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Nonlinear optical corneal collagen crosslinking of ex vivo rabbit eyes

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Abstract

PURPOSE—To determine whether riboflavin-induced collagen crosslinking (CXL) could be precisely achieved in the corneal stroma of ex vivo rabbit eyes using nonlinear optical excitation with a low numerical aperture lens and enlarged focal volume.

SETTING—Gavin Herbert Eye Institute, University of California Irvine, Irvine, California, USA.

DESIGN—Experimental study.

METHODS—The corneal epithelium was removed and the corneas were soaked in 0.5% riboflavin solution. Using a 0.1 numerical aperture objective, a theoretical excitation volume of 150 μm × 3 μm was generated using 1 W of 760 nm femtosecond laser light and raster scanned with 4.4 μm line separation at varying effective speeds over a 4.50 mm × 2.25 mm area. Corneal sections were examined for collagen autofluorescence.

RESULTS—Collagen autofluorescence was enhanced 2.9 times compared with ultraviolet-A (UVA) CXL. Also, increasing speed was linearly associated with decreasing autofluorescence intensity. The slowest speed of 2.69 mm/s showed a mean of 182.97 μm ± 52.35 (SD) long autofluorescent scan lines axially in the central cornea compared with 147.84 ± 4.35 μm for UVA CXL.

CONCLUSIONS—Decreasing dwell time was linearly associated with decreasing autofluorescence intensity, approaching that of UVA CXL at a speed of 8.9 mm/s. Using an effective speed of 8.9 mm/s, nonlinear optical CXL could be achieved over a 3.0 mm diameter area in fewer than 4 minutes. Further development of nonlinear optical CXL might result in safer, faster, and more effective CXL treatments.

Keratoconus is a noninflammatory disease characterized by progressive weakening of the cornea.[1,2] This leads to corneal thinning, a cone-shaped protrusion, and declining visual acuity. Keratoconus affects 1 out of every 2000 people in the general population[3] and is the leading cause of corneal transplantation surgery.[4] It is thought that mechanically strengthening the weakened tissue by cross-linking the collagen fibers can slow or even

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reverse the progression of the disease. One of the newest methods to treat keratoconus, introduced by Spoerl et al.,[5,6] is a crosslinking therapy using ultraviolet-A (UVA) light and corneal collagen crosslinking (CXL).

For this procedure, the cornea is soaked in the photosensitizer riboflavin with the epithelium removed to allow it to penetrate into the stroma and is exposed to 5.4 mJ of 370 nm UVA light with an irradiance[7] of 3 mW/cm^2. The irradiated riboflavin generates free radicals, causing covalent bonds to form between corneal collagen molecules,[8] mechanically stiffening the tissue. Because UVA irradiation and free radical formation are potentially harmful to living tissues, irradiation parameters have been selected to minimize cellular damage to the corneal endothelium, lens, and retina.[9] With the parameters listed above, the irradiance at the endothelial level is one half the known threshold for damage in this tissue and deeper structures as long as the cornea is at least 400 μm thick.[10] Unfortunately, this limits CXL to the anterior cornea and to patients with corneal thicknesses of 400 μm or more,[11] allowing for little control over the treated area and decreasing the number of patients who can benefit from this procedure.

In previous studies,[12] it has been shown that compressed type I collagen hydrogels can be mechanically stiffened to a degree similar to that achieved by UVA CXL by using a nonlinear optical approach to photoactivate riboflavin in precisely defined regions. Nonlinear optical CXL uses highly focused 760 nm femtosecond laser light to induce 2-photon excitation of riboflavin in well-defined focal volumes. Unlike single-photon excitation used for UVA CXL, only the riboflavin within the 2-photon focal volume is excited and generates oxygen free radicals, allowing for very localized and depth-controlled crosslinking. The focal volume can then be scanned throughout the cornea to treat a much larger volume.

