Assessment of UVA-Riboflavin Corneal Cross-Linking Using Small Amplitude Oscillatory Shear Measurements

Ioannis M. Aslanides,[1] Claudia Dessi,[2] Panagiotis Georgoudis,[1] Georgios Charalambidis,[3] Dimitris Vlassopoulos,[2] Athanassios G. Coutsolelos,[3] George Kymionis,[4] Achyut Mukherjee,[1,5] and Theofanis N. Kitsopoulos[6]

[1]Emmetropia Mediterranean Eye Clinic, Crete, Greece
[2]Department of Materials Science and Technology, University of Crete, and Institute of Electronic Structure & Laser, Foundation for Research and Technology Hellas (FORTH), Crete, Greece
[3]Department of Chemistry, Laboratory of Bioinorganic Chemistry, University of Crete, Crete, Greece
[4]Department of Ophthalmology, School of Medicine, University of Athens, Athens, Greece
[5]Cardiff University School of Optometry, South Glamorgan, United Kingdom
[6]Department of Chemistry, University of Crete, and Institute of Electronic Structure and Laser, Foundation for Research and Technology-Hellas, Crete, Greece

Correspondence: Ioannis M. Aslanides, Emmetropia Mediterranean Eye Institute, Plateia Eleftherias 44, Heraklion, 71201, Crete, Greece; i.aslanides@emmetropia.gr.

IMA, CD, PG, and GC contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: August 14, 2015
Accepted: March 6, 2016

Citation: Aslanides IM, Dessi C, Georgoudis P, et al. Assessment of UVA-riboflavin corneal cross-linking using small amplitude oscillatory shear measurements. *Invest Ophthalmol Vis Sci*. 2016;57:2240–2245. DOI:10.1167/iovs.15-17956

Purpose. The effect of ultraviolet (UV)–riboflavin cross-linking (CXL) has been measured primarily using the strip extensometry technique. We propose a simple and reliable methodology for the assessment of CXL treatment by using an established rheologic protocol based on small amplitude oscillatory shear (SAOS) measurements. It provides information on the average cross-link density and the elastic modulus of treated cornea samples.

Methods. Three fresh postmortem porcine corneas were used to study the feasibility of the technique, one serving as control and two receiving corneal collagen cross-linking treatment. Subsequently, five pairs of fresh postmortem porcine corneas received corneal collagen cross-linking treatment with riboflavin and UVA-irradiation (370 nm; irradiance of 3 mW/cm^2) for 30 minutes (Dresden protocol); the contralateral porcine corneas were used as control samples. After the treatment, the linear viscoelastic moduli of the corneal samples were measured using SAOS measurements and the average cross-linking densities extracted.

Results. For all cases investigated, the dynamic moduli of the cross-linked corneas were higher compared to those of the corresponding control samples. The increase of the elastic modulus of the treated samples was between 122% and 1750%. The difference was statistically significant for all tested samples ($P = 0.018$, 2-tailed t-test).

Conclusions. We report a simple and accurate methodology for quantifying the effects of cross-linking on porcine corneas treated with the Dresden protocol by means of SAOS measurements in the linear regime. The measured dynamic moduli, elastic and viscous modulus, represent the energy storage and energy dissipation, respectively. Hence, they provide a means to assess the changing physical properties of the cross-linked collagen networks after CXL treatment.

Keywords: corneal collagen cross-linking, rheology, small amplitude oscillatory shear measurements, linear viscoelasticity, cross-link density, elastic modulus

Corneal stroma is a hydrated structure composed of collagen fibrils with uniform diameter arranged in flat bundles known as lamellae and distributed in a pseudo-hexagonal arrangement, proteoglycans filling the space between collagen fibrils, and the interstitial fluid.[1–3] The cornea biomechanical properties are derived from the intricate and pseudo-regular matrix structure. The decrease of corneal mechanical stability has a critical role in the onset and progression of keratoconus and postlaser-assisted in situ keratomileusis (LASIK) ectasia.[4] Corneal collagen cross-linking (CXL) was first described by Wollensak et al.[5] and is the treatment of choice to stop the progression of keratoconus and ectatic disorders by increasing corneal stiffness.

Corneal collagen cross-linking increases the number of covalent bonds[5,6] (i.e., number of cross-links), but in corneal tissue, the nature of the participants has not been elucidated. The increase in the number of cross-links is directly reflected in elastic modulus increase of the corneal tissue.[7]

The biomechanical behavior of corneal tissue is complex to capture since its stromal microstructure is inhomogeneous and anisotropic[8] with a hyperelastic behavior even under low stress load. In this respect, mechanical parameters of corneal tissues have been studied via strip extensometry,[9–12] pressure inflation,[13] unconfined compression,[14] and inflation testing.[15,16] Technical challenges and reproducibility issues in particular limit strip extensometry. An inherent problem with this technique is alteration of physical properties during processing, particularly sectioning and hydration.

