Chondroitin Sulfate–Based Biocompatible Crosslinker Restores Corneal Mechanics and Collagen Alignment

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PURPOSE. To evaluate the crosslinking effect of functionalized chondroitin sulfate (CS) in an ex vivo rabbit cornea model.

METHODS. Chondroitin sulfate molecules were chemically modified with the N-hydroxysuccinimide (NHS) group. Enucleated rabbit eyes were crosslinked with 2, 5, or 10 mg/mL CS-NHS solution for 30 or 60 minutes. The CS-NHS penetration, corneal swelling ratio, Young's modulus, and ultrastructure of the crosslinked corneas were characterized. In addition, rabbit corneas were further treated with a collagenase–chondroitinase solution to create an ex vivo keratoconus (KC)-like model. The KC model corneas were crosslinked with a standard riboflavin–ultraviolet (UV) method or alternatively with CS-NHS. Corneal mechanics, ultrastructure, and keratocyte gene expression were evaluated after UV and CS-NHS crosslinking.

RESULTS. CS-NHS effectively penetrated into the corneal stroma within 60 minutes of treatment initiation. CS-NHS crosslinking reduced the swelling ratio by 35%, increased Young's modulus by 20%, and increased collagen fibril diameter and density. CS-NHS crosslinking improved corneal mechanics of KC model corneas to levels comparable to those with UV crosslinking. Moreover, CS-NHS crosslinking demonstrated significant downregulation of proinflammatory gene expression of keratocytes, indicating a potential protective effect imparted by CS-NHS during crosslinking.

CONCLUSIONS. Our results demonstrated that CS-NHS can reinforce normal and KC model corneal mechanics, and restore collagen density and alignment in KC model corneas without causing extensive keratocyte apoptosis and proinflammatory gene upregulation. Therefore, CS-NHS crosslinking can potentially provide an effective, safe, and biocompatible means of corneal reinforcement.

Keywords: corneal crosslinking, chondroitin sulfate, corneal biomechanics, ultrastructure

Corneal ectasia is a corneal disorder characterized by progressive corneal axial thinning, which results in irregular astigmatism and decreased vision.[1] The progression of corneal ectasia is more rapid in younger patients and greatly impairs vision and quality of life.[2] Corneal ectasia may result from corneal diseases, for example, keratoconus (KC), or is a complication of photorefractive surgery. It is a consequence of disruption of the normal corneal extracellular matrix (ECM) composition and organization. More than 75% of the corneal stroma is composed of type I collagen, with small amounts of collagen types II, III, V, VI, and XI, and various proteoglycans, which form parallel fibers organized into orthogonally stacked lamellae.[3] In ectatic corneas, complex mechanisms, including abnormal collagenase activity, result in degradation of collagen and loss of proteoglycans, characterized by fibril loosening and weakened mechanics.[4]

Collagen crosslinking is an effective method to slow or halt the progression of corneal ectasia; for example, riboflavin 5′-phosphate (RF) sodium (Photrexa; Avedro, Inc., Waltham, MA, USA) crosslinking[5] was recently approved by the Food and Drug Administration as a clinical treatment for KC. This system uses ultraviolet A (UVA) irradiation to initiate a radical reaction with RF. Riboflavin generates free radicals with UVA irradiation and initiates the formation of carbonyl-based covalent bonds within collagen fibrils and proteoglycans. Numerous studies have demonstrated that collagen crosslinking improves vision quality and slows disease progression.[6] However, UVA irradiation can cause clinical complications, such as keratocyte apoptosis, temporary corneal haze, and, in more serious cases, corneal melting and permanent corneal scarring.[7] Alternative treatments now aim to avoid UV light exposure to the eye. These include Rose Bengal green light crosslinking[8] and core protein decorin crosslinking that does not require any light exposure.[9]

Collagen-based biomaterials also frequently require structural reinforcement through chemical crosslinking. Small-molecule chemical crosslinkers, such as paraformaldehyde and glutaraldehyde, are highly efficient but are also toxic. Their use necessitates additional biomaterial processing steps to ensure complete elimination of these toxic crosslinkers before application in biological systems or for internal use. Carbodiimide

bioconjugation, involving 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), is also widely used in the biomaterials community.[10] The NHS group first interacts with the carboxyl group of the target molecule and then the NHS functionalized target molecule can react with amine groups on other molecules.

Proteoglycans play an important role in collagen fibril formation and lamellar assembly during corneal development.[11] They are composed of glycosaminoglycan (GAG) chains and core proteins. Chondroitin sulfate (CS) is one of the most abundant GAGs in the natural ECM throughout the body, with various biological functions, such as regulating inflammation,[12] reducing collagenase activity,[13] and controlling collagen fiber spacing in the cornea.[14] Chondroitin sulfate is frequently incorporated into biomaterials with collagen[15] or gelatin[16] to fabricate scaffolds for cartilage or corneal substitutes.[17,18] Contact lenses that had CS loaded into them enhanced healing of corneal lesions,[19] purportedly through collagen fibrillogenesis. In this study, CS was functionally modified with a highly reactive amine group, which was then used in conjunction with NHS to form crosslinks with collagens and proteoglycans present in the cornea. This crosslinking process creates strong covalent networks that ultimately stabilize the corneal structure without the use of UV radiation or other radical-initiated chemical reactions. The CS crosslinker was applied to an ex vivo corneal ectatic model developed in the lab, and the resulting biological and structural changes were evaluated.