Although Chai et al.[12] showed this technique's ability to mechanically stiffen collagen hydrogels in a much more precise manner than achievable by UVA CXL, its highly focused laser light required multiple passes at different depths to achieve a large CXL volume. This requires a time commitment of multiple hours and limits its clinical practicality. Adjusting the scanning parameters, mainly focal size and scanning speed, to optimize the approach could address these concerns. Based on equations outlined by Zipfel et al.,[13] a lower numerical aperture should produce increased 2-photon focal volume. With the correct speed, a larger focal volume could theoretically produce results similar to those reported by Chai et al.[12] in a much shorter time.

The purpose of this study was to determine whether nonlinear optical CXL could be achieved in intact ex vivo rabbit eyes using a low numerical aperture objective for rapid single-pass enlarged focal volume CXL.

MATERIALS AND METHODS

Preparation of Eyes

Seventeen rabbit eyes were included in this study. They were shipped to the laboratory fresh (Pel-Freez Biologicals), rinsed in minimal essential medium (Invitrogen), and placed in a

12-well tissue culture plate filled with the same medium to just below the corneal–scleral limbus. They were then incubated in a 5% carbon dioxide (CO_2) humidified incubator at 37°C for at least 1 hour before further preparation. After incubation, a small spot was tattooed at the limbus of each eye to serve as a fiducial marker during treatment using Coomassie blue dye and an 8.0 mm diameter area of the epithelium was scraped from the center of each cornea.

Eyes were segregated into 4 groups. Control 1 eyes (2 eyes) were left completely alone, epithelium intact, no soaking, and no treatment. Control 2 eyes (2 eyes) had the epithelium removed and were soaked in riboflavin-5-phosphate 0.5% solution (Sigma-Aldrich Co.) in phosphate-buffered saline (PBS) (pH 7.2) with high-fraction dextran 20%, molecular weight of 450 to 650 K (Sigma-Aldrich Co.), by dripping the solution onto the corneas every 2 minutes for 30 minutes, but they were not irradiated in any way. Control 3 eyes (2 eyes) were soaked in the same manner in riboflavin 0.1% solution with dextran 20% and placed under a 370 nm UVA lamp, with continued dripping throughout the treatment. The fourth group consisted of 11 eyes soaked in riboflavin (0.5%)–dextran (20%) solution and treated with expanded 2-photon focal volume nonlinear optical CXL at various effective scan speeds. A higher concentration of riboflavin was used in this group because it has been suggested that this can offset lower dwell times.[13,14]

Treatment of Eyes

Prepared eyes were held inside of a glass coverslip bottom Petri dish (Mettek Corp.) and mounted over the objective of a confocal microscope (LSM 510, Carl Zeiss Meditec AG) as shown in Figure 1. The fiducial marker was oriented to the far right and in line with the scan lines to orient the cornea for tissue sectioning and imaging. Using a ×4, 0.1 numerical aperture Apochromat objective (Carl Zeiss Meditec AG), a theoretical 2-photon excitation volume calculated to be 150 μm axial and 3 μm lateral, using the equations for 2-photon focal volume outlined by Zipfel et al.,[13] was generated with 1 W of 760 nm femtosecond laser light (Chameleon, Coherent, Inc.). Corneas were then raster scanned with 4 μm line separation at pixel dwell times varying from 204 μs to 1.63 ms over a 4.50 mm × 2.25 mm area in the central anterior cornea. The effective speed was altered for each eye by changing the combination of the scanning speed and the number of times each line was scanned. The UVA CXL control eyes were irradiated with 370 nm UVA light at 3 mW/cm^2 for 30 minutes as described by Spoerl et al.[10] After treatment, the corneas were excised from the whole eye and fixed overnight in paraformaldehyde 2.0% (PFA) (Mallinckrodit Baker, Inc.) in PBS at 4°C.

