Preservation of the Structure of Enzymatically-Degraded Bovine Vitreous Using Synthetic Proteoglycan Mimics

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Purpose. Vitreous liquefaction and subsequent posterior vitreous detachment can lead to several sight-threatening diseases, including retinal detachment, macular hole and macular traction syndrome, nuclear cataracts, and possibly, open-angle glaucoma. In this study, we tested the ability of three novel synthetic chondroitin sulfate proteoglycan mimics to preserve the structure and physical properties of enzymatically-degraded bovine vitreous.

Methods. Chondroitin sulfate proteoglycan mimics, designed to bind to type II collagen, hyaluronic acid, or both, were applied to trypsin- or collagenase-treated bovine vitreous in situ and in vitro. Rheology and liquefaction tests were performed to determine the physical properties of the vitreous, while Western blots were used to detect the presence and degradation of soluble collagen II (α1). Deep-etch electron microscopy (DEEM) identified the ultrastructure of mimic-treated and untreated enzyme-degraded bovine vitreous.

Results. Proteoglycan mimics preserved the physical properties of trypsin-degraded bovine vitreous and protected against vitreous liquefaction. Although the collagen-binding mimic maintained the physical properties of collagenase-treated vitreous, liquefaction still occurred. Western blots indicated that the mimic provided only marginal protective ability against soluble collagen degradation. Deep-etch electron microscopy, however, showed increased density and isotropy of microstructural components in mimic-treated vitreous, supporting the initial result that vitreous structure was preserved.

Conclusions. Proteoglycan mimics preserved bovine vitreous physical properties after enzymatic degradation. These compounds may be useful in delaying or preventing the pathological effects of age-related, or enzymatically-induced, degradation of the vitreous body.

Keywords: vitreous degeneration, vitreous liquefaction, chondroitin sulfate proteoglycan mimics, vitreous structure

The vitreous body is a highly hydrated (98%–99% water) extracellular matrix that fills the eye. The structure of the vitreous gel derives from interactions between collagens (II, IX, V/XI, and VI), hyaluronic acid, and proteoglycans.[1] In humans, the vitreous body undergoes irreversible age-related liquefaction (also termed vitreous degeneration or synchysis),[2] which often leads to detachment of the vitreous from the inner surface of the retina (posterior vitreous detachment; PVD). Posterior vitreous detachment usually occurs without incident, but stress on the retina resulting from vitreous traction may cause sight threatening vitreoretinal diseases (rhegmatogenous retinal detachment,[3–5] macular hole, macular traction syndrome, epiretinal membrane formation, and myopia-associated retinoschisis).[6–8] Vitreous degeneration is also a risk factor for nuclear cataracts[9–11] and vitreomacular traction can contribute to choroidal neovascularization (CNV) and exudative AMD.[12–14]

However, mechanisms of vitreous liquefaction are still under investigation. One of the major hypotheses is that enzymatic degradation could play a role. Bishop et al.[15,16] hypothesized that synchysis and syneresis may be caused by loss of proteoglycans (in particular, the chondroitin sulfate side chain of collagen IX). Loss of this proteoglycan occurs with aging and is followed by aggregation of collagen fibrils and contraction of vitreous gel, leaving liquid filled spaces (lacunae). Los et al.,[17] also found fragmentation of collagen fibrils in the vicinity of liquefied spaces in light and electron microscopy studies. These results and subsequent studies have suggested the involvement of matrix-related proteolytic enzymes, including matrix metalloproteinases (MMP) found in the vitreous (collagenase MMP-1 and MMP-8; gelatinase MMP-2 and MMP-9),[18,19] trypsin-1 and trypsin-2 in vitreous degeneration.[20,21]

Surgical resolution of PVD-induced vitreoretinal diseases has been through pars plana vitrectomy (PPV) or, more recently, the introduction of ocriplasmin (JETREA; ThromboGenics, Iselin, NJ, USA) to release the traction of symptomatic vitreomacular adhesion (VMA). This treatment often is not successful in resolving VMA and subsequent surgery is required. Surgical procedures are associated with substantial risks (iatrogenic retinal tear or detachment, lens touch), high prevalence of postvitrectomy nuclear sclerotic cataract and