METHODS

Synthesis of Amine-Reactive Chondroitin Sulfate

CS-NHS was synthesized as described previously.[20] Briefly, 10% CS (wt/vol) (25 kDa; New Zealand Pharmaceuticals Ltd, Palmerston North, New Zealand), 67% EDC (wt/vol), and 25% NHS (wt/vol) were combined in a 7:1.5:1.5 (vol/vol/vol) ratio of CS:EDC:NHS in phosphate-buffered saline (PBS) and allowed to react for 10 minutes at 37°C. The mixture was flash frozen to −80°C and then subjected to several rounds of extensive washing with −20°C ethyl alcohol (EtOH). The final CS-NHS product was dried under argon gas flow for 2 hours and subsequently placed in a vacuum desiccator overnight.

Corneal Ectatic Model and Crosslinking

One hundred forty-four fresh albino rabbit eyes (Cat. No. 41211-2) in Dulbecco's modified Eagle's medium (DMEM) were purchased from PelFreez Biologicals (Rogers, AR, USA). Corneas with no treatment (normal) were maintained in DMEM for further crosslinking and characterizacion. Other corneas were treated with an enzymatic digestion solution for 1 hour at 37°C to create a model for KC. The digestion solution contained 10 U/mL collagenase type II (LS004177, 280 U/mg; Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.1 U/mL chondroitinase ABC (C36675U; Sigma-Aldrich Corp., St. Louis, MO, USA) in PBS. The digestion was halted by rinsing the corneas in DMEM with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) multiple times.

Normal corneas were crosslinked by submersing the anterior segment (placing the whole eye globe upside down) in 2, 5, or 10 mg/mL CS-NHS (CS2, CS5, and CS10) solutions at room temperature for 30 or 60 minutes. The KC model corneas were crosslinked in 5 mg/mL CS-NHS solution for 60 minutes. For comparison with other crosslinking techniques, normal and KC model corneas were presaturated for 20 minutes in a 0.1% RF, 15% dextran-500 (Sigma-Aldrich Corp.), and crosslinked using a

365-nm UVA light source (F15T8/BL lamp) at 3 mW/cm² for 30 minutes. One drop of RF was supplemented every 10 minutes.

CS-NHS Penetration Into Corneal Tissue

To track the depth of penetration of CS-NHS into the corneal stroma, the CS-NHS molecule was labeled with tetramethylrhodamine-PEO$_3$-amine (TAMRA-PEO$_3$-amine) (Biotium, Inc., Freemont, CA, USA) dye by mixing 10% CS-NHS and TAMRA-PEO$_3$-amine in PBS in a 1:1 molar ratio for 5 minutes. CS-NHS-TAMRA was washed with −20°C EtOH and dried under argon. The anterior region of each eye globe was immersed in 2 mg/mL TAMRA dye-only solution (background control) for 30 minutes, or varying concentrations (2, 5, or 10 mg/mL) of CS-NHS-TAMRA solution for 60 minutes ($n = 3$ for each test condition). Corneas were subsequently rinsed repeatedly in PBS to minimize nonspecific binding of the dye. Penetration of CS-NHS-TAMRA was observed through fluorescent confocal microscopy (Zeiss LSM 510; Carl Zeiss AG, Jena, Germany). The mean fluorescent intensity was calculated by subtracting the background control intensity at 100-μm depth. The Z-stacking step size was set at 25 μm/section. Penetration depth was calculated by adding the number of Z sections with higher fluorescent intensity compared to the background control.

Corneal Swelling Ratios

Normal, CS2, CS5, and CS10 corneas were stored in 15% dextran-500 PBS solution overnight prior to measurement ($n = 6$ for each test condition). An 8-mm biopsy punch was used to extract the central corneal button. Corneal buttons were weighed and then transferred to PBS. Change in weight due to swelling caused by water uptake was recorded every 30 minutes, up to 120 minutes. The swelling ratio was calculated using the equation

$$q = W_s/W_o,$$

where W_s represents the weight after swelling and W_o represents the original weight before swelling.

Transmission Electron Microscopy (TEM)

After enzyme treatment and crosslinking, cornea samples were stored in 15% dextran-500 overnight before fixation. Samples were fixed in 3% paraformaldehyde, 1.5% glutaraldehyde, 5 mM MgCl$_2$, 5 mM CaCl$_2$, 2.5% sucrose, and 0.1% tannic acid in 0.1 M sodium cacodylate buffer, pH 7.2, overnight at 4°C. They were postfixed in 1% osmium tetroxide. Samples were stained with 2% aqueous uranyl acetate, dehydrated through treatment in increasingly concentrated ethanol, and then embedded in Eponate 12 (Ted Pella, Inc., Redding, CA, USA) resin. Thin sections were cut and grids were stained with 2% uranyl acetate in 50% methanol and visualized with a Philips/FEI BioTwin CM120 TEM at 80 kV (Eindhoven, the Netherlands). Image analysis was carried out using MATLAB (MathWorks, Inc., Natick, MA, USA) as described previously.[21] Original images were binarized using an adaptive thresholding method. Fibril diameter and density were estimated within a randomly localized window (300 × 300 dpi). The number of fibers per unit area was measured and recorded. We repeated this random procedure 200 times and estimated the density of fibers using the median value.

Live–Dead Staining

Fresh corneas (three corneas in each group) were dissected and submersed in PBS, 2, 5, and 10 mg/mL CS-NHS, respectively, for 60 minutes. Positive control corneas were

