summarize the biomechanical effects of crosslinking.

Table 6. Biomechanical Studies of Accelerated Riboflavin / UV-A CXL versus Dresden.

Study	Results
Enzymatic digestion	Dresden CXL provided better resistance to enzymatic digestion than accelerated or pulsed methods.[141] However, another study found that standard and accelerated protocols have similar resistance to enzymatic digestion.[106]
CorVis ST	Corvis ST SP A1 shows weaker stiffening effect for increasingly accelerated protocols.[142]
Acoustic microscopy	No clear difference in acoustic velocity found between Dresden and accelerated[143]
Strip extensiometry	Strip extensiometry shows a weaker stiffening effect for increasingly accelerated protocols[142,144,145]
Brillouin microspectroscopy	Brillouin microspectroscopy showed more superficial stiffening of the cornea for increasingly accelerated protocols.[146]

For instance, the Reichert Ocular Response Analyzer (ORA) – a pneumotonometer which reports corneal biomechanical properties – was used to study the effects of CXL on corneal hysteresis, and in the majority of studies, no significant change in corneal hysteresis was found after crosslinking.[111,121–124] This led some to incorrectly conclude that crosslinking was not inducing a significant mechanical change in the cornea. However, more specific studies of corneal mechanics, including different parameters derived from the ORA, have shown that there is indeed evidence of a significant stiffening effect of crosslinking on the cornea.[107,109]

Further, crosslinking may not occur evenly over a tissue, even if treatment is applied evenly.[119] The spatial distribution of crosslinking formation is an important indicator of the efficacy of a method and useful for predicting what effect this will have on the morphology of the tissue. The various methods of assessing tissue biomechanics are not equivalently capable in this regard, and so the varying ability to resolve spatial information with each method may be an additional consideration when selecting an assessment method.

Comments on various methods which allow for spatially resolving the crosslinking effect may be found in Table 5, in order of decreasing resolving power. Apart from tissue sectioning, these methods generally do not provide any spatial resolution of crosslinking effects: enzymatic digestion, thermal shrinkage, strip extensiometry, inflation testing, and shear rheology. It is also important to highlight that some methods differ not only in capacity for 1, 2, or 3-dimensional spatial sampling but also 1, 2, or 3-dimensional directional sensing (for example, capturing mechanical behaviors in-plane and out-of-plane). Additionally, the time period over which the mechanical response is recorded may be of interest, as long-time methods may probe different aspects of mechanical properties than short-time methods.[125]

Biomechanical Differences Between Crosslink Protocols and their Correspondence to Clinical Outcomes

Some studies have used the same measurement methods to study the efficacy of various crosslinking protocols in a head-to-head comparison. Given the wide array of potentially confounding factors, notably hydration state of the tissue, and general disagreement between biomechanical parameters as measured by different setups, it is not advisable to compare absolute numbers between unrelated studies.

Given the wide array of crosslinking protocols proposed, there are naturally many studies comparing methods, both clinically and with benchtop methods. In nearly all cases, a proposed protocol is compared to the Dresden protocol.

Accelerated Crosslinking

Many clinical studies have sought to determine the efficacy of various accelerated (higher-intensity light for a shorter period of irradiation) protocols for riboflavin / UV-A crosslinking for keratoconus. Accordingly, meta-analyses address the question of clinical effectiveness in terms of long-term morphological outcomes.[135]

A few studies have assessed biomechanical parameters in vivo after Dresden versus accelerated crosslinking. However, they have produced mixed results on the mechanical efficacy of accelerated crosslinking treatments.[136,137] Two recent meta analyses concluded that the evidence was slightly in favor of Dresden crosslinking yielding a more significant increase in corneal

Table 7. Biomechanical Studies Epithelial-on versus Epithelial-off (De-epithelialized) Riboflavin / UV-A Crosslinking

Method	Results
Enzymatic digestion	Epi-on CXL had less resistance to enzymatic digestion[149]
Optical coherence elastography	Using optical coherence elastography, BKC-EDTA transepithelial crosslinking was found to produce a greater (though non-significant) amount of stiffening than Dresden, femto-second assisted transepithelial, or tetracaine transepithelial.[150]
Brillouin microspectroscopy	Epi-on crosslinking resulted in a smaller stiffening effect, as measured by Brillouin microspectroscopy.[151]

Table 8. Mechanical Comparisons of Alternative Crosslinking Protocols to Riboflavin / UV-A