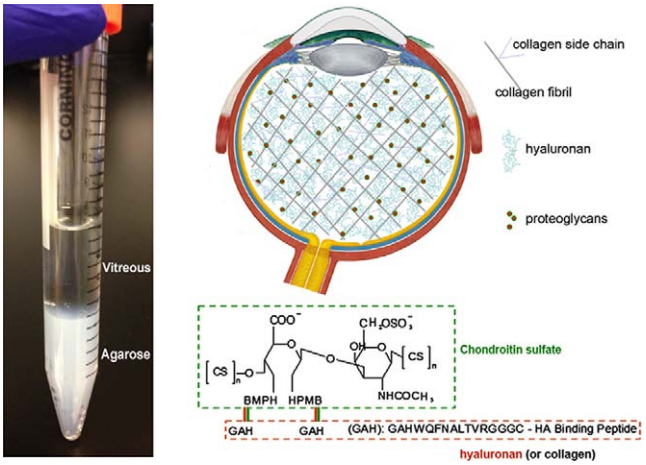

Figure 1. Experiment overview. Storage of vitreous "plugs" in a 15-mL conical tube for in vitro testing (*left*). Schematic diagram of a proteoglycan mimic (*right bottom*) and the binding sites of proteoglycans in the vitreous gel (*right top*).

interventions subsequent to cataract surgery (Nd:YAG laser posterior capsulotomy) for posterior capsule opacification.[22–25] All of these complications have substantial personal and monetary cost and the patients' previous visual acuity may not be restored. Therefore, it would be beneficial if we could avoid the consequences of vitreous degeneration by delaying degradation or restoring the structure of the vitreous gel.

A comprehensive literature search has recently highlighted the limited amount of research aimed at developing and testing therapies to halt or reverse age-related vitreous degeneration.[26] However, biomedical engineers are making efforts to repair connective tissues. The Panitch lab at Purdue University (West Lafayette, IN, USA) has developed synthetic proteoglycan mimics as osteoarthritis (OA) treatments that directly target cartilage. These synthetic proteoglycans function analogous to the proteoglycan aggrecan (AGG), and were able to restore the physical properties of enzymatically degraded cartilage.[27] Studies have also shown the capability of the proteoglycan mimics for improving wound healing by suppressing inflammation and reducing dermal scarring.[28] Since the components of the vitreous body are similar to cartilage, just at a much lower concentration, we applied this novel treatment in an attempt to preserve vitreous structure.

Results from this study showed that collagen type II and hyaluronan-binding chondroitin sulfate (CS) proteoglycan mimics preserved the mechanical properties of enzymatically-degraded bovine vitreous with different efficiency. Deep-etch electron microscopy (DEEM) and Western blot analyses provided additional information about the mechanisms by which the mimics stabilized and preserved vitreous structure. Proteoglycan mimics may have clinical relevance for future prevention of ocular diseases caused by age-related vitreous degeneration.

Methods

Preparation of Proteoglycan Mimics

Synthetic proteoglycan mimics functionalized with peptides designed to bind structural components of the vitreous body (hyaluronan,[27] collagen[28,29]) were synthesized as previously described (Fig. 1). Briefly, the oxidized chondroitin sulfate backbone was conjugated to a linking compound (N-[b-Maleimidopropionic acid] hydrazide trifluoroacetic acid salt [BMPH]) and reacted with synthetic collagen- and/or hyaluronan-binding peptides. Full amino acid sequences for binding peptides are provided in the Table. Proteoglycan mimics (WYR: 30,204 g/mol, collagen binding; GAH: 37,853 g/mol, hyaluronan binding; GAH+WYR: 33,422 g/mol, hyaluronan and collagen binding) were reconstituted in PBS (pH 7.4) to a concentration of 25 μM for subsequent use.

Bovine Vitreous Collection and Preparation

Fresh bovine eyes from a local slaughterhouse (Trenton Processing, Trenton, IL, USA) were transported on ice to Washington University (St. Louis, MO, USA). All specimens were dissected and prepared within 4-hours postmortem, and tested within 24 hours.

