Enzymatic Degradation Identifies Components Responsible for the Structural Properties of the Vitreous Body

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PURPOSE. Vitreous degeneration contributes to several age-related eye diseases, including retinal detachment, macular hole, macular traction syndrome, and nuclear cataracts. Remarkably little is understood about the molecular interactions responsible for maintaining vitreous structure. The purpose of this study was to measure the structural properties of the vitreous body after enzymatic degradation of selected macromolecules.

METHODS. Mechanical properties of plugs of bovine and porcine vitreous were analyzed using a rheometer. Oscillatory and extensional tests measured vitreous stiffness and adhesivity, respectively. Major structural components of the vitreous were degraded by incubation overnight in collagenase, trypsin, or hyaluronidase, singly or in combination. Vitreous bodies were also incubated in hyper- or hypotonic saline. Effects of these treatments on the mechanical properties of the vitreous were measured by rheometry.

RESULTS. Enzymatic digestion of each class of macromolecules decreased the stiffness of bovine vitreous by approximately half ($P < 0.05$). Differential effects were observed on the damping capacity of the vitreous ($P < 0.05$), which was shown to correlate with material behavior in extension ($P < 0.01$). Digestion of hyaluronan significantly decreased the damping capacity of the vitreous and increased adhesivity. Collagen degradation resulted in the opposite effect, whereas digestion of proteins and proteoglycans with trypsin did not alter behavior relative to controls. Osmotic perturbations and double-enzyme treatments further implicated hyaluronan and hyaluronan-associated water as a primary regulator of adhesivity and material behavior in extension.

CONCLUSIONS. Collagen, hyaluronan, and proteoglycans act synergistically to maintain vitreous stiffness. Hyaluronan is a key mediator of vitreous adhesivity, and mechanical damping is an important factor influencing dynamic vitreous behavior.

Keywords: vitreous, enzymatic vitreolysis, dynamic mechanical analysis, mechanical properties, ocular biomechanics, vitreous degeneration, liquefaction

The vitreous body is the structurally homogeneous, transparent gel that fills the space between the retina and the lens. A hydrated network composed predominantly of collagen fibers, hyaluronan, and structural proteoglycans,¹ the vitreous is a viscoelastic mechanical damper that protects surrounding ocular tissues from sudden mechanical impact.² Through mechanisms that are not well understood, the vitreous liquefies (synchysis) with increasing age. During liquefaction, collagen fibrils collapse into bundles (syneresis) and fluid-filled cavities (lacunae) form, beginning in the vitreous core.^{3,4} Later in life, the vitreous may detach from the retina, creating posterior vitreous detachment (PVD). Although PVD is usually benign, persistent local adhesion to the inner retina can result in sight-threatening vitreomacular traction, macular hole, macular pucker, with the subsequent formation of epiretinal membranes. Intervention for such conditions has traditionally been through pars plana vitrectomy, where the vitreous is mechanically disrupted, aspirated, and replaced with saline, silicone oil,

or a gas tamponade. To avoid associated complications, such as retinal tears and detachments, pharmacologic vitreolysis (the injection of agents to digest structural components of the vitreous) to resolve symptomatic vitreomacular tractions has emerged as a potential alternative to surgery. An enzyme (ocriplasmin) developed for this procedure was first approved for use in the United States by the US Food and Drug Administration (FDA) in 2012.⁵

Major goals of pharmacologic vitreolysis are to reduce vitreous stiffness and adhesivity, either as a precursor to vitrectomy surgery or as a stand-alone therapy.³ Several methods exist to gauge the efficacy of vitreolytic agents. Optical coherence tomography, ultrasonography, and slit lamp microscopy are used to noninvasively screen for gross macular pathology or partial vitreous detachments before and after treatment,⁵ while at the microstructural level, electron and light microscopy are used to assess pharmacologic-induced changes at the vitreoretinal interface.⁶ Other approaches

FIGURE 1. Experimental protocol. (A) Anterior tissues are dissected from the eye to expose the vitreous. (B, C) A trephine is used to obtain a cylindrical punch of vitreous that is carefully transferred to the cleated rheometer stage. (D) A cleated parallel plate (20-mm diameter) is lowered such that the vitreous fully occupies the test geometry and normal force on the sample is minimized (gap between stage and parallel plate: 3–6 mm depending on sample size).

include measuring the mass of the gel-like vitreous, relative to the liquid fraction (to assess liquefaction),⁷ and dynamic light scattering, which provides a measure of particle size and diffusion in isolated vitreous.⁸ While useful, current techniques have several limitations. With imaging approaches, the assessment of PVD severity or symptomatic macular pathology may vary between investigators, sites, and modalities, which complicates interstudy comparisons.³ Imaging methods may also be compromised in older eyes by lens opacities. Moreover, while assessing liquefaction or performing dynamic light scattering provides important physical information about the vitreous, mechanical properties (e.g., stiffness, adhesivity) are not directly measured with these techniques.