Sectioning

Corneas were then trimmed and embedded for sectioning. The top two thirds of each cornea was removed perpendicular to the blue marker and embedded straight edge down in low-melting-point agarose 10.0% (Lonza). The samples were then sectioned perpendicular to the blue marker and therefore perpendicular to the direction of the scan to a thickness of 250 μm using a vibratome (Campden Instruments Ltd.). Each slice was floated off and placed in a single well of PFA 2.0% in PBS in a 24-well plate and kept at 4°C until ready for imaging.

Imaging and Quantification

Sections closest to the center of the cornea were then examined for collagen autofluorescence induced by CXL as described by Chai et al.[11] Sections were scanned with the LSM 510 confocal microscope using 2-photon excitation at 760 nm with a ×40 oil immersion objective (1.3 numerical aperture). Emissions were collected in the region of 400 to 450 nm using the Zeiss Meta Detector. Collagen autofluorescence was then quantified using Metamorph image-processing software (Molecular Devices, LLC). Once imported into the software, three 200 pixel × 200 pixel regions of interest were defined in both the treated and untreated portions of each section. The average pixel intensity of each region was reported by the software as an arbitrary value between 0 and 256. The 3 regions within the treated portion were averaged, as were the 3 within the untreated portion. The resulting averages were subtracted from each other to obtain an increase in collagen autofluorescence intensity of the treated region in a given section.

Detection of Chemical Crosslinking

To verify that chemical crosslinking was actually occurring during the nonlinear optical CXL treatment, compressed collagen hydrogels were treated and run through a sodium dodecyl sulfate (SDS) page polyacrylamide gel. Rat tail type I collagen (3 mg/mL)[1] was neutralized with 1 N sodium hydroxide (Acros Organics), poured into a 24-well plate, and left to polymerize at 37°C in a CO_2 5% humidified incubator to form a hydrogel. Gels (3 mL each) were then compressed into sheets roughly 150 μm thick as previously described,[12] and 2.5 mm diameter disks were taken from each sheet. The disks were soaked in riboflavin solution, 0.1% for UVA CXL and 0.5% for nonlinear optical CXL, with no dextran[2] for 30 minutes, and then mounted and treated in a 4.5 mm × 4.5 mm area in the manner described above for the eyes. The focus was aimed at the center of the gel in all directions to achieve maximum crosslinking. Treated gels were then solubilized by boiling in Laemmli SDS buffer, and the resulting solutions were run through an electrophoresis gel to compare the size of the molecules in the various samples.

RESULTS

Figure 2 shows the images taken of the collagen autofluorescence in various eyes. Control eyes soaked in riboflavin alone showed no collagen autofluorescence (Figure 2, *A*), while control eyes receiving UVA CXL for 30 minutes showed increased collagen autofluorescence (Figure 2, *B*). Corneas receiving nonlinear optical CXL and treated at the slowest speed (highest dwell time), with a line separation of 17.6 μm for visual demonstration of the 2-photon focal volume, are shown in Figure 2, *C*. The highest intensity of the collagen autofluorescence signal was measured in corneas treated with nonlinear optical CXL at 2.69 mm/s speed and 4.4 μm line separation (Figure 2, *D*) and showed 2.9 times greater signal than that achieved by standard UVA CXL and reached a comparable depth, with means of 182.97 μm ± 52.53 (SD) for nonlinear optical CXL and 147.84 ± 4.35 μm for UVA CXL. The line width of the nonlinear optical CXL-treated eyes was also measured to be 3 μm. These measurements closely match the theoretical calculations for the size of a 2-photon focal spot in corneal tissue of a 760 nm beam focused through a 0.1 numerical aperture objective.

The arbitrary intensity values, calculated from images similar to those in Figure 2, are compared in Figure 3. This graph shows a linear increase in collagen autofluorescence intensity with increasing pixel dwell time with an R^2 value of 0.9689 after linear regression was performed. Also, nonlinear optical CXL collagen autofluorescence intensity approached UVA CXL collagen autofluorescence intensity at a dwell of 494 μs corresponding to an effective speed of 8.9 mm/s, suggesting that effective therapeutic crosslinking can be achieved at even faster speeds than the standard UVA CXL treatment allows. Analysis of variance performed on this data also showed a significant difference between the different dwell times ($P = .004$).