For in situ tests, trypsin (2.5 mg/mL, Cat. No. 15090046; Life Technologies, Grand Island, NY, USA), mimics, or a mixture of mimics plus trypsin were injected through the pars plana into the center of the vitreous prior to dissection, as described previously.[1] In the case of the mimics + trypsin mixture, mimics and trypsin were injected at same time, because mimics were designed to be insensitive to cleavage by trypsin. In a subset of experiments we tested the effects of adding mimic first (GAH + trypsin) or trypsin first (trypsin + GAH; 1-hour injection interval) on vitreous mechanical properties and liquefaction (pilot experiments confirmed 1 hour was sufficient for a dye, phenol red, to fully infiltrate the vitreous gel). The final injection volume was 300 μL, containing 100 μL trypsin and 200 μL PBS or 200 μL of the 25 μM proteoglycan mimics solution. Sham injections of PBS alone were performed as controls. Samples were kept at room temperature for 1 hour to equilibrate before overnight incubation at 4°C (in a previous study,[1] we found negligible changes in vitreous mechanical properties as a function of incubation temperature when following this protocol). The following morning samples were warmed to room temperature (>2 hours) prior to mechanical testing.

In another set of experiments, we performed in vitro tests using collagenase and mimics. In contrast to the trypsin studies, we found that temperature could have a strong influence on vitreous properties with increasing concentrations of collagenase. Moreover, collagenase-induced damage to surrounding tissues such as the retina and iris during in situ tests could presumably confound the analysis of the vitreous. Hence, to optimize enzyme function, but to limit damage to surrounding tissues, we designed in vitro tests for our collagenase experiments. First, the cornea, iris, and lens were removed using a sharp razorblade, scissors, and forceps. Following dissection, a cylindrical plug of vitreous (~4 mL)

Table. The Amino Acid Compositions, Abbreviations, and Binding Targets for the Three Chondroitin-Sulfate Proteoglycan Mimics Used in This Study

Proteoglycan Mimics	Abbreviation	Binding Target
GAHWQFNALTVRGGGC-BMPH-CS	GAH	Hyaluronan (HA)
WYRGRLGC-BMPH-CS	WYR	Collagen type II
GAHWQFNALTVRGGGC-WYRGRLGC-BMPH-CS	GAH+WYR	HA + collagen type II

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was removed with a trephine (5/8- inch diameter) and gently poured into a 15-mL conical vial with 2 to 3 mL of agarose gel poured in the bottom to protect the vitreous from deformation (Fig. 1, left). For maximal activation of collagenase (from *Clostridium histolyticum*, Product No. C1764; Sigma-Aldrich Corp., St. Louis, MO, USA), all dilutions were performed in PBS containing 0.5 mM $CaCl_2$. The collagenase, mimics, or mimics plus collagenase mixture was then carefully dripped onto the surface of vitreous to minimize any perturbation of vitreous structure. In the case of the mimics plus collagenase mixture, the mimics were dripped 1 hour earlier than collagenase to assure full penetration into the vitreous. The total added volume was 300 μL, containing 200 μL of 25 μM mimics, and the final concentration of collagenase was 0.025 mg/ml. Phosphate buffered saline alone, 300 μL, was added to controls. All in vitro samples were incubated at 37°C overnight.

Vitreous Liquefaction

Liquefaction was quantified prior to the rheological testing of both in situ and in vitro vitreous samples. For in situ experiments, a cylindrical plug of vitreous was isolated with a trephine as described above and transferred into a preweighed dish. This sample was weighed and the gel portion was transferred immediately to the rheometer for viscoelastic analysis. Next, the remainder of the vitreous in the eye cup was dissected from the retina and ciliary body and poured into the same dish. The gel portion of the vitreous was separated, weighed, and added to the earlier value to give the mass of intact vitreous gel (M_{gel}). Next, the residual liquid left in the dish was weighed to provide a measure of liquefied vitreous content (M_{liq}). The percentage of vitreous liquefaction was then calculated as $M_{liq}/(M_{gel} + M_{liq}) \times 100\%$, as previously described.[10]

For in vitro tests, the conical tube containing both liquefied and gel vitreous was emptied into a preweighed dish. The gel portion of the vitreous was gently separated from the liquid portion using forceps and weighed individually and percent liquefaction was calculated using the above formula. The gel portion was then transferred to the rheometer for mechanical testing.