Dynamic mechanical analysis is an approach well suited for the characterization of viscoelastic gels such as the vitreous.^{9–11} In particular, using a cleated, parallel plate geometry on a shear rheometer, Nickerson et al.¹² have delineated oscillatory testing procedures to maximize the accuracy of shear modulus measurements in the linear viscoelastic regime using bovine and porcine vitreous. Building on this study, here we used a similar procedure to show the efficacy of enzymatic vitreolysis in altering the mechanical properties of bovine vitreous. Hyaluronan and collagen were degraded using hyaluronidase and collagenase, respectively, whereas trypsin (a more ubiquitous enzyme) was used to digest chondroitin proteoglycans,^{13,14} and to a lesser degree, some collagens (e.g., chondroitin-associated regions of collagen IX and non-triple helical regions of collagen II).^{15,16} Following oscillatory shear testing, we used an extensional test to gauge vitreous adhesivity. Although the vitreous is known to pull on the retina in cases of symptomatic vitreomacular traction, to our knowledge, the mechanical behavior of the vitreous in tension has not been analyzed previously.

Results from the current study suggest that collagen, hyaluronan, and proteoglycans cooperatively contribute to the structure of the vitreous body, and indicate that hyaluronan (and associated water) is a key mediator of internal adhesivity. This study provided new insight into how macromolecules collectively mediate structure and adhesivity in the vitreous

body, while describing a reproducible, objective method to assess viscoelastic properties that is applicable across species, age, disease groups, and pharmacologic perturbations.

METHODS

Vitreous Preparation

Fresh bovine and porcine eyes were collected immediately after animal sacrifice (Trenton Processing, Trenton, IL) and transported on ice (<1 hour) to Washington University. The cornea and iris were removed using a razor blade, scissors, and forceps. The lens anterior capsule, nucleus, and posterior capsule were carefully dissected to expose the vitreous (Fig. 1A). Next, a trephine (5/8-inch diameter) was used to punch a cylindrical plug of vitreous, extending from the anterior of the vitreous to the vitreoretinal interface, for rheologic analysis (Fig. 1B). For all experiments, mechanical testing was performed on 2 or 3 separate days to confirm repeatability.

In a subset of bovine samples, structural macromolecules were enzymatically digested before dissection. Trypsin (1 mg/mL, catalogue No. 15090046; Life Technologies, Carlsbad, CA), *Streptomyces* hyaluronidase (50 U, H1136, SLBF4583V; Sigma-Aldrich, St. Louis, MO), or collagenase (0.5 mg/mL, C1764, SLBD3343V; Sigma-Aldrich) diluted in PBS including calcium and magnesium chloride was injected (final volume: 200 μ L) through the pars plana into the vitreous core (see Fig. 3A). Separate tests confirmed similar biomechanical effects, using highly purified collagenase (50 U, C0773, SLBB3819V; Sigma-Aldrich).

Additional effects on structure were negligible with higher concentrations of enzymes, using the storage and testing procedure outlined below. In double-enzyme treatments, injection volume was halved and concentration was doubled for each compound. In the case of the collagenase + trypsin mixture, collagenase was injected 1 hour earlier to prevent trypsin from inhibiting collagenase activity, whereas hyaluronidase and purified collagenase were injected at the same time. Samples were kept at room temperature for 1 hour to equilibrate before overnight storage at 4°C. The following morning samples were warmed to room temperature (>2 hours) before dissection, vitreous isolation, and mechanical testing. Separate tests confirmed negligible changes in mechanical properties due to overnight storage, consistent with previous studies.^{12,17}

In a subset of tests, plugs of isolated porcine vitreous were incubated at 37°C for at least 24 hours in a test tube at concentrations of 2.5 mg/mL trypsin, 200 U hyaluronidase, and 1 mg/mL collagenase, and liquefaction was periodically assessed by two observers.