Compared to strip extensometry, shear rheologic techniques have not received much attention. These techniques can

Investigative Ophthalmology & Visual Science

provide reliable data and, more importantly, are capable of directly bridging the gap between macroscopic mechanical properties of corneal tissue and its microstructure. Indeed, to the best of our knowledge only few studies have applied rheologic protocols for the study of corneal biomechanics.[17–19] However, the full characterization of these rheologic measurements with regards to quantitative assessment of the degree of cross-linking of the corneal tissues has not been reported.

We present an experimental study where the viscoelastic behavior and biomechanical effect on network properties of the resultant CXL cross-linked corneal tissue are investigated by means of small amplitude oscillatory shear (SAOS) deformations.

Materials and Methods

Specimen Preparation and Treatment

Initially, three fresh enucleated porcine eyes were used to perform a baseline measurement with the use of one as a control (no CXL treatment) and two eyes receiving CXL treatment. This was done in order to assess the feasibility and initial results of our proposed technique. Subsequent testing was performed using five enucleated pairs of porcine eyes from a local abattoir that were obtained within 2 hours postmortem and transported to the lab on ice. Each pair was obtained from the same animal to ease comparison: one of the two eyes was CXL-treated and the other one was used as a control. The corneal epithelium was removed in all eyes with a hockey blade and corneal buttons of 8 mm diameter were trephined with a corneal trephine. The control porcine corneas (5) were treated with balanced salt solution (BSS) instilled every 5 minutes for 30 minutes. They then were exposed to ultraviolet A (UVA) irradiation (370 nm) with an irradiance of 3 mW/cm^2 for 30 minutes (5.4 J/cm^2). The treated porcine corneas (5) received isotonic solution of 0.1% riboflavin (vitamin B2) photosensitizer and 20% dextran in 1 × PBS buffer every 5 minutes for 30 minutes, and then exposed to the same total UVA irradiation energy by applying the same protocol used for the control cornea samples. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Linear Viscoelastic Measurements

Small amplitude oscillatory shear measurements represent an effective method to investigate the linear behavior of viscoelastic materials.[20] We measured the linear viscoelasticity of a disc-shaped cornea specimen (i.e., their mechanical response was not affected by the extent of imposed time-dependent deformation on the sample) by applying a sinusoidal oscillatory strain $\gamma(t) = \gamma sin(\omega t)$, with γ the strain amplitude and ω the frequency of oscillation. The resulting stress response of the material was $\sigma(t) = \gamma[G'sin(\omega t)+G''cos(\omega t)]$, which provides the strain-independent (see below) storage (or elastic) G' and loss (or viscous) G'' dynamic moduli that characterize the material's viscoelasticity and reflect its structure. For the particular case of network-like corneal tissue which is a viscoelastic solid, the storage modulus G' is proportional to the Young's modulus and reflects the mechanical strength of the material.

From the rubber elasticity theory,[7] the rubbery plateau modulus, i.e., the measured nearly frequency-independent storage modulus G' here, of an elastic network is given by $G' = \upsilon$kT where υ is the number density of cross-links (junctions/m^3), k is Boltzmann's constant (J*K^{-1}) and T the absolute temperature (K). This theory is based on the entropic origin of chain elasticity and considers incompressible and uniform network, as well as affine deformation. The uniformity of the network is reflected in a constant cross-link density. For the present case of cornea physical network this is not necessarily true, hence the present simple analysis provides an estimate of an average cross-link density.

The model originally was developed for rubber-like materials. It was later applied to, and now is widely used for network-forming materials. This includes in particular biological samples, biomacromolecules, such as networks from f-actin or collagen or elastin.[21–23] The model is based on linear viscoelastic response and the definition of modulus (thermal energy over volume). This volume is called correlation volume and represents the stress-carrying volume element in the network as discussed by de Gennes.[24] The additional assumption is that for networks we take an average value for almost homogeneous distribution of network junctions (cross-links). For large macromolecules, as in the present case, this is a sound assumption and the model is universally validated.