Vitreous liquefaction for both in situ and in vitro experiments was measured on 2 or 3 separate days to confirm reproducibility with a minimum of six samples tested per experimental group.

Rheological Testing

To determine viscoelastic mechanical properties of the vitreous, a stress-controlled frequency sweep was performed on a AR G2 rheometer (TA Laboratories, New Castle, DE, USA). A cleated, parallel plate (20-mm diameter) was used to prevent slippage.[30] During testing the plate was lowered until contact was made with the vitreous at a gap between 3 and 6 mm depending on the size of the sample being tested. Preliminary frequency and stress sweeps were performed to determine an appropriate stress-independent storage modulus range (linear viscoelastic regime). For all tests, oscillatory frequency sweeps ($\omega = \frac{\pi}{5}$ to 2π rads/sec) were performed at a fixed shear strain amplitude ($\gamma_0 = 3\%$), similar to previous studies.[30–32] As was done in the liquefaction studies, mechanical testing was performed on a minimum of six samples per experimental group and on a minimum of 2 days.

Western Blot

The presence and degradation of type II collagen after enzymatic degradation and mimics treatment were examined by Western blot analysis. The centrifuged vitreous samples were lyophilized and reconstituted (1 mg/100 ul) in LDS (lithium dodecyl sulfate) sample buffer (10% (wt/vol) glycerol, 2% LDS, 106 mM Tris-HCl, 141 mM Tris base, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM phenol red, pH 8.5; NuPAGE; Invitrogen, Grand Island, NY, USA) containing 50 mM β-mercaptoethanol. After being heated at 95°C for 10 minutes, samples were applied on 4-12% Bis-Tris Gels (NuPAGE Novex; Invitrogen) for protein separation. The separated proteins were then transferred to a nitrocellulose membrane, incubated with preimmune serum (diluted 1:2000 in PBS, ~5 mg IgG/mL) for 1 hour and then affinity-purified rabbit polyclonal antibody to α1 (II) collagen (1:1000; Abcam, Cambridge, MA, USA) at 4°C overnight. After washing with PBS/0.5% Tween 20, IR Dye 800CW-labeled goat anti-rabbit secondary antibody (1:5000 dilution containing 0.5% Tween 20; Li-Cor Biosciences, Lincoln, NE, USA) was applied for 2 hours. Immunoreactive bands were visualized and analyzed with Odyssey infrared imaging system and Image Studio Software (Version 2.1; Li-Cor). Quantified collagen content reflects the densitometric scanning results of both immunoreactive bands at 142 and 165 KDa. All blots were quantified between four and six times for each condition.

Quick-Freeze Deep-Etch Electron Microscopy (DEEM)

Deep-etch electron microscopy was performed according to a published protocol, with modifications.[33] A 10-μL droplet of central core vitreous was placed onto an acid cleaned, air dried 3×3 mm coverglass and sandwiched between a 0.05-mm thick, 3-mm diameter wafer of sapphire glass. The sample was then mounted onto the freezing stage of a "Slam Freezer" and frozen by forceful impact against a pure copper block, cooled to 4 K with liquid helium. Frozen samples were transferred to a liquid nitrogen cooled Balzers 400 vacuum evaporator (Capital Asset Exchange & Trading LLC., Redwood City, CA, USA). Freeze fracture occurred by removing the sapphire cover slip at minus 104°C under vacuum. Samples were etched for 1′ 45" and rotary replicated with approximately 3-nm platinum deposited from a 20° angle above the horizontal, followed by coating with an approximately 10-nm stabilization film of pure carbon deposited from an 85° angle. Replicas were floated onto a dish of concentrated hydrofluoric acid and transferred through a 5-minute rinse in distilled water, a 10-minute float on household bleach, followed by several more water rinses, all containing a loopful of Photo-flo detergent (Eastman Kodak, Rochester, NY, USA). Replicas were picked up on formvar-coated copper grids, and photographed with a JEOL 1400 transmission electron microscope (JEOL USA, Inc., Peabody, MA, USA) with an AMT XR111 digital camera (AMT Corp, Woburn, MA, USA).