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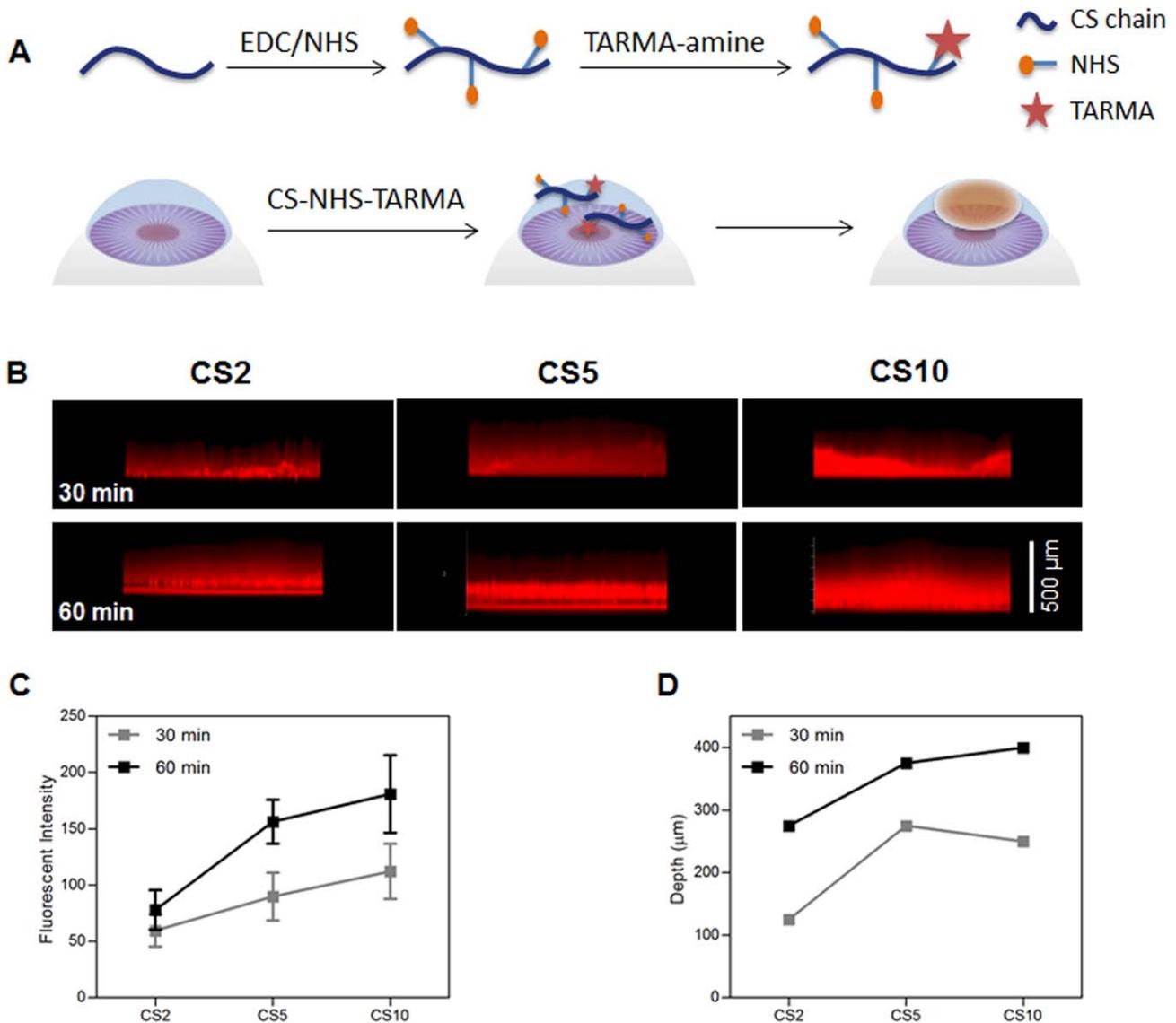
FIGURE 1. Chemical design and penetration of CS crosslinker through the cornea. (**A**) CS-NHS is labeled with TARMA dye by covalent reactions with amine groups in TARMA-(PEO)$_3$-amine to track penetration depth. (**B**) Corneal explants were immersed in varying concentrations of fluorescently labeled crosslinker for 30 and 60 minutes to visualize CS-NHS penetration. Image analysis quantified the (**C**) intensity at 100-µm depth (related to concentration) and (**D**) depth of fluorescently labeled CS-NHS crosslinker. The CS-NHS crosslinker penetrated approximately 250 to 400 µm into the corneal tissue after 60 minutes of exposure.

treated with standard RF-UV crosslinking as described previously in the section "Corneal Ectatic Model and Crosslinking." After treatment, the corneas were stained with LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific L3224) according to standard protocol. The samples were extensively washed in PBS five to eight times to minimize background fluorescence in the stroma. Corneas were laid flat with epithelium facing down, mounted with PBS, and imaged using the Zeiss inverted fluorescence microscope.

Hematoxylin and Eosin (H&E) Staining

Normal, KC model, UV-crosslinked KC model (KC-UV), and 5 mg/mL CS-NHS-crosslinked KC model corneas (KC-CS5) were stored in 15% dextran-500 overnight to minimize swelling, and then fixed overnight with 10% formaldehyde, followed by graded dehydration with 70% to 100% ethanol, and embedded in paraffin. Sections (5 µm) were cut and stained with H&E (Sigma-Aldrich Corp.) according to standard protocol.