This overall approach is the established framework of the molecular level analysis of the viscoelasticity of network-forming synthetic and biological materials. The viscoelastic properties of various tissues in the context of network elasticity are discussed in the following references.[21–23,25] They represent selected examples as the field is wide. However, we note that our approach is a first attempt at correlating these measures with the condition of the cornea tissues, and in this respect it is simplified (albeit useful, we believe). In a next step one should account for the stiffness of the particular tissues (persistence length) which may influence the viscoelastic response. Such an attempt has been made for collagen.[22,26] Note that here we have considered flexible tissues, which is a rough approximation but allows drawing useful correlations. Accounting for the stiffness will be the subject of future work.

We used a commercial rotational rheometer (Kinexus pro+; Kinexus, Malvern, UK) operating in the strain control mode. A parallel plate geometry was used and both plates were covered with sandpaper to avoid wall slip, an issue that might occur when elastic materials are measured.[27] To be consistent with previous studies,[17,28,29] 320 grit sandpaper was used. Temperature control was achieved via a Peltier hood and the setup temperature was maintained at 25°C for all measurements. The risk of evaporation (of water from the corneal tissue) was minimized by means of a custom made trap around the measurement fixture, which created a saturated atmosphere of water vapor. The latter was achieved using a ring-shaped water channel mounted on the bottom plate of the rheometer. The level of water was checked every 30 minutes and maintained constant by refilling. This procedure has been applied successfully with volatile polymer solutions.[30]

The consistency of our measurements confirms the unchanged conditions of corneal samples as explained later. We note that the dynamic measurement accuracy is 10% (maximum allowable variance between consecutive measurements) due to different factors, such as sample positioning, alignment of plates and so forth. All our measurements were within this 10% variance. Each specimen was placed carefully on the bottom plate of a calibrated parallel plate fixture, well-centered, and then the top plate was lowered very slowly until getting in contact with the cornea specimen. This placement shaped the specimen into the form of a disk with curved boundaries at the unconfined edges (i.e., sandwich-like arrangement) as needed for proper measurement.[27] The total normal force applied to the specimen during loading did not exceed 0.1 N. With this protocol, no water was expelled from the sample. The loading procedure typically takes 2 minutes and the specimen then is left to relax for another 15 or 30 minutes before the measurement is initiated for control and

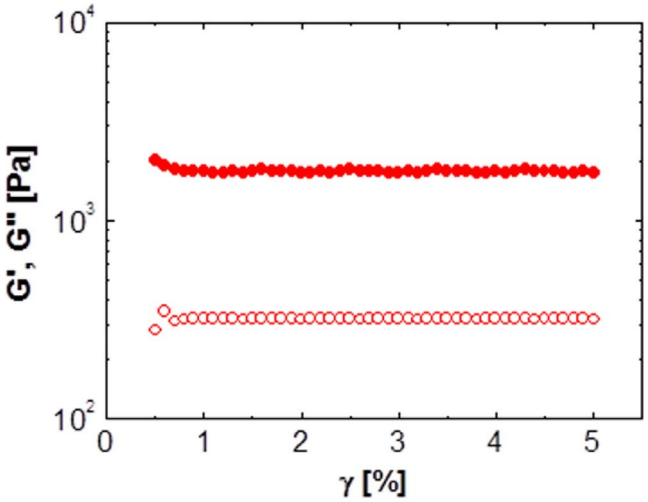

FIGURE 1. Dynamic strain sweep of cornea control sample, depicting the storage (*G'*, *solid symbols*) and loss (*G"*, *open symbols*) moduli as function of strain amplitude (γ). The test was performed at 1 rad/s and room temperature. All subsequent dynamic frequency sweep tests are performed at a strain amplitude of 1%.

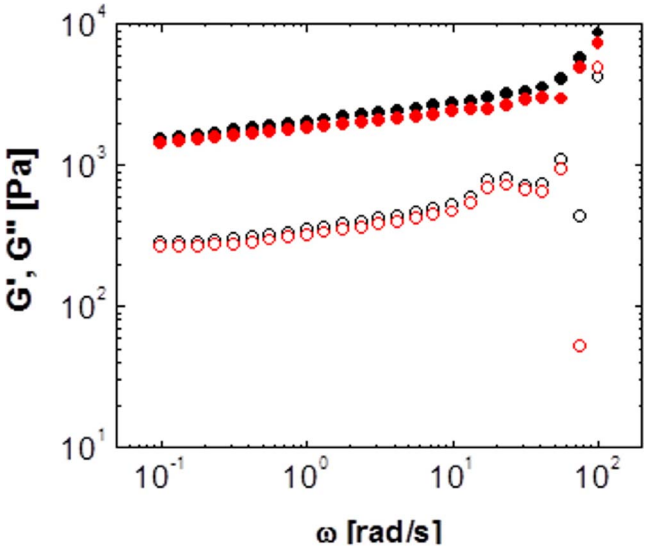

FIGURE 2. *G'* (*closed symbols*) and *G"* (*open symbols*) as function of frequency for a cornea control sample. The first dynamic frequency sweep test was performed at room temperature, *black symbols*, and repeated after 15 minutes, *red symbols*.