Image Analysis

Deep-etch electron microscopy images showed a complex milieu of fibers and circular globules with varying orientation and density in different treatment conditions (control, trypsin, collagenase, WYR; $n \geq 6$ in all cases). Prior to analysis, images acquired at ×25,000 magnification were cropped, down-sampled, smoothed, and contrast-enhanced in Fiji [in the public domain http://fiji.sc/Welcome]. Fiber and globule density were then estimated by thresholding bright pixels (kept constant across all images) in Fiji.

To detect globules alone, we applied the Circular Hough Transform using the Matlab routine CircularHough_Grd.m (in the public domain www.mathworks.com/matlabcentral/fileexchange). Briefly, edges were first detected from image features and then perpendicular lines were taken to the edges, with line intersection points indicating circle centroids. Dark

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circles were excluded (globules always appeared as bright spots on a dark background), while an adaptive threshold was used to screen remaining false-positives (e.g., light gray background surrounded by bright fibers). Visual inspection confirmed the success of the algorithm in identifying globules while limiting false positives in more than 95% of cases. Computed globule area was subsequently subtracted from the fiber and globule threshold density (calculated above) to give a measure of fiber density alone. Please see Figure 8 in the results for more details.

Fiber alignment was assessed using an intensity gradient algorithm as described in Karlon et al.[34] Angle measurements were separated into 10 bins (18° increments) and the measurement frequency in each bin was recorded. To estimate fiber alignment in each image, the highest angular frequency in a particular bin was divided by the total number of measurements to give an alignment ratio. Using this definition, an alignment ratio approaching one or zero would indicate fiber alignment or isotropy, respectively.

Data Analysis and Statistics

All data were analyzed by ANOVA, with post hoc tests using Student-Newman-Keuls Multiple Comparisons (Instat; GraphPad Software Co., La Jolla, CA, USA). Results are presented as average ± SD or as raw data with the mean indicated by a black bar. Statistical significance was considered to be P less than 0.05.

Results

Proteoglycan Mimics Preserve the Storage Modulus of Trypsin-Treated Bovine Vitreous and Prevent Vitreous Liquefaction

Previous studies validated our mechanical testing method via a rheometer frequency sweep at 3% shear stress to be a reliable approach to describe the viscoelastic behavior of the vitreous.[1,31] In the current study, we used storage modulus (G', Pa), a parameter for evaluating the stiffness of the vitreous gel, to measure the mechanical properties of vitreous treated in different manners. Stiffness showed minor changes after injection of PBS or the three types of proteoglycan mimics (G' ≈ 9.6–15.2 Pa), as all values were not statistically different from the controls (G' = 11.1 ± 2.9 Pa, Fig. 2). Injection of 2.5 mg/mL trypsin (to digest proteoglycans and other proteins) significantly decreased the stiffness of the vitreous by approximately one-half ($P < 0.05$). Injection of each of the three mimics with 2.5 mg/mL trypsin preserved stiffness values similar to the control group (G' ≈ 10.4–12.4 Pa). Finding that the mimics prevented trypsin-induced loss of mechanical stiffness, we next tested whether vitreous liquefaction could be similarly prevented using the mimics.

Because hyaluronan is present throughout the vitreous, we used the hyaluronan binding mimic (GAH) to test whether the mimics could prevent global vitreous liquefaction caused by enzymatic proteoglycan digestion. In these in situ experiments, the composition of control and PBS-injected vitreous was approximately 10% liquid (Fig. 3). Liquefaction increased by approximately 50% if trypsin was injected alone or if trypsin was injected 1 hour prior to the addition GAH mimic (TRYP+GAH; $P < 0.01$). However, if the mimic was injected 1 hour prior to trypsin (GAH+TRYP), vitreous liquefaction was similar to controls. Additional experiments ($n = 6$ per group) showed negligible changes in storage modulus relative to controls despite whether trypsin was injected 1 hour prior to the GAH mimic (G' = 10.8 ± 4.7 Pa) or if the experiment was performed in the reverse order (G' = 11.3 ± 3.2 Pa), these two

