

# REVIEWS

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MAGDALENA KUBRAK-KISZA<sup>A-D</sup>, KRYSZTIAN JERZY KISZA<sup>B,C</sup>,  
MARTA MISIUK-HOJŁO<sup>A,F</sup>

## Corneal Cross-Linking: An Example of Photoinduced Polymerization as a Treatment Modality in Keratoconus

Department and Clinic of Ophthalmology, Wrocław Medical University, Wrocław, Poland

**A** – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation;  
**D** – writing the article; **E** – critical revision of the article; **F** – final approval of the article

### Abstract

The cornea is one of the principal refractive elements in the human eye and plays a crucial role in the process of vision. Keratoconus is the most common corneal dystrophy, found mostly among young adults. It is characterized by a reduced number of collagen cross-links in the corneal stroma, resulting in reduced biomechanical stability and an abnormal shape of the cornea. These changes lead to progressive myopia, corneal thinning, central scarring and irregular astigmatism, causing severely impaired vision. Hard contact lenses, photorefractive keratectomy or intracorneal rings are the most common treatment options for refractive error caused by keratoconus. However, these techniques do not treat the underlying cause of the corneal ectasia and therefore are not able to stop the progression of the disease. Riboflavin photoinduced polymerization of corneal collagen, also known as corneal cross-linking (CXL), has been introduced as the first therapy which, by stabilizing the structure of the cornea, prevents the progression of keratoconus. It stiffens the cornea using the photo-sensitizer riboflavin in combination with ultraviolet irradiation. This is a current review of the CXL procedure as a therapy for keratoconus, which relies on photoinduced polymerization of human tissue. We have focused on its biomechanical and physiological influences on the human cornea and have reviewed the previous and current biochemical theories behind cross-linking reactions in the cornea (*Polim. Med.* 2016, 46, 1, 89–94).

**Key words:** cornea, keratoconus, polymerization, cross-linking.

A polymer is defined as a chain of monomeric material – either a synthetic polymer or a biologic molecule, such as a protein. Cross-linking is a creation of bonds that connect one polymer chain to another. The bonds can be covalent or ionic [1].

Conducting a polymerization process of a biologic molecule is a major challenge, as the whole process must be accompanied by a thorough understanding of the anatomy, physiology and biochemistry of the targeted tissues.

In 1992, Hettlich et al. [2] investigated possible ways to use polymerization of a monomer in the eye. Their idea was to change a liquid, synthetic monomer, which had been injected into an empty lens capsule, into a solid-state polymer to recreate the intraocular

lens, by exposing it to light. One example of biologic tissue polymerization used in ophthalmology today is cross-linking of the cornea (CXL). It is now the leading therapy in keratoconus that is aimed at halting the progression of this corneal ectatic dystrophy, resulting in a significant decrease in the need for the corneal transplantations which the disease previously necessitated.

Other uses of cross-linking in ophthalmology include the arrest of post-LASIK ectasia and pellucid marginal degeneration progression. CXL has also been shown to be effective in the treatment of corneal infections and management of various forms of corneal edema.

This article will be focused on the structural and biomechanical changes in the cornea induced by CXL.

## Anatomy of the Cornea

The human cornea plays a crucial role in the process of vision. It is the principal refractive element of the eye, contributing 45 out of the 63 total dioptres of its unaccommodated refractive power [3]. Maintenance of a shape as maximally spherical as possible is essential for the cornea's refractive role. The human cornea is comprised of 5 layers: 3 cellular layers (epithelium, stroma and endothelium) and 2 interfaces (the Bowman's membrane and the Descemet's membrane) [4].

The corneal epithelium is an anterior squamous layer 50  $\mu\text{m}$  thick. It acts as a protective barrier of the cornea and consists of several layers of cells which are constantly undergoing mitosis.

The corneal endothelium consists of a thin cell monolayer that does not regenerate. Its essential function is to regulate corneal hydration and to maintain the 78% water content in the stroma. The endothelium also ensures the uptake of nutrients and waste release in the cornea [5]. The difference between the regenerative capacity of the epithelium and endothelium is crucial in the planning of any corneal surgery. Damage to the epithelial layer is rapidly repaired by regeneration. By contrast, the endothelium, if damaged by surgery or disease, cannot be regenerated.