Tensile Testing

Uniaxial tensile testing was carried out on a Bose EnduraTEC ELF3200 (TA Instruments, Waters LLC, ElectroForce Systems Group, New Castle, DE, USA) to determine the stress–strain behavior of the corneas before and after crosslinking. All test samples for tensile testing were stored in 15% dextran-500 solution overnight to minimize swelling. Corneas ($n = 6$ in each test group) were cut into strips of around 5 mm each, and the exact width and thickness of each strip were recorded. The corneal strip ends were affixed to the clamps on the tensile tester using super glue to avoid slippage. Load and displacement measurements were recorded by WinTest software (TA Instruments, New Castle, DE, USA).
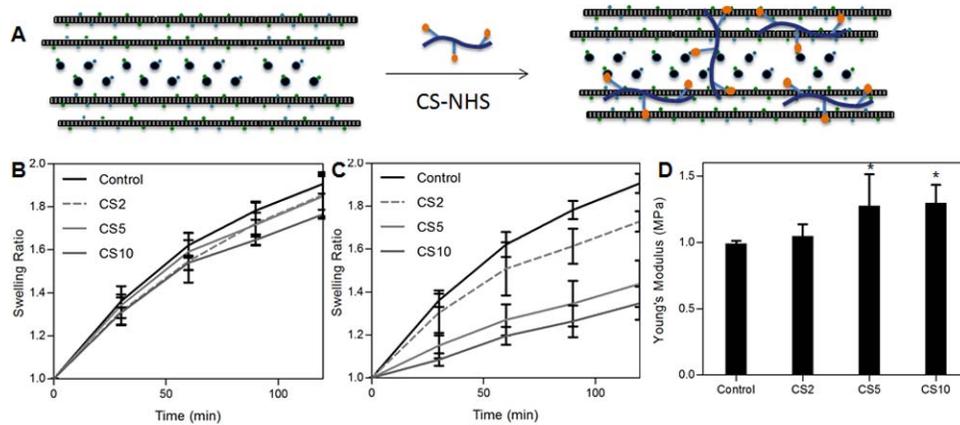
FIGURE 2. Normal corneal swelling and biomechanical properties after crosslinking. (**A**) CS-NHS reacts with collagen molecules and forms a strong covalent network. (**B**) The decrease of swelling ratio was in direct response to crosslinking time and CS-NHS concentration after 30 and 60 minutes of crosslinking; (**C**) the enhancement of corneal biomechanics was demonstrated with Young's modulus increase after 60 minutes of crosslinking. $N = 6$ in each group and *$P < 0.05$ compared to control corneas.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR)

Immediately following enzyme digestion for KC model corneas and treatment via crosslinking, corneas ($n = 6$ for each test condition) were flash frozen in liquid nitrogen and ground using precooled ceramic mortars and pestles. The powdered corneal tissue samples were treated with TRIzol (Thermo Fisher Scientific), and total RNA was collected using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). The mRNA was quantified using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific), and then reverse-transcribed using a High Capacity cDNA Kit (Thermo Fisher Scientific). Q-RT-PCR was performed with the SYBR Green qPCR Master Mix (Applied Biosystems, Foster City, CA, USA), and expression levels were normalized using the housekeeping gene *Gapdh*. The primers of the targeted genes are listed below.

Gapdh forward: 5′-CTCTGGAGTGGATGTT-3′
Reverse: 5′-CCATGGGTGGAATCATACTG-3′
Tnfα forward: 5′-GTAGTAGCAAACCCGCAAGT-3′
Reverse: 5′-GGTTGTCCGTGAGCTTCAT-3′
Mmp9 forward: 5′-AGTACCGAGGAAAGCCTACTT-3′
Reverse: 5′-TGCAGGATGTCAAAGTCTAC-3′
Aldh forward: 5′-GACGATAACTGCAGAGCACG-3′

Reverse: 5′-ACTCATTCGACAAGCAGACAG-3′
Biglycan forward: 5′-GCATCCCCAAAGATCTGCC-3′
Reverse: 5′-CAACTTGGAGTATCGGAGCAG-3′

Statistical Analysis

Student's *t*-test was used to statistically examine differences between normal corneas and crosslinked corneas in terms of collagen fibril diameter and fibril density. Swelling ratio, Young's modulus, and gene expression were analyzed with Prism 6.01 software (GraphPad Software, Inc., San Diego, CA, USA) using 1-way analysis of variation (ANOVA). All experiments were repeated twice, with $n = 6$ corneas in each experimental group. P values < 0.05 were considered statistically significant.

RESULTS

CS-NHS Penetration Into the Cornea

As CS is a large molecule (~25 kDa molecular weight), it was important to validate its penetration capacity into the corneal stroma before assessing its crosslinking efficacy. The NHS
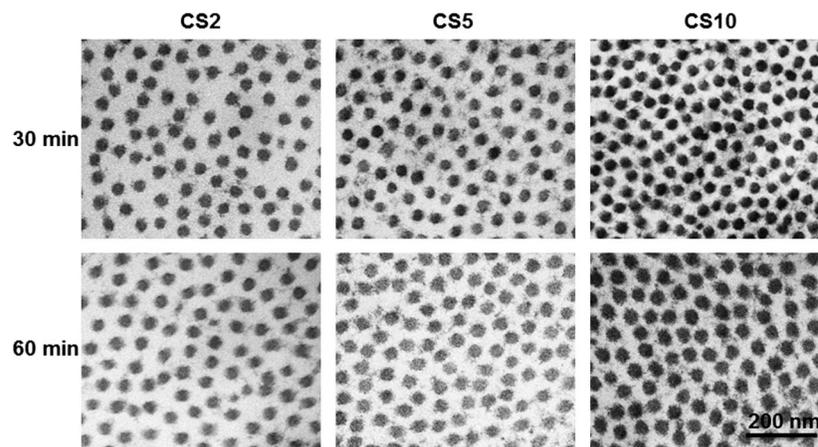
FIGURE 3. Normal corneal ultrastructures after crosslinking. Crosslinking time and crosslinker concentration significantly impact corneal ultrastructure. Collagen diameter and fibril density increased gradually with increase in crosslinking time (30 and 60 minutes) and concentration (from CS2 to CS10).
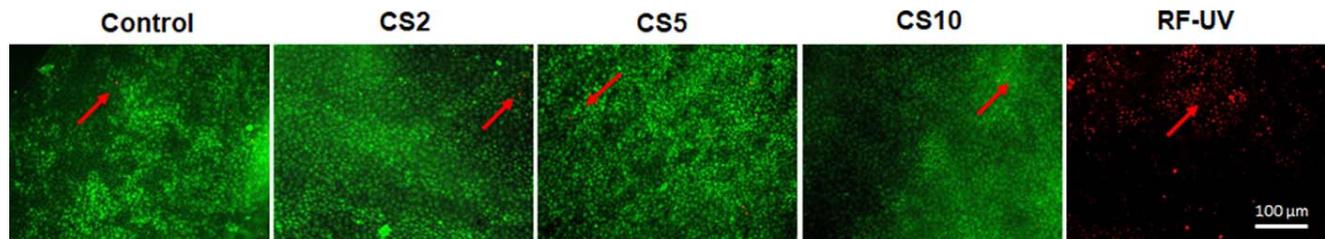
FIGURE 4. Cell viability after crosslinking. Corneal cell viability after CS-NHS crosslinking was evaluated with live–dead staining. The *red arrows* indicate apoptotic cells. No significant cell apoptosis was observed following 60 minutes of CS-NHS crosslinking for all concentrations. Positive control corneas were treated with standard RF-UV crosslinking, with 0.1% RF presaturation for 20 minutes and UVA crosslinking for 30 minutes.