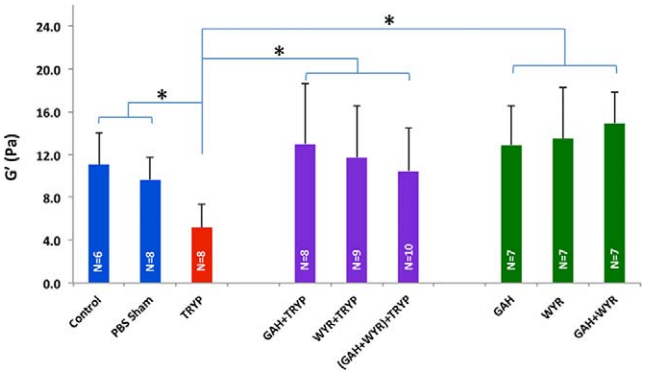

Figure 2. In situ test of bovine vitreous treated with 2.5 mg/mL trypsin, and incubated at 4°C overnight. All samples were characterized using rheometry to evaluate the ability of the three proteoglycan mimics (WYR: collagen binding; GAH: hyaluronan binding; GAH+WYR: hyaluronan and collagen binding) to preserve the physical properties of the vitreous. Phosphate-buffered saline (sham) injection is similar to controls ($P > 0.05$). Adding mimics alone (*green bars*) did not change the stiffness of vitreous compared with control group or PBS-sham group (*blue bars*, $P > 0.05$). However, the stiffness of the vitreous (G') significantly decreased after trypsin treatment (*red bar*, $P < 0.05$ compared with control or PBS-sham group). Adding mimics together with trypsin (*purple bars*) preserved the stiffness of the vitreous at control levels ($P > 0.05$). TRYP: 2.5 mg/mL trypsin; N = sample numbers; $^{*}P < 0.05$.

groups were not significantly different ($P > 0.05$). Together, these results suggested that although the chondroitin sulfate proteoglycan mimics could not restore previously liquefied vitreous, the mimics could protect against further trypsin-induced vitreous liquefaction and loss of mechanical stiffness.

Proteoglycan Mimics Preserve the Stiffness of Collagenase-Treated Bovine Vitreous but Do Not Protect Against Vitreous Liquefaction

The next set of experiments was designed to test the efficacy of the mimics when the important structural component of the vitreous, collagen II, was partially degraded. To maintain the activity of the collagenase and reduce damage to surrounding

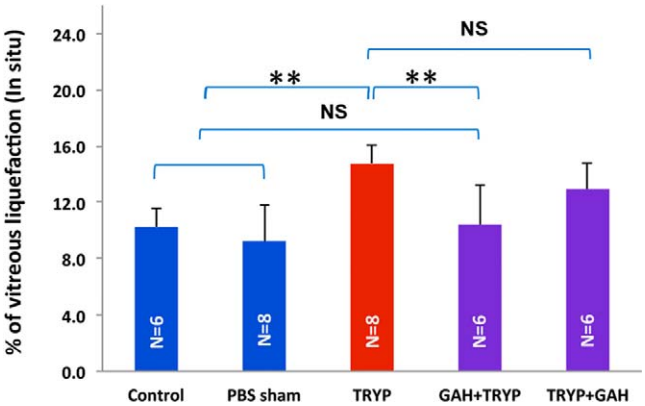

Figure 3. Trypsin-induced vitreous liquefaction. Control and PBS sham–injected vitreous were approximately 10% liquefied in these in situ experiments (*blue bars*). Adding trypsin alone (*red bar*) or trypsin 1 hour prior to the GAH mimic (TRYP+GAH, *purple bar*) significantly increased vitreous liquefaction ($P < 0.01$). If the mimic was added prior to trypsin (GAH+TRYP, *purple bar*), however, liquefaction was similar to controls ($P > 0.05$). TRYP, 2.5 mg/mL trypsin; NS, no significant difference; N = sample numbers; $^{**}P < 0.01$.