To assess the effect of osmotic swelling on the mechanical properties of the vitreous, bovine eyes were dissected as above, but with the lens posterior capsule left intact (Fig. 4A). The eye was submerged in isotonic PBS and the sclera, choroid, and retina were gently removed with scissors and forceps. The isolated vitreous was transferred to a Petri dish (6-cm diameter) and was either kept dry or submerged in de-ionized water (dH₂O), isotonic PBS, or a hypertonic salt solution (2.5% NaCl). Samples were rinsed (3 $\times$) in each solution, sealed with Parafilm (American Can Co., Chicago, IL), and stored overnight (4°C) before testing.

Dynamic Mechanical Analysis

Cylindrical plugs of vitreous were transferred directly to the cleated stage of a stress-controlled AR-G2 rheometer (TA Laboratories, New Castle, DE; Fig. 1C). Previous investiga-

FIGURE 2. Oscillatory shear testing of bovine and porcine vitreous. (A, B) Frequency sweeps for bovine and porcine vitreous at fixed strain magnitude ($\gamma_o = 3\%$). Error bars indicate standard deviation. Linear viscoelastic region (modulus independent of frequency) is taken from $\frac{\pi}{5}$ to π rad/s. (C, D) Average storage modulus ($\bar{G}$) and loss tangent ($\tan \delta$) in the linear viscoelastic region (overbars are omitted in subsequent figures). Bovine vitreous is significantly stiffer (higher storage modulus; $P < 0.001$) but behaves less elastically (higher loss tangent; $P < 0.001$) than porcine vitreous.

tions have shown the importance of cleated geometries in suppressing wall slip to reduce testing variability in highly hydrated biological samples such as the vitreous body.¹⁸ A cleated parallel plate (20-mm diameter) was gradually lowered until the cylindrical plug of vitreous was in full contact with the upper plate. To minimize normal forces, the gap between the upper plate and the stage varied between 3 and 6 mm depending on the size of the sample being tested (Fig. 1D). Because preliminary results confirmed little variation in vitreous properties with environment,^{9,19} all samples were tested at room temperature to minimize experimental variability and associated intravitreal temperature fluctuations.

Consistent with previous investigations,^{10,12} we found a 3% oscillatory strain amplitude to apply sufficient torque for accurate modulus determination while maintaining linear viscoelastic behavior. Hence, after a 1-minute equilibration, an oscillatory frequency sweep ($\omega = \frac{\pi}{5}$ to 2π rad/s) at fixed shear strain amplitude ($\gamma_o = 3\%$) was performed to measure the dynamic shear modulus (see Fig. 2). Following another 1-minute equilibration, the upper plate was raised at a fixed rate (250 $\mu\text{m/s}$) to exert tensile forces on the vitreous plug (load cell sensitivity: ± 1 mN; see Fig. 5A). As this study marked a first attempt at measuring the viscoelastic behavior of the vitreous in extension, rate-dependent responses were not considered in the current analysis.

Viscoelastic Properties

Oscillatory sweeps applied a frequency-dependent, sinusoidal stress, $\sigma_o \sin(\omega t)$, that subjected the vitreous to the dynamic shear strain

$$\gamma = \gamma_o \sin(\omega t + \delta), \quad (1)$$

where ω is oscillation frequency, t is time, δ is the phase angle,

and γ is the strain amplitude. The storage modulus (elastic component of the shear modulus) is

$$G'(\omega) = \frac{\sigma_o}{\gamma_o} \cos \delta, \quad (2)$$

and the loss modulus (viscous component of the shear modulus) is

$$G''(\omega) = \frac{\sigma_o}{\gamma_o} \sin \delta. \quad (3)$$

For a perfectly elastic solid, deformation is in phase with applied stress, whereas in an ideal viscous fluid, strain lags stress by 90° . For viscoelastic materials that fall between these limits, it is convenient to define the loss tangent (or loss factor),

$$\tan \delta = \frac{G''}{G'}, \quad (4)$$

which provides a measure of the elastic relative to the viscous behavior of the material. Practically, we interpret the storage modulus as a measure of the stiffness, and the loss tangent as a measure of the damping capacity of the vitreous.²⁰

Data Analysis and Statistics

For extension experiments, normal force exerted on the test geometry was plotted as a function of axial stretch distance (see Fig. 5B). A central weighted moving average filter was applied to highlight mean trends (calculated using `movingstd.m`; in the public domain at www.mathworks.com/matlabcentral/fileexchange) and the data were masked for further analysis. Adhesive force (F_A), axial stretch length at first detachment (L_D), and the change in axial length during detachment (ΔL_D) were measured from these plots.