To further verify these results, compressed collagen hydrogels were treated with both nonlinear optical CXL and UVA CXL and then run through an SDS page electrophoresis gel (Figure 4). After CXL in the UVA treatment and nonlinear optical treatment, all 3 collagen bands,—β, α1, and α2,—showed a decrease in intensity that was visually detectable. The intensity of the β, α1, and α2 bands was calculated to be 10.4%, 26.3%, and 9.1% of the control (Figure 4, *A,* lane 1) when subjected to 10 minutes of UVA treatment, shown in lane 2 of Figure 4, *A.* Nonlinear optical CXL treatment at the slowest speed, shown in lane 1 of Figure 4, *B,* had similar results of 8.9%, 41.6%, and 9.3% compared to the control. The collagen gels treated at faster speeds, shown in lanes 2 and 3 of Figure 4, *B,* were less affected by the procedure, showing 44.2%, 55.7%, and 81.5% of the control for an effective speed of 5.37 mm/s and 27.5%, 52.5%, and 61.4% of the control for an effective speed of 10.74 mm/s.

DISCUSSION

Chai et al.[12] showed that multiphoton activation of riboflavin using 760 nm femtosecond laser focused infrared light through a 0.75 numerical aperture lens produces mechanical stiffening of collagen hydrogels comparable to that shown for standard UVA CXL using 370 nm light. The high numerical aperture, however, produces crosslinking in a very small volume, 0.5 μm lateral and 2.5 μm axial. Although this increases precision, potentially addressing the limitations of UVA CXL, the length of the procedure is impractical in a clinical setting.

This study found that it is possible to achieve a similar degree of mechanical stiffening by using a low numerical aperture lens that produces a 3 orders of magnitude larger 2-photon focal volume. Using a 0.1 numerical aperture lens, single-pass nonlinear optical CXL induced greatly enhanced collagen autofluorescence within ex vivo rabbit corneas that was of equal depth in the cornea compared with standard UVA CXL. Chemical data also verified the ability of nonlinear optical CXL to induce molecular crosslinking of collagen molecules, as evidenced by the loss of the individual collagen α1 and α2 chains that form the triple helical collagen molecule upon polymerization. Zhang et al.[15] showed that collagen subjected to UVA CXL had a marked decrease in the amount of α and β chains when subjected to gel electrophoresis. In our study, SDS page electrophoresis gel was performed with both an untreated control and a standard UVA-treated gel for comparison. Because crosslinking of the collagen results in much larger molecules, the smaller molecules, clearly visible in the untreated control collagen hydrogel, are fewer or nonexistent in crosslinked

collagen. This suggests that after nonlinear optical crosslinking, covalent bonds form between α1 and α2 chains, creating larger molecular weight molecules that are unable to migrate into the gel.

Interestingly, oxygen concentration within the cornea has been shown to be depleted within 15 seconds of the onset of standard UVA CXL treatment, returning to normal levels 3 to 4 minutes after crosslinking. This depletion is much more dramatic in accelerated UVA CXL (30 mW/cm^2), occurring within 5 seconds, and has thus hampered the ability to significantly shorten crosslinking time for individual patients.[8] It is likely that nonlinear optical CXL follows the same principles as traditional UVA CXL with regard to oxygen dependency because it is still dependent on riboflavin excitation. However, the small excitation volume and shortened excitation time using nonlinear optical CXL might limit oxygen depletion in the surrounding tissue, thus facilitating faster corneal crosslinking, unlike UVA CXL. Specifically, nonlinear optical CXL only excites a very small focal volume of tissue for no more than 1.6 milliseconds, thus limiting oxygen depletion and/or allowing sufficient oxygen diffusion from the surrounding tissue. These factors might help explain why nonlinear optical CXL might be able to achieve increased collagen autofluorescence, suggesting greater corneal CXL in a shorter time compared with UVA CXL.