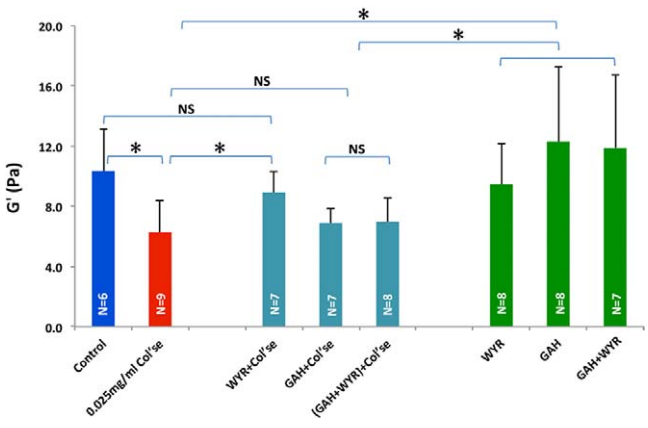

Figure 4. In vitro test of bovine vitreous treated with collagenase (0.025 mg/mL), and three proteoglycan mimics. Treatment with 0.025 mg/mL collagenase (*red bar*) significantly decreased the stiffness of the vitreous compared with controls (*blue bar*, $P < 0.05$). Among the three mimics plus collagenase treatment groups (*light blue bars*), only WYR + collagenase significantly preserved vitreous stiffness ($P < 0.05$ compared with collagenase treated group). The three mimics alone (*green bars*) showed no significant differences compared with the control group ($P > 0.05$). Col'se, 0.025 mg/mL collagenase; N = sample numbers; $^{*}P < 0.05$.

tissues, bovine vitreous was isolated with a sterilized trephine and incubated overnight at 37°C prior to rheometry. In these tests, 0.025 mg/mL collagenase softened the vitreous gel to approximately 40% of the stiffness of the control group ($P < 0.05$, Fig. 4). Importantly, the stiffness of control vitreous tested in vitro (G' = 10.3 ± 1.3 Pa, Fig. 4) was similar to control vitreous tested in situ (G' = 11.1 ± 2.9 Pa, Fig. 2), suggesting no major biomechanical differences between these methods. Addition of each of the mimics alone did not significantly change vitreous stiffness relative to controls ($P > 0.05$). After the addition of each of the mimics with collagenase (mimics added 1 hour earlier, then collagenase), only the WYR (the collagen II-binding mimic) plus collagenase group preserved vitreous stiffness at values similar to controls ($P > 0.05$). The other two mimics (GAH and GAH+WYR) + collagenase groups were significantly softer, similar to vitreous treated with

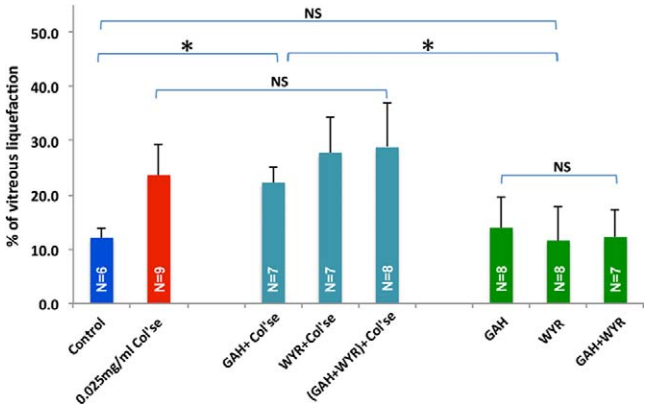

Figure 5. In vitro test of bovine vitreous liquefaction after treatment with collagenase (0.025 mg/mL) and proteoglycan mimics. There is no significant difference between the control (*blue bar*) and the three mimics groups (*green bars*, $P > 0.05$). Adding collagenase alone (*red bar*) or mimics plus collagenase (*light blue bars*) showed similar amounts of vitreous liquefaction ($P > 0.05$). Vitreous liquefaction was significantly higher in groups treated with collagenase compared with those with no enzyme treatment ($P < 0.05$). Col'se, 0.025 mg/mL collagenase; $^{*}P < 0.05$.