group in CS-NHS and the amine group in the fluorescent dye TARMA react to form the TAMRA-labeled CS-NHS entity (Fig. 1A), which allows visualization of the penetration depth and intensity of CS-NHS in the corneal stroma. Corneas treated with CS-NHS-TARMA had a clear fluorescent band. The fluorescent intensity was stronger with longer treatment durations and higher CS-NHS concentrations (Fig. 1B). The mean fluorescent intensity at 100 μm in stroma increased significantly with crosslinking time and concentration (Fig. 1C). At 60 minutes of crosslinking, the penetration depth reached up to 275 μm (2 mg/mL crosslinker), 375 μm (5 mg/mL crosslinker), and 400 μm (10 mg/mL crosslinker), respectively (Fig. 1D), with an average total corneal thickness of over 700 μm resulting from swelling.

Impact of CS-NHS Crosslinking on Corneal Swelling and Biomechanics

CS-NHS reacts with amine groups in collagen and proteoglycans in the cornea, forming crosslinked covalent networks (Fig. 2A). Therefore, the effectiveness of the crosslinking treatment can be estimated by differences in corneal swelling ratios. The decrease in corneal swelling ratios after CS-NHS treatment indicated that CS-NHS was able to effectively crosslink corneal ECMs (Fig. 2B). A 30-minute treatment with 2 and 5 mg/mL CS-NHS slightly reduced the swelling ratio, while a 15% decrease in swelling ratio was observed with 10 mg/mL CS-NHS treatment. A 60-minute crosslinking treatment with CS-NHS significantly decreased the swelling ratio in all treatment groups. A decrease in swelling ratio of up to 35% in CS5 and CS10 groups was observed (Fig. 2C). To further characterize the enhancement of corneal mechanics through corneal crosslinking, Young's modulus of treated and untreated corneas was evaluated by tensile testing (Fig. 2D). After 60 minutes of crosslinking treatment, CS5 and CS10 groups demonstrated significantly higher Young's modulus that was up to 20% greater than in groups that did not receive crosslinking treatment.

Normal Corneal Ultrastructure After Crosslinking

Normal corneas were crosslinked to determine the optimal treatment time and crosslinker concentrations. Following CS-NHS crosslinking, treated corneas exhibited larger fibril diameters and densities compared to normal corneas (Fig. 3; Table). The formation of a tighter covalent network due to CS-NHS crosslinking increased fibril density when compared to that in normal corneas. CS5 and CS10 crosslinking demonstrated a significant increase in fibril density, even at lower crosslinking durations (30 minutes). Following crosslinking for 60 minutes, CS5 and CS10 groups also presented a larger mean fibril diameter of 55 ± 13.40 and 59 ± 11.56 nm, respectively, compared to 44.24 ± 11.89 nm in the untreated normal group. Overall, higher crosslinker concentrations and crosslinking

time significantly increased fibril diameter and density. The morphologic and mechanical data taken together suggest that CS5 (60 minutes) crosslinking provided significant mechanical reinforcement while minimally changing the collagen fibrillar dimensions compared to the native cornea. Therefore, CS5 was chosen as the optimal crosslinking condition for treatment of KC model corneas.

Cell Viability in CS-NHS Crosslinking

Corneal cells maintained viability after 60 minutes of cross-linking with few apoptotic cells (Fig. 4, red arrow) in all CS-NHS crosslinking concentrations used, as evidenced by qualitative live–dead staining. No visible difference among 2, 5, and 10 mg/mL CS-NHS crosslinking concentrations was observed, and there were no significant differences in cell viability compared to that in noncrosslinked cornea controls. The positive dead cell staining alone has been presented for RF-UV corneas due to the interference from RF autofluorescence in the 488-nm channel. The positive control demonstrated considerable cell apoptosis compared to the rest of the groups.

Ectatic Corneal Morphologies and Biomechanics Following CS-NHS Crosslinking

The efficacy of CS-NHS crosslinking versus the gold standard UV crosslinking was evaluated in an ectatic cornea model. KC model corneas exhibited damaged lamellar structures after enzyme treatment. Following CS-NHS crosslinking treatment, the corneas recovered the compact stromal structure seen in native tissue (Figs. 5A, 5B). No major histologic differences

TABLE. Ultrastructural Analysis of Corneas Before and After Cross-linking

	Fibril Diameter, nm	Density of Fibril, per 300 × 300 dpi
Normal	44.24 ± 11.89	16.6 ± 0.55
CS2 30 min	45.76 ± 10.57	17.2 ± 0.53
CS2 60 min	52.37 ± 9.42	18.4 ± 1.10*
CS5 30 min	49.32 ± 12.43	20.3 ± 0.74*
CS5 60 min	55.77 ± 13.40*	20.9 ± 0.98*
CS10 30 min	52.78 ± 13.23*	28.0 ± 1.02*
CS10 60 min	59.51 ± 11.56*	22.8 ± 0.89*
KC	45.38 ± 14.66	14.1 ± 0.68*
KC-UV 30 min	46.42 ± 11.39	19.1 ± 0.97†
KC-CS5 60 min	50.35 ± 13.74	19.6 ± 1.07†

Normal group refers to rabbit corneas without any treatment. Data presented as means ± standard deviation. Student's *t*-test was used to statistically examine differences.