We found that a larger 2-photon focal volume can provide rapid nonlinear optical CXL that could be used to treat a circular 3.0 mm diameter area of the cornea in fewer than 4 minutes. Besides more rapid crosslinking, nonlinear optical CXL has additional advantages over UVA CXL because the excitation beam can be scanned over the cornea, unlike that of UVA CXL. We propose that custom corneal crosslinking modeled on the patient's preoperative corneal topography could be achieved by modifying both 2-photon focal volume and laser intensity during the scanning procedure. In this manner, nonlinear optical CXL could produce regional differences in corneal stiffening that might more precisely correct corneal astigmatism resulting from keratoconus. In addition, the ability to control the depth of crosslinking would allow this procedure to be performed on much thinner corneas than currently recommended for UVA CXL and could also be used to treat post-laser in situ keratomileusis ectasia. Further development of nonlinear optical CXL might therefore provide not only safer and more effective corneal crosslinking but also potentially greatly expand the capability of crosslinking to treat refractive errors.

In conclusion, nonlinear optical CXL can provide a controlled, safe, and effective treatment for keratoconus and other forms of corneal ectasia.[12] This study found that it is possible to rapidly perform this treatment using a low numerical aperture objective, making it a clinical possibility. Future work has to be performed to fully assess the effects of this treatment.

Acknowledgments

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WHAT WAS KNOWN

- The Dresden protocol for CXL and stiffening the corneal stroma uses UVA light to activate riboflavin within the corneal stroma to induce production of oxygen-free radicals, leading to covalent crosslinking of collagen fibrils.

- This method is clinically effective but it has very little precision, has a long procedure time, and poses the risk for damaging the corneal endothelium.

WHAT THIS PAPER ADDS

- Use of nonlinear optical excitation of riboflavin by infrared 760 nm femtosecond laser light achieved faster and higher precision corneal CXL.