The corneal stroma makes up 90% of the corneal thickness [6]. It is a connective tissue mainly composed of a collagen type I matrix in which mesenchymal cells, the keratocytes, are embedded. Type I collagen makes up 90% of the corneal collagen. Type V collagen constitutes only 10%. Collagen fibrils in the stroma have a uniform diameter of 25–35 nm. These fibrils run parallel to each other, forming flat lamellar bundles. In the anterior third of the stroma, the thin lamellae are more narrowly interwoven than in the posterior two thirds and run mostly obliquely to the corneal surface. In the posterior one third, the lamellae are described as thicker than those in the superficial layers and are usually parallel to the corneal surface [6]. The corneal stroma is a perfect example of an extracellular matrix that is dense and precisely ordered. It consists of very small-diameter collagen fibrils surrounded by a special array of four types of proteoglycans: three core proteins containing glycosaminoglycan (GAG) chains of keratan sulfate (lumican, keratocan, and mimecan) and one bearing GAG chains of chondroitin/dermatan sulfate (decorin). These core proteins are classified as small leucine-rich repeat proteins [7].

Molecular models of the corneal stroma, based on the most recent research, suggest that the proteoglycan core proteins wrap themselves laterally around the collagen fibrils in such a way that their hydrophobic domains fold inside, against the collagen fibrils [7].

By contrast, it is thought that the highly sulphated GAG chains protrude laterally away from the sides of the collagen fibrils, with the result that they form an

exterior hydrophilic structure. Its thickness matches that of the space surrounding adjoining fibrils. This produces precise, center-to-center spacing between the collagen fibrils, characteristic of corneal stroma and necessary for its transparency [8, 9].

## Keratoconus – Disease and Therapy

Whenever the anatomic structure of the cornea is disrupted, abnormal corneal refractive function occurs and vision deteriorates. Keratoconus is an example of such a situation. A reduced number of collagen cross-links and increased activity of proteinase enzymes, causing increased stromal protein digestion [10], resulting in reduced biomechanical stability, have been suggested as possible explanations for an overall structural weakness and thinning of the corneal tissue in keratoconus, resulting in a stiffness of only 60% of that of a normal cornea [11].

These structural changes of the cornea in keratoconus may result in changes in the corneal radius of curvature and localized reduction in thickness, resulting in progressive myopia, corneal thinning, central scarring and irregular astigmatism, causing impaired vision, ghosting and polyopia. This relatively uncommon condition, whose prevalence varies in different geographic regions but numbers about 54.5 cases per 100,000 people [12], is typically bilateral but often asymmetrical. Despite its rarity as a condition, it is the most common corneal dystrophy [13].

Its etiology is not fully understood and includes biochemical, physical and genetic factors, with no sole proposed theory elucidated. It is probable that keratoconus is a manifestation of several various conditions, possibly being induced by repeated surface ocular trauma or eye rubbing. It usually appears as an isolated condition, but has also been associated with a number of ocular and systemic disorders, including allergic eye disease, magnesium deficiency, connective-tissue disorders and many others [14, 15].

Usually, the condition starts at puberty, progressing in approximately 20% of cases to such an advanced stage that corneal transplantation is required to prevent corneal perforation [13, 16].

Hard contact lenses are the most common treatment for the refractive error caused by keratoconus. In some cases, photorefractive keratectomy or intracorneal rings are considered. However, these techniques do not treat the underlying cause of the corneal ectasia and therefore are not able to stop the progression of keratoconus.

Riboflavin photo-induced polymerization, also known as collagen cross-linking (CXL), has been introduced as the first therapy which, by influencing the changes to the structure of the cornea, prevents the progression of keratoconus. It stiffens the cornea using the photosensitizer riboflavin in combination with ultraviolet irradiation.

The standard treatment protocol, known as the Dresden Protocol, after the Technical University of Dresden where it was first described by Wollensak et al. [17], consists of the following steps:

1. Anesthetizing the eye with a local anesthetic in drop form;

2. Removing the central 7–9 mm of the corneal epithelium. Corneal epithelium with a thickness of approximately 50  $\mu\text{m}$  is a limiting factor. Removal of this tissue enhances the penetration of the photosensitizer and allows its proper absorption into the cornea. Due to the high mitotic index of the corneal epithelium, it takes 3–4 days after the therapy for the cornea to be re-epithelialized;

3. Before commencing with the UV-light illumination, the stroma is soaked with a photosensitizer: riboflavin A (vitamin B12). This is applied in the form of a 0.1% riboflavin 5-phosphate and 20% dextran solution to the de-epithelialized cornea every 5 min for 30 min;

4. A further application of the above solution, again every 5 min for 30 min, in combination with exposure to UVA (370 nm, 3  $\text{mW}/\text{cm}^2$ ) radiation. Using a wavelength of 360–370 nm, with a UV intensity of 3  $\text{mW}/\text{cm}^2$  and 5.4  $\text{J}/\text{cm}^2$ , ensures the exposure to UV light on the cornea is below harmful levels. To avoid damage to the endothelium caused by UVA light, effective CXL should only occur in the first 200–250  $\mu\text{m}$  of the corneal stroma. The cross-linking effect is strongest in the anterior half of the stroma because of the rapid decrease in UVA irradiance across the corneal stroma as a result of riboflavin-enhanced UVA absorption;

5. Application of a soft bandage contact lens with good oxygen permeability. This is kept in place for 3–4 days until the re-epithelialization process is complete, at which point it is removed. Immediately after surgery, a course of topical antibiotics in drop form is applied for the next few days. In addition, topical steroid therapy is introduced for the next few months.