* $P < 0.01$ compared with the normal corneas.

† $P < 0.01$ compared with the KC corneas.
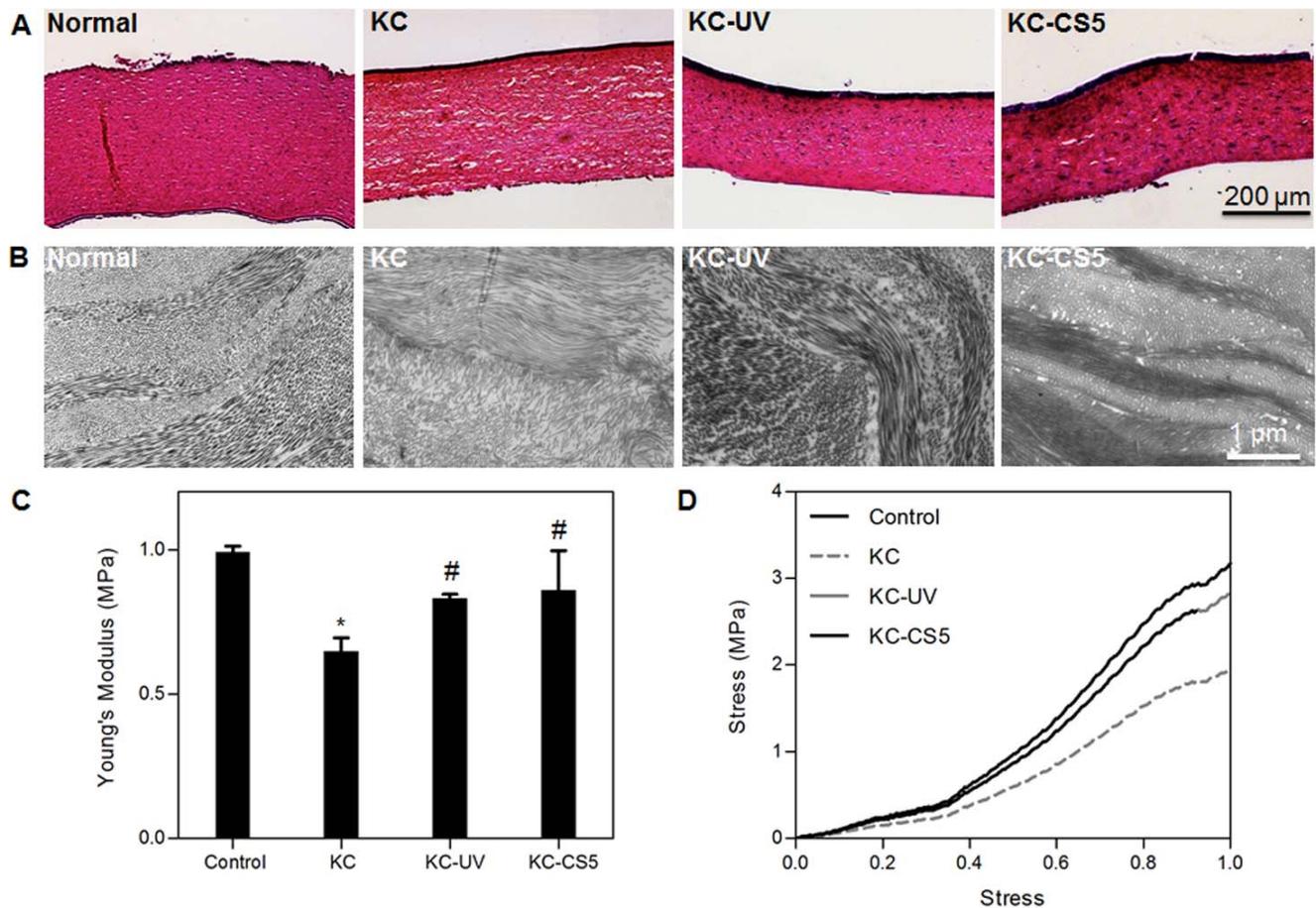
FIGURE 5. Ectatic corneal morphology and biomechanics after crosslinking. (**A**) Histologic images of corneas undergoing different treatments, and (**B**) TEM images at ×9700 magnification of KC corneas after crosslinking. Images from *left to right* represent normal, enzyme-treated (KC), UV crosslinked KC (KC-UV), and 5 mg/mL CS-NHS–treated KC (KC-CS5) corneas. (**C**) Young's modulus and (**D**) stress–strain curves of normal and ectatic corneas after crosslinking. $N = 6$ in each group; $*P < 0.05$ compared to normal corneas, and $\#P < 0.05$ compared to KC corneas.

were observed between UV and CS-NHS crosslinked explant corneas (Fig. 5A).

TEM imaging (Fig. 5B) corroborated that collagen fibers in KC model corneas were disrupted, with decreased fiber diameter and increased interfibrillar spacing. Treatment with CS-NHS and UV increased collagen fibril density and restored lamellar spacing. Analysis of TEM images of ectatic corneas demonstrated the efficacy of UV versus CS-NHS crosslinking. The standard 30-minute UV irradiation increased collagen density with no significant change in fibril diameter. On the other hand, 60 minutes of 5 mg/mL CS-NHS crosslinking increased corneal collagen fibril diameter and density (Table).