Eosin Y/Green Light	Rose Bengal/Green	WST11/NIR	Glyceraldehyde	Genipin	Transglutaminases
Eosin Y had faster crosslink formation[34]	Rose Bengal causes more superficial crosslinking, due to the strong affinity between Rose Bengal and collagen, limiting diffusion[38,152]	Found that the two methods equivalently increase resistance to enzymatic digestion[153]	Glyceraldehyde stiffened similarly, but slightly more, than riboflavin / UV-A[96]	Equivalently resistant to inflation after crosslinking[47]	Transglutaminase was found to induce a higher tangent modulus than riboflavin / UV-A crosslinking[56]

resistance.[135,138] However, both analyses also concluded that both standard and accelerated crosslinking were effective at halting keratoconus progression up to a year postprocedure. It remains to be seen if the different biomechanical effects portend differences in the efficacy of the methods over longer periods of time. Finally, a recent study which used accelerated crosslinking with the addition of supplemental oxygen found a significant increase of corneal resistance factor in the case of accelerated CXL and not a significant increase in the case of Dresden CXL.[139] This may mean that oxygen supplementation or other cutting-edge protocols may further enhance the stiffening effect of accelerated crosslinking to perhaps exceed that of Dresden crosslinking.

This lack of conclusive evidence may be the result of ambiguous measurement. The ORA is the most commonly used tool to measure corneal biomechanics in vivo, but it may be less sensitive to differences caused by CXL than newer options, such as the Corvis ST. Due to its increased data collection (a video-rate Scheimpflug image of the deforming cornea, as compared to the surface-reflected beam of the ORA) the Corvis ST has been shown to more reliably measure biomechanical changes in vivo.[140]

Table 6 highlights published studies which assess the direct mechanical effects of Dresden protocol versus accelerated crosslinking.

Transepithelial Crosslinking

In addition to the study of accelerated crosslinking protocols, there has been considerable study in the field on the comparative effects of epithelial-on (transepithelial) versus epithelial-off (de-epithelialized) riboflavin / UV-A crosslinking protocols.[147] These studies have concluded that although transepithelial CXL results in reduced healing time and improved best-corrected visual acuity, it is less effective (in most implementations studied) at halting the progression of keratoconus.

A few clinical studies (summarized elsewhere ref. 148) have specifically looked at biomechanical parameters of transepithelial CXL. Table 7 highlights published studies which assess the direct mechanical effects of epithelial-on versus epithelial-off (de-epithelialized) riboflavin / UV-A crosslinking.

Alternative Crosslinking Methods

Finally, there have been a limited number of studies reporting the efficacy of non-riboflavin methods of crosslinking to riboflavin / UV-A crosslinking. To our knowledge, no clinical trials have been completed assessing Dresden CXL to these alternative methods. Biomechanical results are summarized in Table 8. In addition, an important consideration of alternative protocols is reduced cytotoxicity.

Predicting the Mechanical and Morphological Outcomes of Crosslinking

In some cases, such as keratoconus, the desired outcome of crosslinking is primarily the stiffening effect. However, in other cases, such as myopia and refractive correction, the desired outcome is a morphological change.[154] In some ways, this is an easier output metric to measure than stiffening – corneal topographers and tomographers are commonplace in refractive clinics, and can be used to determine the morphology of the cornea to a high degree of precision. However, titrating treatment for a desired morphological outcome is more difficult than titrating treatment simply to prevent keratoconus progression, where a broad range of outcomes may be equally acceptable. Visual acuity is sensitive to the morphology of the cornea on the order of 40 nm.[155] Thus, morphological outcomes must be very tightly controlled to produce the desired improvement in visual acuity.

To help close this gap, a number of mechanical models of the cornea and ocular globe have been devised. These models allow for changes to the mechanical properties of constituent tissues to be modeled and the predicted morphological outcomes analyzed. To produce useful results, this type of modeling relies on a thorough understanding of the mechanical changes induced by crosslinking. If changes in mechanical properties of the tissues as a result of crosslinking are well-characterized, the morphological outcomes may be predicted and optimized for a desired effect.[156]

Examples of work in this direction include a 2013 study which demonstrated an inverse, finite-element driven model for predicting morphological changes in the cornea due to riboflavin / UV-A crosslinking.[157] A further paper demonstrated that laterally patterning the crosslinking can be used to fine-tune the refractive effect, helping to reduce aberrations.[64] A 2017 study demonstrated an algorithm which predicts the stiffening effect of corneal crosslinking given riboflavin diffusion and irradiation parameters.[158] Such modeling may serve as useful input to finite element-driven modeling of corneal morphology, to prescribe a CXL treatment pattern and protocol meeting the required stiffening profile to generate a given morphology.

Conclusion

Crosslinking using exogenous methods of "light" and "dark" varieties is a rapidly evolving area of translational interest. These approaches are being applied to an increasing number of vision-related diseases, such as keratoconus, progressive myopia, and glaucoma. Biomechanical measurement methods, which differ in spatial resolution, mechanical sensitivity, suitability for detecting crosslinking subtypes, and translational readiness, are central to the effort to understand the mechanistic link between crosslinking methods, the desired effects in tissue, and clinical outcomes of candidate therapies.

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