Internal Tension: A Novel Hypothesis Concerning the Mechanical Properties of the Vitreous Humor

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Summary: The vitreous humor is a soft viscoelastic gel composed of a complex network of biomolecules (primarily collagen and polyhyaluronic acid, "hyaluronan"). While extensive progress has been made in identifying the components of the vitreous, lack of sufficient experimental methods has hampered previous efforts to quantitatively define its mechanical properties. To address this issue, we have developed a novel "cleat" tool geometry for dynamic shear rheometry. Using this geometry we find that the shear moduli of vitreous decline by roughly a factor of five to steady-state values within an hour after removal from the eye. Steady-state values (Porcine: $G' = 2.6 \pm 0.9$ Pa, and $G'' = 0.65 \pm 0.41$ Pa, n = 9; Bovine: $G' = 6.5 \pm 2.0$ Pa, and $G'' = 2.0 \pm 0.6$ Pa, n = 17) are significantly larger than previously reported. The decrease in modulus also correlates with a decrease in mass (65 ± 11 %, n = 8) that occurs spontaneously after the vitreous is extracted. Rheological findings, taken in combination with biochemical analyses suggest that hyaluronan, a ubiquitous polyelectrolyte in connective tissue, contributes to vitreous stiffness by inducing tension in the network in vivo.

Keywords: biomechanics; biopolymers; hyaluronan; networks; vitreous humor

Introduction

The vitreous humor is the transparent gel within the eye that fills the space between the lens capsule and the retina. It is composed primarily of a hydrated double-network of type II collagen and hyaluronan, a ubiquitous extracellular matrix polyanion (Figure 1). Its functions in healthy eyes have been summarized as: developmental—mediating proper growth of the eye; optical—maintaining a clear path to the retina; mechanical—supporting the various ocular tissues during physical activity; and metabolic—providing a repository of various small molecules for the retina.[1] Fulfilling the first three of these functions

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would not be possible without the unique mechanical properties of this delicate tissue. Interest in understanding the biomechanics of the vitreous has risen in the past few decades as its importance in retinal health, particularly in diabetics and the elderly, has become more apparent.

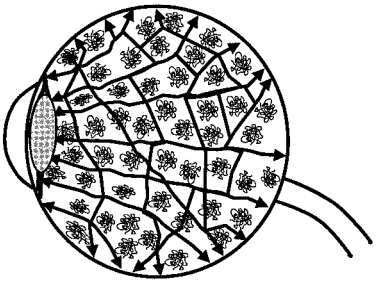

Figure 1. Schematic representation of the vitreous humor of an intact eye. The vitreous network fills the center of the eye. Collagen type II fibrils are shown as heavy black lines spanning the full diameter of the eye. Hyaluronan molecules are shown as coils with large hydration spheres filling the space between the fibrils.

Lack of sufficient experimental methods has been the primary obstacle to quantitatively defining its mechanical properties.[2] While experimental data from standard rheometry fixtures has been published,[3] when we duplicate these experiments wall slip is clear: the sample moved as a solid body relative to the stationary upper tool and the torque was below the sensitivity of the instrument-even if the tool surfaces are roughened or sandpaper is used. The vitreous is also very delicate; application of even a small normal force squeezes fluid out of the network. To address these issues and measure the moduli of the vitreous, we have devised the "cleat" geometry,[4] a novel tool for overcoming wall slip in shear rheometry (Figure 2).

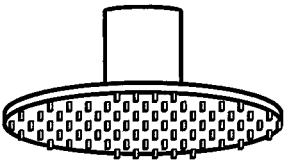

Figure 2. Schematic representation of the cleated rheometry tool. The actual cleat density is ~100/cm^2.

Using the cleat geometry we have succeeded in measuring the shear moduli of the vitreous directly and in tracking the softening that occurs after the vitreous is extracted from the eye. These results, in conjunction with observations of mass changes and analytical biochemistry suggest a modified view of the molecular basis of the mechanical properties of the vitreous. Overcoming the obstacles to measuring the shear moduli of the vitreous represents an essential step in understanding the vitreous' unique properties, relevant to transport of molecules (e.g. metabolites and potential therapeutics) to the retina, pharmacological vitrectomy agents, and target mechanical properties of potential vitreous replacement materials.