- Nonlinear optical excitation limited photoactivation of riboflavin to the focal volume as defined by the delivery optics allowing for highly controlled crosslinking in the *x*, *y*, and *z* directions.
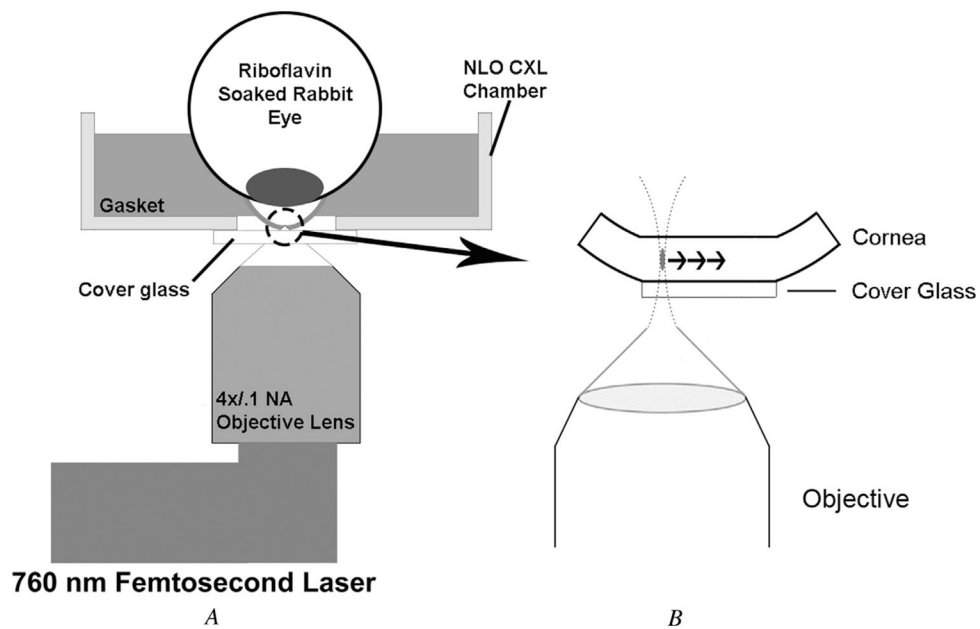
Figure 1.
Setup of nonlinear optical CXL-treated eyes. For these experiments, prepared eyes were positioned above the ×4, 0.1 numerical aperture objective of a confocal microscope (*A*). A spot was generated using 760 nm femtosecond laser light focused approximately 150 μm into the corneal tissue (*B*). The spot was then raster scanned over 2 tiles, totaling a 2.25 mm × 4.50 mm area (NLO CXL = nonlinear optical collagen crosslinking; NA = numerical aperture).
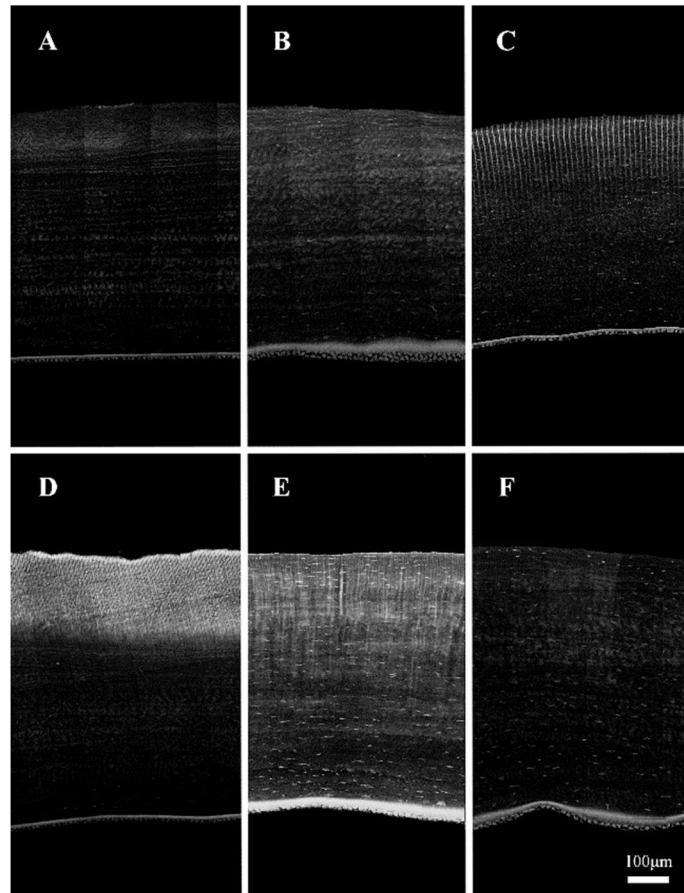
Figure 2.