FIGURE 3. Enzymatic vitreolysis. (A) Enzyme solutions (200 μ L) are injected into the bovine vitreous core through the pars plana and incubated overnight. (B) Storage modulus (G') determined via oscillatory shear testing. Phosphate-buffered saline sham injection ($G' = 8.6 \pm 2.4$ Pa) is similar to controls (10.7 ± 3.2 Pa; see Fig. 2C). Enzymes targeted to structural components of the vitreous (trypsin, 1 mg/mL; hyaluronidase, 50 U; collagenase, 0.5 mg/mL) significantly decrease the storage modulus ($P < 0.05$). Enzyme cocktails (C + H = collagenase, 0.5 mg/mL + hyaluronidase, 50 U; C + T = collagenase, 0.5 mg/mL + trypsin, 1 mg/mL) do not significantly decrease stiffness relative to individual treatments. (C) Loss tangent ($\tan \delta$) is similar between PBS and trypsin-treated vitreous. Hyaluronidase decreases, while collagenase increases, the loss tangent ($P < 0.05$ between these two groups). Loss tangent of vitreous treated with C + H is significantly less than C + T mixture ($P < 0.05$).

Combining these data with the storage modulus (G') and loss tangent ($\tan \delta$) determined from oscillatory shear sweeps gave five experimental variables for pairwise correlation analyses.

Four of the five parameters (F_A , ΔL_D , G' , $\tan \delta$) exhibited normal distributions and were compared with a Spearman correlation (Sigmaplot 12.0; Systat, Chicago, IL; similar to Pearson correlation but more robust to outliers; see Fig. 6). The stretch length at first detachment (L_D) parameter exhibited bimodal behavior and was analyzed using the mixtools package²¹ for finite mixture models in R 2.15.1 (in the public domain at <http://www.r-project.org>; see Fig. 5C). Briefly, data were separated into two normal distributions using a maximum likelihood analysis and statistically compared with a two-tailed Student's t -test.

One-way ANOVA, with post hoc pairwise comparison made with the Bonferroni-Dunn test, was used to compare storage modulus and loss tangent data, following enzymatic and osmotic perturbations. A two-tailed Student's t -test was used to compare differences between bovine and porcine vitreous, as well as double enzymatic digestions. Results are either plotted as mean $\pm$ SD, or as raw data, with $P < 0.05$ for statistical significance.

FIGURE 4. Osmotic perturbations. (A) Isolated vitreous is kept dry or submerged in solution and stored overnight before frequency sweep ($\gamma_o = 3\%$). (B) Bovine vitreous storage modulus in different osmotic conditions. Hypertonic (2.5% NaCl) and dry conditions tend to decrease stiffness relative to isotonic (PBS) and hypotonic (dH₂O) solutions ($P < 0.05$ for dH₂O relative to dry case). (C) Average loss tangent. Although no significant differences are observed, the loss tangent tends to be lower in the dry condition.

RESULTS

Bovine Storage Modulus and Loss Tangent Are Higher Than Porcine Vitreous

Previous investigators have performed oscillatory frequency sweeps on bovine and porcine vitreous with care taken to maximize measurement accuracy in the linear viscoelastic regime.¹² To validate our current approach, we repeated this procedure on cylindrical plugs of bovine and porcine vitreous isolated with a trephine (Figs. 1, 2). In both species, the storage modulus was relatively constant until oscillation frequency exceeded approximately 5 rad/s, at which point nonlinear viscoelastic (e.g., inertial) effects became significant, similar to past results.^{10,12} Hence, the plateau region ($\omega \leq \pi$ rad/s) was taken as the linear viscoelastic region (shear moduli are frequency independent), from where the average storage

FIGURE 5. Vitreous adhesivity assay. (A) After shear testing and equilibration, enzymatically treated bovine vitreous was stretched axially at a fixed rate (250 $\mu\text{m/s}$). (B) Bimodal vitreous behavior in tension. In the more common case (blue), the vitreous gradually sloughed from the upper plate early in the assay. In the other case (red), the vitreous demonstrated stretch and snap behavior, remaining strongly attached before quickly releasing from the plate. Adhesion force (F_A), axial stretch length at first detachment (L_D), and axial stretch length during detachment (ΔL_D) were quantified for all enzymatic treatments ($n = 39$). (C) Bimodal distribution of L_D measurement. Each point is color coded to denote most probable component of membership. Estimated component means (black dots) plotted with 90%, 95%, and 99% bivariate normal distribution contours. Stretch and snap (red) generates significantly more adhesive force than sloughing (blue) vitreous. (C') Respective component membership for each enzyme treatment.