Since the Dresden protocol for corneal cross-linking therapy was first introduced, several new propositions for its modification have been proposed. This includes protocols with higher intensities and shorter treatment times and epi-on CXL.

The higher-intensity protocols are based on the Bunsen-Roscoe law of reciprocity which states that a certain biological effect is directly proportional to the total energy dose, irrespective of time. Based on this, it has been concluded that the following: 10  $\text{mW}/\text{cm}^2$  for 9 min, 18  $\text{mW}/\text{cm}^2$  for 5 min, 30  $\text{mW}/\text{cm}^2$  for 3 min or 45  $\text{mW}/\text{cm}^2$  for 2 min, at a constant dose  $E$  of 5.4  $\text{J}/\text{cm}^2$ , may have the same biological effect as traditional CXL at 3  $\text{mW}/\text{cm}^2$  for 30 min. *Ex vivo* experiments have shown biomechanical stiffening of the corneal tissue after exposure to 10  $\text{mW}/\text{cm}^2$ , for a duration of 9 min, correlating with the outcomes seen after treatment with standard CXL [18]. The response to irradiances

between 3 and 90  $\text{mW}/\text{cm}^2$  with illumination times between 30 s and 1 min respectively was investigated in an extensive *ex vivo* study of porcine eyes. A steady increase in stiffness after exposure to illumination intensities of 40–45  $\text{mW}/\text{cm}^2$  was observed. However, no statistically significant increase in stiffness in intensities between 50 and 90  $\text{mW}/\text{cm}^2$  was found [19]. This suggests that higher-intensity cross-linking may not be as effective if illumination duration is less than 7 min. An *ex vivo* study of human eyes was conducted in order to compare CXL with standard (3  $\text{mW}/\text{cm}^2$  for 30 min) versus accelerated (9  $\text{mW}/\text{cm}^2$  for 10 min) protocol. This revealed that there were no differences in corneal stiffness results between the groups [20]. This area of research is still ongoing.

Despite a large number of studies showing favorable outcomes with no evidence of endothelial cell density changes during a 6-month follow-up [21], no uniform protocol for accelerated CXL has so far been proposed. This could be investigated by further study.

Another example of a different protocol was focused on corneal de-epithelialization, which is performed during the standard procedure. As an alternative to this, the epi-on procedure, without the removal of the cornea, has been proposed [22–24]. Different studies have shown that epi-on CXL does affect the biomechanical properties of the cornea. However, corneas without the epithelium seem to benefit more compared to corneas with it [22–24]. Noticeably better results were obtained with epi-on CXL using iontophoresis, but the relative efficacy of that technique compared to standard epithelium-off CXL still remains to be determined [25].

There are many different protocols which are still being investigated and it has yet to be determined which one is the most appropriate. Although great progress has been made since the introduction of the Dresden protocol, further research is needed in this area, with longer follow-up times. It is hoped that more information will be available in the near future.

## The Idea of Cross-Linking Bonds

Cross-linking reagents are molecules that contain two or more reactive ends that are capable of chemically attaching to specific functional groups such as primary amines on proteins or other molecules.

In corneal cross-linking, the precise location of the cross-links at a molecular level is as yet undetermined. In the 1970s, Siegel et al. [26] discussed cross-linking reactions in which the formation of cross-linking aldehydes in collagen and elastin were catalyzed by lysyl oxidase. Several years later, in 1997, Spoerl and Seiler [27] at the University of Dresden developed photochemical cross-linking with riboflavin and UVA. Riboflavin would absorb UVA and act as a photosensitizer and produce free radicals that would activate the natural

lysyl oxidase pathway to induce cross-linking between collagen fibers. According to this hypothesis, riboflavin molecules absorb energy and reach an excited state when exposed to ultraviolet radiation. Riboflavin can, in its excited state, produce singlet oxygen molecules or other free radicals. These generate reactive oxygen species which, in turn, cause the intermolecular, cross-linking dityrosine bonds to form.

Recently McCall et al. [28] have proposed that the singlets do not instigate cross-linking by lysyl oxidase. Other mechanisms have been proposed instead:

1. Production of imidazolone, which can attach to molecules, such as histidine, to form new covalent bonds;

2. Endogenous populations of carbonyl groups in the extracellular matrix (allysine, hydroxyallysine) being triggered, with the resultant formation of cross-links;

3. The riboflavin molecule itself breaking down, with the subsequent release of 2,3-butanedione. This could further react with the endogenous carbonyl groups of the stromal proteins.