Collagen autofluorescence images of corneal sections. *A*: A collagen autofluorescence image of an eye that had its epithelium removed and was soaked in riboflavin 0.5%, dextran 20% solution for 30 minutes but was not treated with either CXL method. *B*: A collagen autofluorescence image of an eye treated with the standard UVA CXL protocol. *C* to *F*: Eyes that were all treated with nonlinear optical CXL with varying parameters. The section shown in *C* had a 2.69 mm/s speed (1638.4 μs pixel dwell time) and line separation of 17.6 μm (all others had a line separation of 4.4 μm). The section shown in *D* was also treated with a speed of 2.69 mm/s, *E* with a speed of 5.37 mm/s (819.2 μs dwell), and *F* with a speed of 10.74 mm/s (409.6 μs dwell).
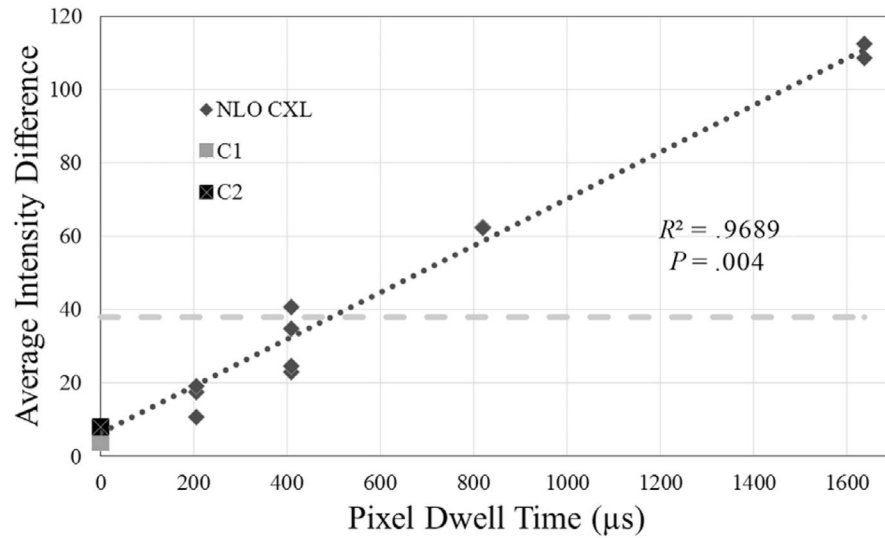
Figure 3.

Mean intensity difference of collagen autofluorescence between treated sections and untreated sections in nonlinear optical and UVA CXL. The mean intensity increase, arbitrary units, was plotted for control eyes and treated eyes to compare the effects of increasing dwell time (which corresponds to decreasing scanning speed) on collagen autofluorescence. The data were found to match the line $y = 0.0636\times + 6.5323$ with an R^2 value of 0.9689 and a significance of $P = .004$. The mean collagen autofluorescence for UVA-treated eyes was plotted as a horizontal dashed line. Collagen autofluorescence from nonlinear optical CXL exceeds this amount at dwell times greater than 494 μs, corresponding to a speed of 8.9 mm/s (C1 = control 1; C2 = control 2; NLO CXL = nonlinear optical collagen crosslinking.

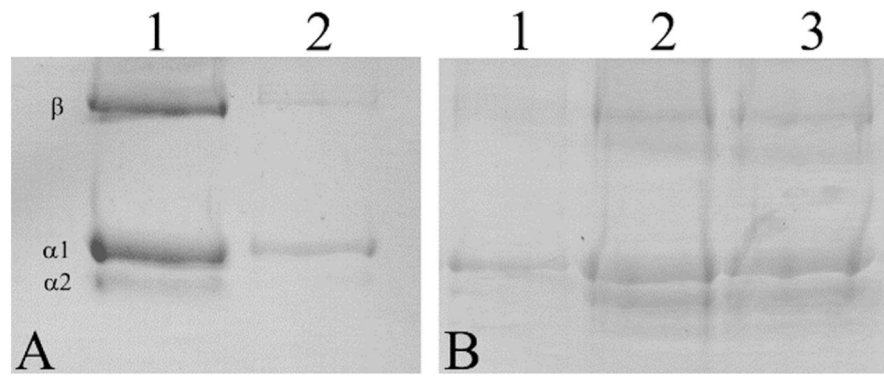**Figure 4.**
The SDS page of UVA CXL-treated and nonlinear optical CXL-treated compressed collagen gel. Panel A shows an SDS page of compressed collagen hydrogels treated using UVA CXL. Lane 1 shows a control collagen hydrogel, with no CXL. Lane 2 shows a collagen hydrogel treated with UVA CXL for 10 minutes. Panel B shows a collagen hydrogel treated with nonlinear optical CXL. Lanes 1, 2, and 3 represent hydrogels treated with increasing effective speeds of 2.69 mm/s, 5.37 mm/s, and 10.74 mm/s, respectively.