modulus ($\bar{G}'$; Fig. 2C) and loss tangent ($\overline{\tan \delta}$; Fig. 2D) were calculated. (For convenience, overbars are omitted in the remainder of the article, with the understanding that measurements refer to the average material behavior in the linear viscoelastic regime.) Consistent with Nickerson et al.,¹² we found that (1) steady-state moduli are on the order of 1 to 10 Pa, (2) the storage modulus is approximately 2.5 times greater in bovine than porcine vitreous ($P < 0.001$), and (3) the loss tangent is lower in porcine relative to bovine vitreous ($P < 0.001$; Fig. 2). In other words, porcine vitreous is less dissipative despite being softer than bovine vitreous. Good agreement between our results and past studies suggested our

FIGURE 6. Enzymatic vitreolysis correlation analysis. Adhesion force (F_A), axial stretch length during detachment (ΔL_D), loss tangent ($\tan \delta$), and storage modulus (G') were compared using Spearman correlation analysis. *Left:* y-axis variables and ranges; *Bottom:* x-axis variables and ranges; *Lower right:* colors denote sample treatment ($n = 39$). Numeric correlation coefficients shown for positive (red) and negative (blue) correlations (light shading indicates $P < 0.05$; dark shading indicates $P < 0.01$). Storage modulus did not correlate with loss tangent or material behavior in extension (F_A or ΔL_D ; $P > 0.05$). A decrease in loss tangent (e.g., increasing elastic-like behavior) strongly correlated with an increase in vitreous adhesion force ($P < 0.01$). F_A and $\tan \delta$ also correlated with ΔL_D ($P < 0.05$).

experimental method was a reliable approach to describe the viscoelastic behavior of the vitreous.

Enzyme Treatment Decreases the Storage Modulus but Has Variable Effect on the Loss Tangent

Next, we tested the hypothesis that changes in vitreous structure following enzymatic vitreolysis could be quantified using dynamic mechanical analysis. For these tests, we used bovine vitreous because we hypothesized that enzymatic perturbations would be more readily identifiable in samples with higher initial moduli. Enzymes targeted to structural components of the vitreous (trypsin: ubiquitous protease to primarily digest proteoglycans; hyaluronidase: hyaluronan; collagenase: type II and XI collagen backbone and supporting type IX side chains¹) were injected into the vitreous core, samples were incubated overnight, and tested the following morning (Fig. 3A). Compared to a PBS (sham) injection, the storage modulus (or stiffness) of the vitreous significantly decreased after digestion with each enzyme (Fig. 3B). Injection of enzyme cocktails (collagenase + trypsin, or collagenase + hyaluronidase) decreased the storage modulus further, but differences were not statistically significant compared to individual injections (Fig. 3B). This result suggested that hyaluronan, collagen, and proteoglycans all function as important biomechanical components of the vitreous body. Because structural effects were not additive, as indicated by the double-enzyme treatments, dynamic interactions between these macromolecules are likely to be involved in maintaining vitreous structure. Qualitatively, we note that in cases of extreme enzyme exposure (reduced vitreous volume and longer incubation times), collagenase (but not trypsin or hyaluronidase at the concentrations given in the Methods) could induce complete gel liquefaction.

While all enzyme treatments decreased the storage modulus, variable effects were observed on the loss tangent. PBS

sham, and trypsin-treated samples were similar to controls ($\tan \delta \approx 0.43 \pm 0.05$), but hyaluronidase and collagenase exposure decreased and increased the loss tangent, respectively (differences between these treatments were statistically significant; $P < 0.05$). Opposing effects were also observed in the double-enzyme treatments, with hyaluronidase + collagenase decreasing, and trypsin + collagenase increasing the loss tangent ($P < 0.05$). Together, these results indicated that digesting hyaluronan causes the vitreous to behave more elastically, while digesting collagen fibrils has the opposite effect, leading to a more viscous, or damped material response. Treating with both collagenase and hyaluronidase resulted in a lower loss tangent, suggesting hyaluronan as the key mediator of this parameter. Because a major biomechanical role for hyaluronan is binding water to lubricate and strengthen extracellular matrices, as another test for the structural role of hyaluronan, we next investigated how osmotic swelling affected the viscoelastic properties of the vitreous.