Stress–strain curves from uniaxial tensile testing demonstrated that, on average, KC model corneas had a 40% lower Young's modulus value when compared to normal corneas. Efficacy of UV crosslinking and CS-NHS crosslinking was comparable, and both treatments increased the Young's modulus of KC model corneas by approximately 40% (Figs. 5C, 5D).

Keratocyte Gene Expression After Crosslinking

Maintaining keratocyte phenotype without triggering proinflammatory gene activation is important to minimize clinical complications, such as corneal haze and scarring. The proinflammatory genes, *Tnfα* and *Mmp9*, as well as the keratocyte marker genes, *Aldh* and *Biglycan*, were evaluated in ex vivo rabbit corneas after UV and CS-NHS crosslinking (Fig. 6). Enzyme treatment (KC) did not significantly affect inflammatory gene expression; however, it downregulated keratocyte marker expression. UV crosslinking substantially upregulated inflammatory gene production (*Tnfα* and *Mmp9*) in both normal and KC model corneas. Conversely, CS-NHS crosslinking did not upregulate *Tnfα* expression, and, moreover, significantly downregulated *Mmp9* expression. Both UV and CS-NHS crosslinking enhanced *Aldh* expression in KC model corneas. Crosslinking and enzyme treatment reduced *Biglycan* expression, but CS-NHS crosslinking preserved higher *Biglycan* expression as compared to UV crosslinking in KC model corneas (Fig. 6). Therefore, CS-NHS crosslinking induced less inflammation and better preserved keratocyte phenotype when compared to UV crosslinking.

DISCUSSION

UV light–initiated corneal crosslinking, the leading clinical strategy to delay or even halt KC progression,[22] utilizes photogenerated free radicals to strengthen intrafibrillar binding within collagen fibrils.[23] However, the cytotoxicity owing to extended periods of UV irradiation causes keratocyte depletion and can also potentially harm corneal endothelial cells.[24] Glycosaminoglycans, such as CS, have been proven to provide biological benefits in corneal tissue engineering, such as promoting corneal wound healing.[19] Therefore, CS was
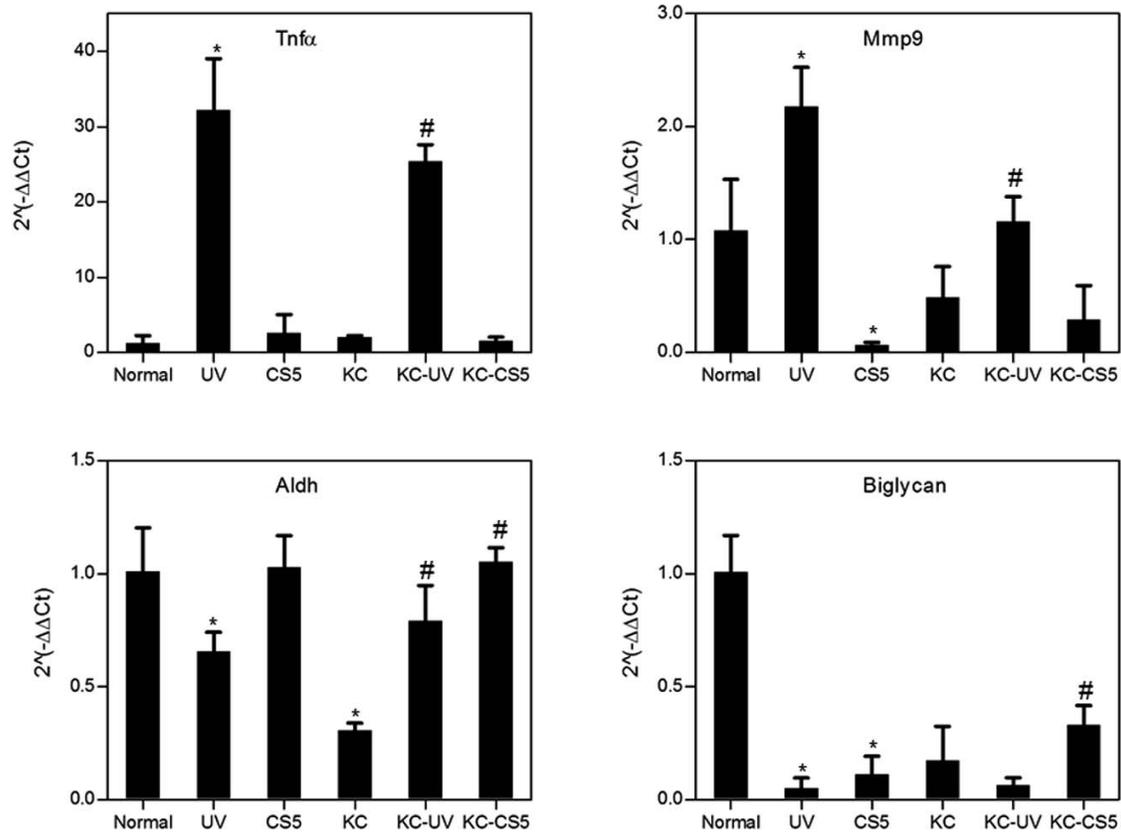
FIGURE 6. Gene expression of keratocytes after crosslinking. Inflammatory markers *Tnfα* and *Mmp9*, keratocyte markers *Aldh* and *Biglycan* were evaluated among normal, KC, and crosslinked KC corneas (KC-UV and KC-CS5). $N = 6$ in each group, *$P < 0.05$ compared to normal corneas; #$P < 0.05$ compared to KC corneas.

designed to be the backbone of the biocompatible crosslinker by modifying it with NHS, an amine-reactive moiety, which allowed us to apply CS-NHS for collagen fibril crosslinking in animal corneas.