treated cornea samples, respectively. During this time the tare stress relaxed to zero and the resulting equilibrium thickness of specimens was taken as the loading gap. To obtain reliable measurements in the linear viscoelastic regime, first we performed dynamic strain sweep tests to determine the linear strain range to apply and tested the consistency of the measurements. Figure 1 depicts typical results of dynamic strain sweeps which allow determining the linear viscoelastic regime, as already mentioned. For the particular case of untreated cornea sample tested at a frequency of 1 rad/s, *G'* and *G"* are independent of the imposed strain amplitude (γ), hence the response is linear in this range of γ (0.5%–5%). The subsequent SAOS measurements were performed at a chosen strain amplitude of 1% within a frequency window between 0.1 and 100 rad/s. Data collected between 100 and 10 rad/s were affected by either sample stiffness or wall slip. For this reason these data were not taken into account in our analysis. The consistency of the measurements, hence time–independent viscoelastic properties of the cornea specimens, is demonstrated in Figure 2. It depicts the frequency-dependent viscoelastic moduli for a cornea control sample measured at two different times with an interval of 30 minutes. The time of 30 minutes is the typical relaxation time for cornea control samples, corresponding to the relaxation of the normal force signal after specimen loading. The discrepancy of experimental data between the two tests is less than 5% (well within the measurement specifications). This meticulous protocol ensured high quality consistent measurements confirming that the sample's condition remained unchanged during testing. The analysis of the linear viscoelastic moduli is based on the theory of rubber elasticity[7] which is discussed in the materials and methods section.

RESULTS

The results demonstrated a change in the elastic and viscous properties of the treated corneas.

We used SAOS measurements as a means to assess the condition of the measured sample. The result of the initial testing of the first three porcine corneal samples that were used to perform a baseline measurement (one control and two treated samples) to assess the feasibility and initial results of our proposed technique is depicted in Figure 3 with the storage modulus *G'* as a function of frequency. The value of *G'* at a frequency of 1 rad/s, is observed to change from 2×10^3 to 1.5×10^4 and to 3×10^4 Pa from the control cornea sample to treated 1 to treated 2 cornea samples, respectively. Since the thermal unit kT is kept constant by taking into account a single temperature value for all of the measurements, v increases almost 10-fold from control to treatment 1 and then by a factor of 2 to treatment 2. The control sample at room temperature (25°C), has a value of 4.86×10^{14} junctions/mm^3. The typical distance between two junctions, ξ, is estimated to be approximately 13 nm by considering $\xi \approx [kT/G']^{1/3}$.

Subsequent testing was performed using five pairs of corneal samples. Each pair originated from the same animal, one cornea serving as control and the contralateral cornea receiving CXL treatment.

The potential to improve the mechanical properties of corneal tissue by UV treatment, the improvement itself depending on the type of the treatment applied can be

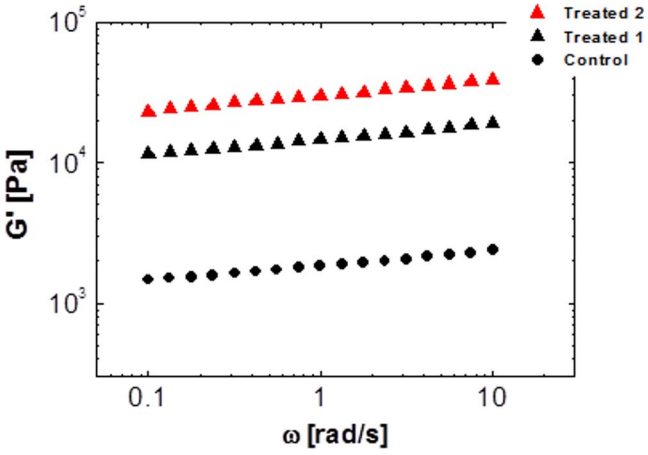

FIGURE 3. Frequency-dependent *G'* of a control (untreated) corneal sample compared to two treated corneal samples (Treated 1 and Treated 2).