Experimental

Fresh bovine and porcine eyes were gently dissected to remove the vitreous with minimal disruption. All experiments were conducted at 20-22 °C and within 60 hours post mortem. Mechanical measurements were made on disc-like sections cut from the intact vitreous with the axis of the disc coinciding with the anterior-to-posterior axis of the eye (typically 1.5-2.5 g). These sections were initially loaded into traditional smooth, then roughened parallel plate geometries but significant wall slip occurred. Therefore, measurements were repeated using a novel "cleat" test geometry[4] designed to prevent slip. The cleat test geometry was made by adding rows of cleats (450x450 μm square cross-section, 600 μm tall, 900 μm center-to-center distance) to the upper fixture of a traditional parallel plate test geometry and affixing a glass frit to the lower fixture. The cleats penetrated the lubricating boundary layer to measure the mechanical responses of the tissue. The bottom fixture did not require cleats because gravity forces the aqueous layer into the glass frit. The entire test assembly was mounted on an ARES-RFS fluids rheometer and encased in a modified hydration chamber to allow visual observation of the sample during measurements. Shear moduli were monitored as samples were subjected to oscillatory strain (amplitude = 3%) at a fixed frequency (10 rad/sec) for a period of two hours. The conditions for these "time sweep" experiments were chosen based on the results of variable frequency (strain = 3%, frequency = 100-0.1 rad/sec) and variable strain (frequency = 10 rad/sec, strain = 0.5 – 50%) experiments.

In conjunction with rheological measurements, we measured the weight of vitreous samples as a function of time during the first few hours post mortem. Vitreous specimens were gently removed as described above and weighed immediately. Specimens were then

either left as intact bodies or the anterior and posterior sections were gently cut away, leaving a disc of central vitreous. The vitreous samples were then exposed to one of four conditions denoted as A-D. In condition A, an intact vitreous body was placed in a dry Petri dish and covered. In conditions B-D, central discs of vitreous were placed in covered Petri dishes that were either dry (B), filled with isotonic saline (C) or filled with mechanically liquefied vitreous from other eyes (D). The mass of each vitreous sample was measured 0, 5, 10, 15, 30, 45, 60, 90 and 120-150 minutes after dissection.

Results and Discussion

Shear moduli of sections of vitreous were monitored under oscillatory strain (amplitude = 3%) at a fixed frequency (10 rad/sec) in a hydrated atmosphere for up to two hours. Initial attempts to measure the modulus of the tissue with traditional tools failed due to wall slip: measurements with smooth tools were impossible (wall slip prevented the rheometer from detecting sufficient torque to make any meaningful measurements), and roughened tools prevented slip inconsistently and only when compression was applied (data not shown due to excessively large standard deviations). With the cleated tools we observed a monotonic modulus decay period that reached a significantly lower, steady-state value within one hour and persisted thereafter. The initial G′ and G″ of bovine vitreous were 30 ± 12 Pa (mean ± SD) and 16 ± 7 Pa (n = 17) respectively and for porcine vitreous 9.5 ± 1.9 Pa and 3.6 ± 0.8 Pa (n = 9) respectively. The large standard deviations reflect the rapid initial changes, making the observed moduli sensitive to the precise time from dissection to loading. Smaller standard deviations for porcine samples were achieved by using a consistent loading time. The final (steady-state) G′ and G″ values for bovine vitreous were 6.5 ± 2.0 Pa and 2.0 ± 0.6 Pa respectively and for porcine vitreous were 2.6 ± 0.9 Pa and 0.65 ± 0.41 Pa respectively. These storage modulus values are significantly higher than any estimates found in the literature,[3,5-7] in some cases by orders of magnitude (Figure 3). Thus, efforts to develop vitreous replacement materials should target stiffer materials than previously thought.

Because prior mechanical investigations of the vitreous are unsatisfactory, we cannot independently verify the accuracy of our modulus values. The sample dictates the gap and is destroyed by compression, precluding the usual procedure to test for slip (varying sample gap or diameter). We observed that, near the tools, heterogeneities in the tissue

moved with the tool surface; therefore, we believe that residual slip is negligible. Nevertheless, the values we measure represent a lower bound.

The modulus values here pertain to the central vitreous, the bulk of the tissue. Different moduli would characterize the tissue near the anterior (stiffer) pole.

Upon removal of the test samples from the rheometer two obvious changes in the tissue suggest that the steady-state moduli we measure are lower than the moduli of the vitreous *in vivo*. First, the vitreous appears to sag, elongating far more when lifted with forceps. This observation suggests that the equilibrium moduli seen after approximately one hour represent a new, post-dissection steady-state of the network which is softer than the native vitreous tissue. Hence, the initial moduli may be closer to the moduli of the vitreous *in vivo* and the steady-state moduli represent a minimum value—perhaps as much as five times lower than the *in vivo* moduli. Nevertheless, these steady-state moduli are significantly *greater* than previously reported values. The reason for the modulus decrease with time may be found in the second major observation: a small puddle of liquid is left behind on the instrument where aqueous material seeped out of the vitreous.