Corneal ectasia causes stromal thinning, resulting in weakening and distortion of the underlying stromal collagen ultrastructure.[25] Therefore, a major indicator for successful crosslinking is the restoration of the collagen fibril arrangement and spacing in diseased corneas.[26] The mechanism of UV crosslinking has been reported to be mostly intrafibrillar, as evidenced by previously reported small- and wide-angle X-ray scattering results, demonstrating no changes to interfibrillar spacing after crosslinking. Thus, crosslinking occurred predominately at the collagen fibril surface and in the protein network surrounding the collagen, and the UV crosslinking slightly increased fibril diameter, but did not reduce swelling ratio.[27] In our present study, after 60 minutes of CS-NHS crosslinking, the swelling ratio was reduced and, more importantly, when carried out with high crosslinker concentrations, collagen fibril diameter and fibril density were significantly increased. This indicates that with multiple NHS functional groups on a single CS chain, crosslinks between CS-NHS and collagen fibrils occur at both intrafibrillar and interfibrillar levels.

Our previously established KC model[21] demonstrated that controlled enzymatic treatment of the corneal ECM triggers collagen fibril degradation, resulting in an increase in interfibrillar spacing and corneal thinning. The KC model corneas also showed lamellar undulations representing disruption in corneal collagen organization, mimicking physical and ultrastructural changes in severely ectatic corneas. Tissue stiffness of normal corneas increased significantly after cross-

linking with CS-NHS, particularly at high crosslinker concentrations. CS-NHS crosslinking also largely ameliorated the loss of mechanical integrity of enzymatically weakened KC model corneas, as observed via mechanical testing. Therefore, covalent crosslinking at the fibrillar level, which decreases interfibrillar spacing and increases fibril diameter, led to an increase in bulk stiffness. Histologic analysis confirmed that increase in fibril thickness and tighter packing of collagen fibrils resulted in corneal densification, as evidenced by change in the gross thickness.

Since the reinforcement of corneal biomechanics is the overarching goal of corneal crosslinking, it was important to compare CS-NHS crosslinking with traditional UV-based crosslinking, which demonstrated some of the same outcomes.[26] UV and CS-NHS crosslinking treatments on KC model corneas resulted in a statistically significant increase in tissue stiffness compared to untreated KC model corneas. Moreover, the difference in efficacy of treatment between UV and CS-NHS crosslinking was not significant.

Nevertheless, any improvement to mechanical stability or vision is of no clinical value if the safety of the treatment is not ensured. Although KC is generally considered a noninflammatory disease, previous studies have shown that the levels of degradative enzymes, such as *Mmp9*, were elevated in keratoconic corneas.[28] Moreover, UV crosslinking also triggers proinflammatory cytokine production, including *Tnfα*, which affects cell signaling in corneal stroma and eventually induces keratocyte apoptosis and, in severe cases, permanent corneal scarring.[29,30] We therefore examined the expression of proinflammatory markers *Tnfα* and *Mmp9*, as well as key keratocyte markers *Aldh* and *Biglycan*, in normal and KC

model corneas subjected to the different crosslinking techniques. UV crosslinking treatment in both normal and KC model corneas elicited drastic *Tnfα* and *Mmp9* upregulation, direct evidence of the inflammatory response caused by UV irradiation. In comparison, CS-NHS crosslinking did not show upregulation of these inflammatory markers and, in fact, elicited a downregulation of *Mmp9*. Furthermore, CS-NHS crosslinking was able to protect the keratocyte phenotype by maintaining *Aldh* expression. *Biglycan* was downregulated in all crosslinked and KC model groups, but CS-NHS crosslinked corneas had higher *Biglycan* expression in comparison to UV crosslinked corneas. The gene expression results demonstrate the biocompatible and potentially protective nature of CS-NHS as a corneal crosslinker.

In summary, CS-NHS provides a safe and effective potential alternative option to treat or prevent corneal ectasia. The enzyme-mediated ex vivo model we utilized to characterize crosslinking efficacy presented loss of mechanical strength, loss of total protein, and ultrastructural damage, which represented several characteristics of KC. There are caveats to the KC model that may preclude its ability to mimic in vivo function and efficacy of CS-NHS crosslinking. Keratoconus is an idiopathic disease and can have several potential pathophysiologies. However, this ex vivo model still provides an affordable and versatile approach to evaluate new crosslinking methods before initiating preclinical animal studies. Further, enucleated ex vivo eyes undergo swelling, which may impair the epithelial barrier function, allowing deeper CS-NHS penetration. Additionally, CS-NHS also potentially binds to the epithelial layer ECM. Therefore, although the study was carried out without epithelial cell removal, the transepithelial penetration of CS-NHS needs further examination in vivo. Finally, due to the high reactivity of the NHS functional group, CS-NHS has to be prepared fresh before use and could be challenging to maintain on the eye for an extended duration. Future in vivo studies will be designed after careful evaluation of the transepithelial diffusion rate and crosslinking efficacy of CS-NHS.

In conclusion, the ability to slow corneal ectasia and KC progression should not come at the cost of damage or injury to healthy cells in the cornea. To circumvent potential harm from exposure to UV in traditional UV crosslinking techniques, we developed a safe and biocompatible crosslinking approach that maintains the efficacy standards of the traditional treatment. CS-NHS induced both intrafibrillar and interfibrillar crosslinking, promoted lamellar stabilization, restored mechanical properties in disease model corneas, and reduced proinflammatory gene (*Tnfα* and *Mmp9*) expression while maintaining higher levels of keratocyte marker gene expression compared to UV crosslinking. In conclusion, the CS-NHS crosslinking technique was demonstrated to be an effective tool to repair tissue degeneration in an ex vivo rabbit model, presenting several potential advantages over current and traditional therapies. Future studies of the CS-NHS crosslinking method will be carried out to test the efficacy of the crosslinking technique and safety in vivo.

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