TABLE. The Young's Modulus E, Average Cross-Linking Density ν, and Junction Distance ξ for Control and CXL-Treated Cornea Samples, and the Relative Improvement for the Young's Modulus Was Calculated for Five Samples Receiving the Same UVA Irradiation Treatment

	E, Pa		ν, junct/mm^3		ξ, nm		
Sample	Control	Treated	Control	Treated	Control	Treated	Relative Improvement, *E*, %
1	9902	82,401	8×10^{14}	7×10^{15}	11	5	700
2	4734	93,439	3.8×10^{14}	7.6×10^{15}	14	5	1750
3	11,150	25,300	9×10^{14}	2×10^{15}	10	8	122
4	8530	62,421	7×10^{14}	5×10^{15}	11	6	607
5	20,742	51,351	1.7×10^{15}	4×10^{15}	8	6	160

demonstrated and measured reliably with the proposed technique. Furthermore, the above simple methodology can be used to compare corneal samples undergoing the same treatment as shown in Figure 4. Whereas the treatment is very effective, leading to an unambiguous increase of modulus *G'* which can be as high as a factor of 20 (sample 2), it is apparent that there is a nonnegligible variance (the lowest increase is factor of approximately 2.5 for samples 3 and 5). This result suggests that there is variability in the effect of the treatment. However, an unambiguous change can be measured reliably with the described technique.

In Figure 5, we summarize the results obtained in Figure 4 in the form of a quantitative improvement of the mechanical properties (*G'* at 1 rad/s) of the treated corneal samples over the reference (untreated control sample). Results show the elastic modulus *G'* increase at 1 rad/s. These simple metrics compare samples from different animals and demonstrate the range of variance, which should relate to the difference among animals, with the most likely factor being age.

The Young's modulus *E* was calculated as three times of the rubbery plateau modulus *G'* according to the theory of linear viscoelasticity[27] and it was 11,011 ± 5947 Pa for the control corneal samples and 62,982 ± 26,740 Pa for the treated corneal samples. This difference is statistically significant with a *P* value of 0.018 (paired *t*-test). Values of average cross-linking density ν and junction distance ξ for control and CXL treated corneal samples are reported in the Table together with Young's modulus and its relative improvement.

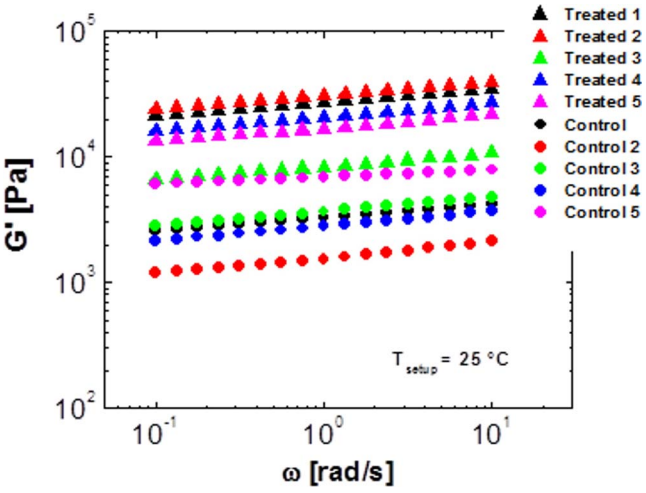

FIGURE 4. Comparison of frequency-dependent *G'* of cornea control samples and respective samples treated with riboflavin applied for 30 minutes. *Solid symbols* refer to control samples and *open symbols* to treated samples. Sample numbers from *top* to *bottom*: 2 (*red*), 1 (*black*), 4 (*blue*), 5 (*rose*), 3 (*green*).

We point out that values of ν and ξ for control corneal samples do not have their original physical meaning of numbers of actively junction points per unit volume and distance between two adjacent junction points, respectively. They are material parameters for chemical cross-linked network systems. Stromal structure of control corneal samples consists of temporary bridges between collagen fibrils via proteoglycan chains in an antiparallel fashion.[31] For this reason values of ν and ξ for control corneal samples must be considered more qualitatively than quantitatively as reference point.

DISCUSSION

The effect of corneal collagen cross-linking in porcine cornea samples has been measured mostly by means of strip extensometry, although other techniques have been described in the literature as well.[32] Strip extensometry involves cutting the corneal tissue in equal sized strips, then mounting it on an appropriately modified extensometer[9,12,33] and eventually measuring the Young modulus until the sample breaks up.[34] Although the technique is standardized, it can prove difficult to obtain consistent measurements due to several issues, such as ensuring equal tissue strip sizes to measure and avoiding slippage as the tissue is stretched.[12] Furthermore, measurements must be performed quickly enough to avoid dehydration of the corneal sample, which can alter the results of the measurement itself. Hence, two crucial issues are maintaining a constant water content of the sample and the nonlinear deformation imposed, leading to fracture. The former already