This conclusion was arrived at after a series of experiments during which possible chemical mechanisms of cross-linking were tested. After giving careful consideration to the anatomical, histological, biochemical and molecular structure of the cornea, McCall [28] has concluded that the intrafibrillar, fibrillar-extracellular and interlamellar bonds within the corneal stroma are promoted by and play a crucial role in the riboflavin-UVA catalyzed cross-linking process. The intrafibrillar bonds form inside individual collagen fibrils when the amino groups of the lysine, with residues in one of the tropocollagen chains, reacts with a residue within an adjacent chain of a second tropocollagen molecule [29, 30]. The fibrillar-extracellular matrix bonds, due to their molecular proximity, promote covalent bonding between the activated residues along tropocollagen molecules and appropriate residues in a proteoglycan core protein [7, 31]. The interlamellar bonds are capable of increasing the mechanical strength of the cornea by physically linking entire adjacent lamellae of the corneal stroma, in a similar way that the sutural fibers of elasmobranch corneas do. Interactions might occur between separate collagen fibrils within an individual ply and between adjacent plies [28].

Despite all uncertainty and discussion as to where and how cross-linking takes place, publications with prospective case series with follow-ups from 1 to 4 years report stabilization of keratoconus after CXL. In some of them, improvements in visual acuity and higher-order aberrations and a reduction of keratometry values have also been reported [32–34].

Peer-reviewed literature also reports structural changes in the cross-linked corneas, reflected in increased collagen fiber diameter [35], an increase in shrinking temperature [36] and an increase in enzymatic digestion [37].

## Cross-Linking and Biomechanical Changes in the Cornea

In 2004, Wollensk et al. [38], using electron microscopy, found a morphologic change after cornea cross-linking therapy in New Zealand White Albino rabbits. The published results revealed that in the anterior stroma, the collagen-fiber diameter in the treated eyes was significantly increased by 12.2% (3.96 nm), and in the posterior stroma by 4.6% (1.63 nm), compared to the eyes in the control group.

In the same year Spoerl [36] compared the maximum shrinkage temperature of untreated fresh porcine cadaver eyes with ones treated with riboflavin/UVA irradiation. This study demonstrated an increased maximum shrinkage temperature in these cross-linked corneas compared to those that were not cross-linked. The maximum shrinkage temperature was 70°C for the untreated corneas, 75°C for the corneas cross-linked with riboflavin/UVA and 90°C for corneas cross-linked with glutaraldehyde. The difference in the degree of cross-linking was clearly demonstrated by comparing the anterior and posterior portions after heating: a mushroom shape was observed at 70°C when only the posterior, non-cross-linked portion was contracted, while a cylinder shape could be seen at 75°C, when both the anterior and posterior portions were denatured by heat [36].

In a study conducted by Seiler, Spoerl and Wollensak [37] the researchers observed an impressive twofold increase in the digestion time following pepsin, trypsin and collagenase digestion in corneas cross-linked with riboflavin and UVA at 3 mW/cm<sup>2</sup> compared to the controls. This conclusion is of great importance as resistance to collagenase digestion may be a vital aspect in the efficacy of cross-linking treatment in corneal ulceration and also in keratoconus treatment, as studies of tear-fluid samples from keratoconus patients have been found to contain levels of collagenase-induced degradation products (telopeptides) 2.5 times higher than normal [39].

Gregor Wollensak [40] conducted a study to observe the biochemical changes in cross-linked corneal collagen by comparing the electrophoretic pattern of Type I collagen in cross-linked porcine corneas with a control group. In this trial he used 40 porcine corneas collected 24 h *post mortem*. In the controls, the typical collagen pattern of a normal cornea was found to contain 1 gamma trimer band, 2 β dimer bands, and 2 α monomer bands. In the cross-linked corneas, a strong band of high-molecular-weight collagen polymers was shown to be the biochemical correlate of the cross-linking effect, demonstrating the efficiency of this procedure. The cross-linked polymer product was remarkably chemically stable, as shown by such resistance.



A remarkable level of chemical stability was observed in the cross-linked polymer product, which was resistant to mercaptoethanol, heat, and pepsin treatment. Its molecular size was estimated to be at least 1000 kDa.

In all the above reports, a significant influence of cross-linking therapy on corneal parameters was confirmed, at the same time indicating a different degree of cross-linking between the anterior and posterior part of the cornea after therapy.

## Conclusion

Cross-linking may be an illustration of a successful multidisciplinary collaboration where *in vivo* photoinduced polymerization of biological tissue is used as a disease modifying therapy for patients with keratoconus as well as other corneal disorders.

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**Address for correspondence:**

Magdalena Kubrak-Kisza  
ul. Gen. Hallera 17/4  
53-319 Wrocław  
Poland  
E-mail: mkubrakster@gmail.com

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