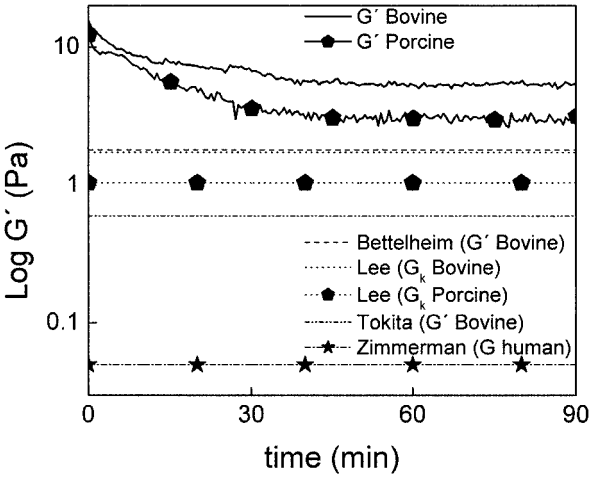

Figure 3. Typical time-dependent behavior of G' at fixed strain amplitude (3%) and frequency (10 rad/sec) for bovine and porcine vitreous. Estimates of the modulus based on prior literature are indicated for reference.

To quantify the liquid-loss, we obtained fresh vitreous specimens and placed them under the conditions A-D listed above. Condition A was chosen because the sample is not cut and we are able to observe the intact network. Condition B duplicates the conditions of the sample on the rheometer (central vitreous section placed in hydrated atmosphere) and shows the physical effects of cutting the network. Condition C duplicates the rheometer condition, but eliminates both the effect of hydrostatic pressure driving fluid loss and evaporation. Condition D duplicates the rheometer condition, but completely eliminates the concentration gradients and hydrostatic pressure. Regardless of the conditions under which the vitreous is placed, the mass drops by at least $8 \pm 1\%$, $n \geq 3$ (Porcine vitreous, Condition D) after 120 min., without weighing at intermediate time intervals. In order to probe the kinetics of weight loss, more frequent weight measurements were made. Under conditions A and B, the weight loss is larger in magnitude ($65 \pm 11\%$, $n = 8$) to a near-equilibrium value in both bovine and porcine vitreous but the kinetics are the same. The mass-decay starts immediately after the vitreous body is removed from the eye and stabilizes within the first two hours. The vitreous wets any surface on contact and weeps fluid until it reaches a steady-state hydration level. The mass loss correlates with this loss of fluid. The seeping fluid is rich in hyaluronan but contains very little protein as determined by enzyme-linked hyaluronic acid binding protein assay and Ninhydrin amino acid assay respectively.

The loss of hyaluronan-rich fluid from the vitreous did not follow standard Fickian diffusion kinetics; weight loss was linear with ln(t) rather that with $t^{1/2}$. Nor is the weight loss driven by diffusion down a gradient; ~10% is lost even in the absence of a chemical potential difference at the boundary of the vitreous (condition D). Nor is the efflux driven by gravity: it occurs even when saline or homogenized vitreous fill the dish up to the top of the specimen. The rate and magnitude of fluid loss are also independent of sample surface area. These observations combine to strongly suggest that hyaluronan is not simply diffusing out of the vitreous but, rather, that it is *driven* out. The correlation between fluid loss and modulus decrease suggests that they may have a common cause. We propose that hyaluronan trapped in the vitreous *in vivo* increases the modulus of the vitreous by placing the collagen network under internal tension as it swells to find a Donnan equilibrium hydration state. Tension on the network would reduce its ability to deform and, thereby, increase the modulus. Tension release would provide the driving

force for fluid expulsion and modulus reduction when the vitreous is removed from the eye and hyaluronan is no longer trapped.

Conclusions

We have demonstrated a method to measure the shear moduli of the vitreous network using a novel "cleat" geometry. Our results indicate that the shear moduli of the vitreous are higher than previously reported and provide new target properties for potential synthetic vitreous substitutes. The moduli decay following dissection, concurrent with a loss of mass due to the efflux of a hyaluronan-rich fluid from the tissue. Together, the results indicate that hyaluronan contributes significantly to the modulus of the vitreous by keeping the collagen fibrils under tension. Ongoing research uses the ability to quantify the vitreous modulus to explore the molecular interactions that maintain the mechanical integrity of the vitreous and to quantify the effects of pharmacological vitrectomy agents.

[1] J. Sebag, "*The Vitreous - Structure, Function, and Pathobiology*", Springer-Verlag Inc., New York 1989, p. 59.
[2] H. Weber, G. Lendwehr, *Ophthalmic Res.* **1982,** *14,* 326.
[3] M. Tokita, Y. Fujiya, K. Hikichi, *Biorheology* **1984,** *21*, 751.
[4] C. Nickerson, J. Kornfield, accepted, *J. Rheol.* **2005.**
[5] F. A. Bettelheim, T. J. Y. Wang, *Exp. Eye Res.* **1976**, *23*, 435.
[6] B. Lee, M. Litt, G.. Buchsbaum, *Biorheology* **1994**, *31*, 327.
[7] R. L. Zimmerman, *Biophys. J.* **1980**, *29*, 539.