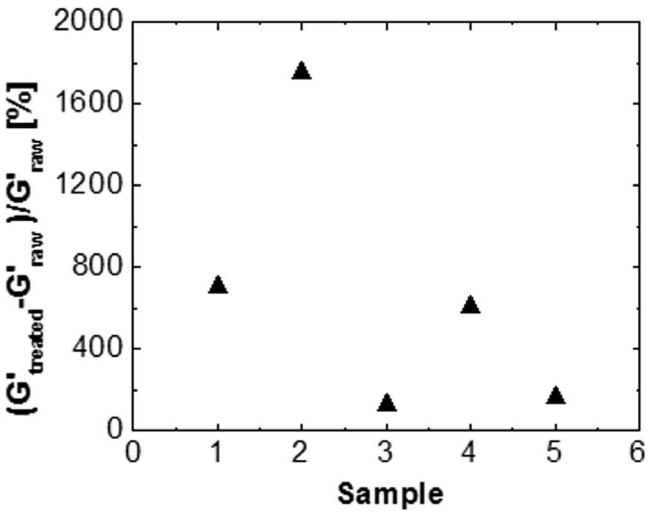

FIGURE 5. Relative improvement of elastic moduli of treated corneal samples over control samples (Samples 1–5).

has been addressed, whereas the latter affects the sample's microstructure significantly, as will be discussed further below.

Recently, there have been efforts to develop noninvasive measurements, such as Brillouin,[18] and ultrasound microscopy.[35] Despite the fact that these techniques provide quantitative information about tensile/compressive material properties (such as Young's/Longitudinal modulus, Poisson ratio) of cornea and some of them can be applied even in vivo,[36,37] they have limitations and, moreover, they do not always yield consistent results.[38] Hence, a simple but reliable biomaterial-dedicated experimental procedure is needed.

Compared to strip extensometry, SAOS measurements can offer a wider range of experimental parameter values (specifically the oscillatory frequency) for describing the biomechanical changes of the viscoelastic properties of the cornea. They also require minimal manipulation of the treated sample, making it more likely to produce consistent and, hence. more reliable results. Another advantage of SAOS measurements, and more particular of the proposed procedure, is that the corneal sample is less likely to be dehydrated due to the fact that it is effectively "sandwiched" between two metal plates of the device, hence leaving only a very small surface of the tissue exposed to air.

In SAOS measurements, a fixed diameter corneal button that can be produced consistently with a set diameter corneal trephine, ensuring consistent results, can be easily produced accurately. The oscillatory shear technique does not require any specific modification of the device and is straightforward to perform in conditions of controlled humidity that ensure unchanged properties of the cornea sample over time, since the importance of corneal stroma hydration during measurements for tissue biomechanical properties has been reported in the literature.[39,40]

In contrast, strip extensometry requires that cutting of a strip of tissue ensuring accurate cut and equal size[9,12] which, in our experience, can be challenging. It also requires a modification of the strip extensometer to mount the strips of tissue and prevent slippage. Moreover, the continuous deformation accumulates strain in the material and becomes nonlinear, hence affecting its microstructure, with the constituting collagen network constituents being oriented in the flow direction, stretched, and eventually broken (sample fracture).

A similar SAOS-based measuring technique has been reported in the literature.[19] The measurements were performed in bovine corneas. However, the corneal samples were cut into strips and preconditioned before the frequency sweep measurements were performed. Hence, no specific protocol was used aiming at preserving the original microstructure and water content of the cornea samples.

Although there is an apparent change, our results for CXL-treated corneal samples in terms of Young's modulus values from shear modulus are not comparable to those ones from tensile modulus in the past literature.[9,12] Our values under shear deformations are one order of magnitude smaller than those under small tensile deformations. Conversely, there is a good agreement for control corneal samples in terms of shear modulus between our work and Hatami and Marbini's study.[17] A possible explanation for the difference in corneal behavior in shear and tensile modes of deformation might be given in terms of the microstructure. The corneal extracellular matrix is composed of stacks of collagen lamellae with a parallel-to-the-surface distribution, each comprising bundles of thin collagen fibrils and proteoglycans. The gap between the collagen fibrils is filled with a network of proteoglycans which are responsible to maintain the uniform spacing of the fibrils.[1–3] In compression studies,[14] a difference of two orders of magnitude already was observed between in-plane (compressive) and out-of-plane (transverse) Young's modulus. From the same point of view, when subjected to uniaxial tensile strain in the strip testing method, the collagen lamellae are mainly loaded in tension. It is known that collagen fibrils have a nonlinear stress-strain behavior. Thus, a strain-hardening response (associated with collagen fibril stretching) is expected with increasing imposed deformation. In previous studies[12,13] the calculation of Young's modulus was made at different strains of a uniaxial extensional test where the stress-strain behavior of the cornea sample already was nonlinear (i.e., strain hardening was observed). On the other hand, when subjected to shear deformation the proteoglycan matrix mainly provides the shear stiffness. Adjacent lamellae just slide to each other and a lower shear stiffness is obtained. In summary, the described SAOS testing procedure ensured nearly equilibrium measurements of the viscoelasticity of the measured cornea samples while maintaining their microstructure and water content intact. Despite the above discussion, however, further studies will be needed to fully elucidate the physical origin of cornea's viscoelastic behavior and the issues raised.