Water Loss Decreases the Storage Modulus of the Vitreous Body

Isolated vitreous was kept dry or submerged in dH₂O (hypotonic), PBS (isotonic), or a hypertonic salt solution (2.5% NaCl) overnight before testing (Fig. 4A). Relative to the PBS treatment, dH₂O slightly increased, while the high salt and dry conditions decreased the storage modulus (differences between dH₂O and dry conditions were statistically significant; $P < 0.05$; Fig. 4B). The loss tangent was relatively consistent across groups, although dry conditions tended to decrease this value (Fig. 4C; water loss in dry conditions: 3.1 ± 0.4 g, $\approx 15\%$ – 20% of total vitreous weight). This set of experiments supported a role for osmotic swelling and overall water content in affecting the viscoelastic properties of the vitreous. In general, water loss decreased vitreous stiffness, but increased the elastic behavior of the gel, similar to hyaluronidase treatments.

Loss Tangent, Not Storage Modulus, Predicts Vitreous Behavior in Extension

As described in Methods, in the enzymatic vitreolysis experiments, oscillatory frequency sweeps were followed by uniaxial extension (Fig. 5A). We simplified this analysis to a constant loading rate (250 $\mu\text{m/s}$), and quantified adhesive force (F_A), axial stretch length at first detachment (L_D), and change in length during detachment (ΔL_D) during vitreous extension (Fig. 5B). These experiments were characterized by two types of material behavior. In the first mode, the vitreous gradually sloughed from the upper plate with little normal (adhesive) force generation (Fig. 5B, blue), whereas the second mode was characterized by significant axial stretch, an increase in adhesive force, and sudden release (Fig. 5B, red). Data analysis confirmed bimodal behavior, as L_D clustered at 1.4 ± 1.0 mm and 11.9 ± 2.1 mm (blue and red dots, respectively; Fig. 5C), with significantly greater adhesion force in the latter cluster ($P < 0.05$; Fig. 5C). Collagenase and trypsin treatment always resulted in the first mode ($n = 14/14$), which also predominately occurred in controls ($n = 5/7$; Fig. 5C'). Hyaluronidase exposure more frequently resulted in the second mode ($n = 4/8$), which also occurred after treatment with collagenase plus hyaluronidase ($n = 1/4$; Fig. 5C'). These data suggested that enzymatic vitreolysis could alter the intrinsic adhesivity and fundamental material behavior (gradual sloughing versus stretch and snap) of the vitreous body in extension. To provide further insight into these results, we performed pairwise comparisons between the four normally distributed param-

eters determined from oscillatory (G' , $\tan \delta$) and extensional testing (F_A , ΔL_D ; Fig. 6).

In oscillatory shear tests, the storage modulus (G') did not correlate with the loss tangent ($\tan \delta$), as would be predicted from earlier findings (all enzymes decreased the storage modulus but had opposing effects on the loss tangent; see Figs. 3B, 3C; Fig. 6, lower left). In extension tests, adhesion force increased as the length during detachment decreased (Fig. 6, upper right; $P < 0.05$). This result reflects the differences between stretch and snap (high adhesion force, quick detachment) and gradual sloughing (low adhesion force, slow detachment) behavior across a more gradual (as opposed to binary, see Fig. 5C) continuum. Across mechanical tests (shear versus extension), the loss tangent was an excellent predictor for adhesive force (F_A , Fig. 6, upper middle; $P < 0.01$) and to a lesser degree, stretch length during detachment (ΔL_D , Fig. 6, center middle; $P < 0.05$). Conversely, the storage modulus (G') did not correlate with parameters measured in the extension tests (Fig. 6, upper and middle left panels). Together, these data indicated that material behavior in extension, or intrinsic adhesivity, is well predicted by the loss tangent (damping), but not the storage modulus (stiffness), following enzymatic vitreolysis.

DISCUSSION

In this study we demonstrated how dynamic mechanical analysis (in both shear and extension) can be used to quantitatively and objectively probe enzyme-induced changes in vitreous stiffness and adhesivity. Results suggest that collagens, proteoglycans, and hyaluronan cooperatively regulate the stiffness of the vitreous body, and further implicate hyaluronan (and associated water content) as a key regulator of vitreous adhesivity. Moreover, we found that enzymatic degradation of vitreous components differentially affects viscoelastic damping, which can lead to dramatic differences in material behavior (sloughing versus stretch and snap) during in vitro extensional tests.