As mentioned, one very interesting aspect of the oscillatory shear technique is the potential to estimate the average number of cross-links and their related distance. This information may prove very useful in our endeavor to clarify the molecular basis of the corneal cross-linking reaction.

Conclusions

We have described the application of a technique using SAOS measurements in a commercial rotational rheometer to compare the results of corneal collagen cross-linking treatment in porcine corneas. It provides quantitative information regarding the change in the viscoelastic properties of the corneal tissue undergoing the treatment. This technique measures the elastic and viscous moduli of the treated tissue and can offer a valuable research tool in observing the effect of riboflavin and UVA corneal collagen cross-linking in corneal tissue samples.

In conclusion, having performed strip extensometry and SAOS measurements, we find that the latter technique requires minimal sample manipulation, is consistent, and can reliably measure the change in the cornea biomechanical properties. Further work in this direction is needed to optimize the technique.

Acknowledgments

Supported in part by CLEAR an NSRF EU Greece-China research project 12CHN406 (IMA, PG, GC, AGC, TNK) and the Greek General Secretariat for Research and Technology (program Thalis, project COVISCO, for CD, DV).

Disclosure: **I.M. Aslanides**, None; **C. Dessi**, None; **P. Georgoudis**, None; **G. Charalambidis**, None; **D. Vlassopoulos**, None; **A.G. Coutsolelos**, None; **G. Kymionis**, None; **A. Mukherjee**, **T.N. Kitsopoulos**, None

References

1. Maurice DM. The structure and transparency of the cornea. *J Physiol*. 1957;136:263–286.
2. Maurice DM. The cornea and sclera. In: Davason H, ed. *The Eye*. Cambridge: Academic Press; 1984.
3. Lewis PN, Pinali C, Young RD, Meek KM, Quantock AJ, Knupp C. Structural interactions between collagen and proteoglycans are elucidated by three-dimensional electrontomography of bovine cornea. *Structure*. 2010;18:239–245.

4. Dupps WJ. Biomechanical modeling of corneal ectasia. *J Refract Surg*. 2005;21:186–190.
5. Wollensak G. Crosslinking treatment of progressive keratoconus: new hope. *Curr Opin Ophthalmol*. 2006;17:356–360.
6. Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. *Exp Eye Res*. 1998;66:97–103.
7. Rubinstein M, Colby RH. *Polymer Physics*. Oxford: Oxford University Press; 2003.
8. Meek KM. The cornea and the sclera. In: Fratzl P, ed. *Collagen: Structure and Mechanics*. Berlin: Springer Science; 2008.
9. Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A induced cross-linking. *J Cataract Refract Surg*. 2003;29:1780–1785.
10. Avila MY, Navia JL. Effect of genipin collagen crosslinking on porcine corneas. *J Cataract Refract Surg*. 2010;36:659–664.
11. Hammer A, Richoz O, Arba Mosquera S, Tabibian D, Hoogewoud F, Hafezi F. Corneal biomechanical properties at different corneal cross-linking (CXL) irradiances. *Invest Ophthalmol Vis Sci*. 2014;2;55:2881–2884.
12. Richoz O, Kling S, Zandi S, Hammer A, Spoerl E, Hafezi F. A constant-force technique to measure corneal biomechanical changes after collagen cross-linking. *PLoS One*. 2014;9:1–5.
13. Elsheikh A, Wang D, Pye D. Determination of the modulus of elasticity of the human cornea. *J Refract Surg*. 2007;23:808–818.
14. Hatami-Marbini H, Etebu E. An experimental and theoretical analysis of unconfined compression of corneal stroma. *J Biomechanics*. 2013;46:1752–1758.
15. Kling S, Remon L, Pérez-Escudero A, Merayo-Lloves J, Marcos S. Corneal biomechanical changes after collagen cross-linking from porcine eye inflation experiments. *Invest Ophthalmol Vis Sci*. 2010;51:3961–3968.
16. Lombardo M, Serrao S, Rosati M, Ducoli P, Lombardo G. Biomechanical changes in the human cornea after transepithelial corneal crosslinking using iontophoresis. *J Cataract Refract Surg*. 2014;40:1706–1715.
17. Hatami-Marbini H. Viscoelastic shear properties of the corneal stroma. *J Biomechanics*. 2014;47:723–728.
18. Scarcelli G, Kling S, Quijano E, Pineda R, Marcos S, Yun SH. Brillouin microscopy of collagen crosslinking: noncontact depth-dependent analysis of corneal elastic modulus. *Invest Ophthalmol Vis Sci*. 2013;54:1418–1425.
19. Hatami-Marbini H, Rahimi A. Collagen crosslinking treatment effects on corneal dynamic biomechanical properties. *Exp Eye Res*. 2015;135:88–92.
20. Barnes HA, Hutton JF, Walters K. *An Introduction to Rheology*. Philadelphia: Elsevier; 1989.
21. Waigh TA. *Applied Biophysics*. New York: John Wiley & Sons; 2007.
22. MacKintosh FC, Käs J, Janmey PA. Elasticity of semiflexible biopolymer networks. *Phys Rev Lett*. 1995;75:4425–4428.
23. Clark AH, Ross-Murphy SB. Structural and mechanical properties of biopolymer gels. *Adv Polym Sci*. 1987;83:57–192.
24. de Gennes PG. *Scaling Concepts in Polymer Physics*. New York: Cornell University Press, 1979.
25. Hinner B, Tempel M, Sackmann E, Kroy K, Frey E. Entanglement, elasticity, and viscous relaxation of actin solutions. *Phys Rev Lett*. 1998;81:2614–2617.
26. Storm C, Pastore JJ, MacKintosh FC, Lubensky TC, Janmey PA Nonlinear elasticity in biological gels. *Nature*. 2005;435:191–194.
27. Macosko CW. *Rheology: Principles, Measurements and Applications*. New York: John Wiley & Sons; 1994.
28. Nickerson CS. *Engineering the Mechanical Properties of Ocular Tissue* [PhD thesis]. Pasadena, CA: California Institute of Technology, 2005.
29. Petsche SJ, Chernyak D, Martiz J, Levenston ME, Pinsky PM. Depth-dependent transverse shear properties of the human corneal stroma. *Invest Ophthalmol Vis Sci*. 2012;53:873–880.
30. Sato J, Breedveld V. Evaporation blocker for cone-plate rheometry of volatile samples. *Appl Rheol*. 2005;15:390–397.
31. Scott JE. Extracellular matrix, supramolecular organisation and shape. *J Anat*. 1995;187:259–269.
32. Ahearne M, Yang Y, Then KY, Liu KK. Non-destructive mechanical characterisation of UVA/riboflavin crosslinked collagen hydrogels. *Br J Ophthalmol*. 2008;92:268–271.
33. Hatami-Marbini H, Rahimi A. Effects of bathing solution on tensile properties of the cornea. *Exp Eye Res*. 2014;120:103–108.
34. Elsheikh A, Alhasso D. Mechanical anisotropy of porcine cornea and correlation with stromal microstructure. *Exp Eye Res*. 2009;88:1084–1091.
35. He X, Liu JA. Quantitative ultrasonic spectroscopy method for noninvasive determination of corneal biomechanical properties. *Invest Ophthalmol Vis Sci*. 2009;50:5148–5154.
36. Dorronsoro C, Pascual D, Perez-Merino P, Kling S, Marcos S. Dynamic OCT measurement of corneal deformation by an air puff in normal and cross-linked corneas. *Biomed Opt Exp*. 2012;3:473–487.
37. Scarcelli G, Yun SH. In vivo Brillouin optical microscopy of the human eye. *Opt Express*. 2012;20:9197–9202.
38. Scarcelli G, Besner S, Pineda R, Yun SH. Biomechanical characterization of keratoconus corneas ex vivo with brillouin microscopy. *Invest Ophthalmol Vis Sci*. 2014;55:4490–4495.
39. Hatami-Marbini H, Etebu E. Hydration dependent biomechanical properties of the corneal stroma. *Exp Eye Res*. 2013;116:47–54.
40. Hatami-Marbini H, Rahimi A. Evaluation of hydration effects on tensile properties of bovine corneas. *J Cataract Refract Surg*. 2015;41:644–651.

Investigative Ophthalmology & Visual Science