A Network of Macromolecules Regulates Vitreous Mechanical Properties

While it is known that large collagen fibrils (composed of types II, IX, and V/XI collagen) are important structural components of the vitreous body,^{1,4,10,22} biomechanical roles for supporting glycosaminoglycans and proteoglycans are less well understood. Previously, Nickerson et al.¹² proposed a “network tension hypothesis” where hyaluronan-induced swelling stiffens the vitreous by stretching collagen fibrils, an effect that has been qualitatively observed at the microstructural level with both transmission and scanning electron microscopy.^{23,24} Results from the current study also support a structural role for hyaluronan in the vitreous, as hyaluronidase treatment significantly decreased vitreous stiffness (Fig. 3B), while osmotic swelling and dehydration tended to increase and decrease vitreous stiffness, respectively (Fig. 4B). In addition to hyaluronan, our results also indicated a biomechanical role for noncollagenous proteins and proteoglycans, as revealed by a significant decrease in storage modulus, following trypsin exposure compared to PBS-injected controls (Fig. 3B). Supporting this result, Bishop et al.¹⁵ have found a significant decrease in the N-terminal proteoglycan region of type IX collagen and associated chondroitin sulfate side chains in aged human vitreous. We speculate that enzymatic degradation of these macromolecules, in addition to other potential cross-linking proteins, such as fibrillin and opticin, compromise the lateral reinforcement of major collagen fibrils (type II, IX, and

FIGURE 7. Summary of results and hypothesis for vitreous structure and vitreolytic mechanisms. In normal vitreous (*left*), collagen fibrils are separated by the proteoglycan side chains of type IX collagen, proteoglycans, and hydrophilic hyaluronan macromolecules. Trypsin digests proteoglycans and cross-linking proteins, but larger collagen fibrils remain intact. Loss of lateral reinforcement leads to a decrease in stiffness but adhesivity is largely unaffected. Hyaluronidase induces water loss and the vitreous collapses into a more flaccid structure. Remaining collagens and proteoglycans are intact and concentrated, leading to an increase in adhesivity. Collagenase digests collagen fibrils into smaller fragments, but the hyaluronan network remains intact. Stiffness decreases owing to collagen loss, as does adhesivity, as material behavior becomes more viscous. Total collagen degradation completely liquefies the vitreous gel.

V/XI) to reduce vitreous stiffness. Proposed effects of collagen, hyaluronan, and proteoglycan digestion on vitreous structure are summarized in schematic form in Figure 7.

Mechanical Damping Is an Important Component of Vitreous Viscoelasticity

The loss tangent provides a measure of the viscoelastic damping ability of the vitreous. Although all enzyme treatments reduced stiffness (Fig. 3B), different effects were observed on the loss tangent (Fig. 3C). Because of the high water binding capacity of hyaluronan, we propose that digesting this glycosaminoglycan (or incubating the vitreous in dry conditions) caused water loss that concentrated remaining collagens and proteoglycans (Fig. 7), which decreased the loss tangent. Corresponding extensional tests showed how this decrease can lead to greater adhesivity (Fig. 6) and stretch and snap behavior during uniaxial testing (Fig. 5). Collagenase, on the other hand, digested the fibrillar backbone of the vitreous, leaving the hyaluronan network intact, resulting in more viscous behavior (increase in loss tangent) and a decrease in overall intrinsic adhesivity (Fig. 7).

These findings are supported by Lai et al.²⁵ who have found a decrease in loss tangent as collagen concentration increases in a rheologic study of collagen gels. For comparison, collagen concentration has been reported to average 60 $\mu\text{g/mL}$ ¹ for bovine vitreous ($\tan \delta = 0.44 \pm 0.05$; Fig. 2D), whereas for pure, fibrillar type I/III collagen gels at 2 mg/mL, the frequency-independent loss tangent is considerably lower ($\tan \delta = 0.19$).²⁶ Across a range of species (goat, cow, pig, sheep, rabbit) but a limited number of samples, Mensitieri et al.²⁷ have noted a vitreous loss tangent between 0.2 and 0.5 at a 1-Hz sampling frequency. Consistent with these initial data, we found the loss tangent for bovine and porcine vitreous to fall within this window, with significantly more damping observed in bovine vitreous (Fig. 2D). At an evolutionary level, future studies might investigate interspecies differences in vitreous stiffness (storage modulus) and damping (loss tangent)

to determine whether these parameters correlate with biochemical composition and biomechanical factors such as eye size and movement frequency.

Our data also showed how enzymatic vitreolysis can lead to fundamentally different modes of material behavior in extension, suggesting that the effects of damping on vitreous deformations *in vivo* warrant further investigation.¹⁹ In particular, results from this study provide baseline data for computational modeling efforts to more accurately identify how altered viscoelastic properties influence vitreous dynamics and associated retinal pathologies.^{28–30} For example, biomechanical modeling could help determine how vitreous damping and stiffness influence the local stresses that develop at tractional vitreoretinal adhesions and lead to retinal detachments. Although spatiotemporal resolution limitations currently preclude detailed investigations of altered damping in patients (e.g., near vitreous lacunae or at sites of vitreomacular traction), we note that advancements in noninvasive, dynamic, ultrasonography³¹ and magnetic resonance-based³² imaging of the vitreous have shown promise in this regard.

“Liquefactants” Do Not Necessarily Liquefy the Vitreous

Vitreolytic agents are commonly classified as liquefactants if the compound induces liquefaction, or causes water loss from the vitreous (both hyaluronidase and collagenase are considered liquefactants).³ However, results from this study showed that hyaluronidase and collagenase have opposing effects on the viscoelastic behavior of the vitreous. Treatment with hyaluronidase concentrates the remaining macromolecules, inducing elastic-like behavior ($\tan \delta$ decreases), while collagenase treatment causes the gel to act more like a viscous fluid ($\tan \delta$ increases). Quantitative mechanical testing with additional enzymes (e.g., plasmin, ocriplasmin, chondroitinase) should aid in better determining whether these compounds liquefy or concentrate the vitreous gel. Because most

researchers and clinicians are interested in the effects on the vitreous gel following vitreolysis (rather than what is lost from it), the current definition of liquefactant may be insufficient. If water is lost from the vitreous after pharmacologic vitreolysis, such an effect has no bearing on whether or not the remaining gel is, in fact, liquefied. Moreover, although we did not observe this effect, it is conceivable that excessive water loss could collapse remaining structural proteins beyond a critical density, at which point the stiffness of the gel could increase. In such a case, the term “liquefaction” could refer to an increase in structure. Careful biomechanical analyses will help better define these potentially important differences in future studies.

Clinical Implications and Future Directions

Results from this study also shed light on the *in vivo* effects of vitreolytic agents. For example, consider the case of treating symptomatic vitreoretinal traction with hyaluronidase to soften the vitreous before surgical vitrectomy. (Note that hyaluronidase, otherwise known as Vitrase [Ista Pharmaceuticals, Irvine, CA], successfully reached phase III FDA clinical trials before being disapproved.)³³ Our results suggest that the vitreous will contract as hyaluronan is digested and the water-binding capacity of the vitreous is lost (Fig. 7). Since proteins spanning the vitreoretinal interface are not affected by hyaluronidase, this treatment could exacerbate traction, leading to retinal damage and vision loss.

The inability to quantitatively assess the effect of enzymatic vitreolysis on vitreoretinal adhesion forces is a limitation of this study. Owing to spatial gradients and possible age-related changes in proteins spanning the vitreoretinal interface,²² spatiotemporal differences in adhesion forces exist in the eye. Such forces are better suited for study using localized methods (e.g., micro-aspiration), as opposed to the rheologic approach taken here. In the future, coupling local adhesion force measurements with imaging methods that detect changes in macromolecule morphology at the vitreoretinal interface could provide important multiscale information as to how adhesion and microstructure are regulated and altered during vitreolysis.^{34,35}

Beyond locally symptomatic regions, however, this study showed the effects of enzymatic vitreolysis on the bulk of the vitreous body. As outlined above, computational models will be useful in applying these experimental data to better understand how pharmacologic vitreolysis affects intravitreal transport. For example, an increase in transport following gel degradation could alter pharmacokinetic profiles of injected intravitreal drugs,³⁶ as well as increase oxygen transport between the retina and the lens.³⁷ In the latter case, pharmacologic vitreolysis (plasmin and microplasmin treatment) has been shown to increase oxygen content in the vitreous and lens,^{38–40} a known risk factor for the development of nuclear cataracts.⁴¹ It is important to note this effect when considering how potential side effects from pharmacologic vitreolysis, such as lens subluxation due to digestion or weakening of the zonules, could complicate later cataract surgeries. To help protect the lens from cataracts post vitreolysis, research strategies that restore the structure of enzyme-treated vitreous warrant further attention.

CONCLUSIONS

In this study we described a reproducible, objective method to quantitatively assess changes in vitreous stiffness and adhesivity after pharmacologic vitreolysis. Results indicate that vitreous macromolecules act synergistically to maintain structure, with hyaluronan and associated water content acting as a

major mediator of intrinsic adhesivity. Future studies should consider adapting this approach to help better understand changes in macromolecule morphology and vitreoretinal adhesion forces following pharmacologic vitreolysis.

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