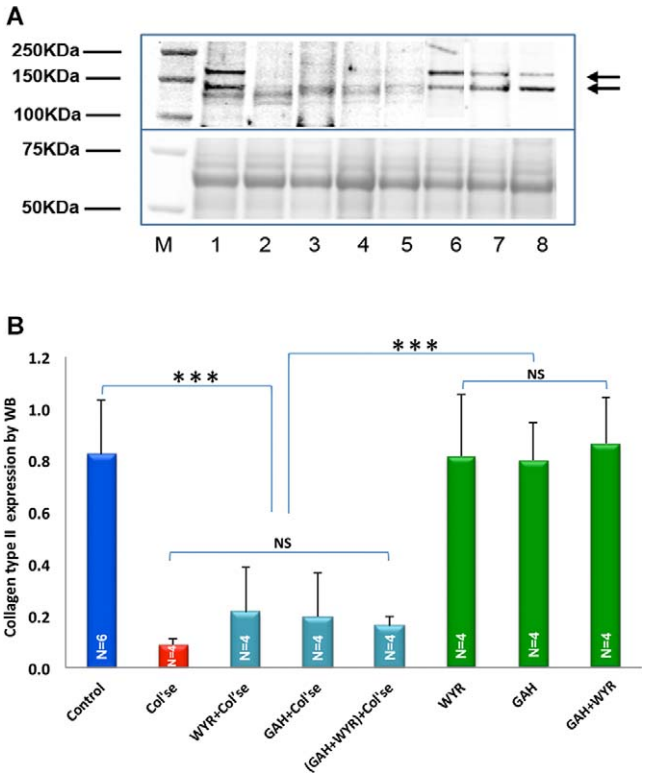

Figure 6. The effect of proteoglycan mimics on the degradation of soluble collagen type II (α1) in control and collagenase-treated bovine vitreous. (**A**) Immunoreactive collagen bands are present at 142 and 165 kDa (*arrows*) in the control and mimics only groups (lanes 1, 6–8). Collagenase treatment results in the loss of the upper band at 165 kDa and a marked decrease in the lower band (lane 2). The three mimics + collagenase groups showed loss of the upper band and varied amounts of the lower 142 KDa band (lanes 3–5). M: Marker; 1: Control; 2: 0.025 mg/mL collagenase; 3: WYR + collagenase; 4: GAH + collagenase; 5: GAHWYR + collagenase; 6: WYR; 7: GAH; 8: GAHWYR. (**B**) Normalized densitometric scanning of the collagen II (α1) bands ($n = 4$–6 for each group). There are no statistically significant differences between the control (*blue bar*) and the three mimics only groups (*green bars*, $P > 0.05$). Collagenase greatly degraded soluble collagen II (*red bar*, $P < 0.001$ compared with control). Adding proteoglycan mimics before collagenase (*light blue bars*) slightly increased soluble collagen content compared with collagenase alone, but these differences were not statistically significant. Col'se, 0.025 mg/mL collagenase; N = sample numbers; $^{***}P < 0.001$.

collagenase alone ($P > 0.05$ when compared with the collagenase only group).

Next, we calculated how these treatments impacted vitreous liquefaction (Fig. 5). The percentage of vitreous liquefaction showed no statistically significant difference between controls and each of the three mimics (11.6%–13.9%, $P > 0.05$). However, 0.025 mg/mL collagenase alone or collagenase combined with the mimics groups increased the extent of vitreous liquefaction to a similar degree (22.2%–28.9%; $P > 0.05$). These results indicated that the mimics did not prevent collagenase-induced vitreous liquefaction, not even in the WYR plus collagenase group.

Together these results suggested that the collagen binding proteoglycan mimic alone preserved vitreous stiffness, but none of the mimics protected against vitreous liquefaction in experimental conditions designed to optimize the enzymatic activity of collagenase. To provide further insight into this finding, we next tested for collagen protein degradation in each of the experimental conditions.

Proteoglycan Mimics Provide Marginal Protection Against Soluble Collagen II Degradation

Western blotting was used to determine the amount of degradation of soluble collagen II (α1) in the vitreous after treatment with collagenase or collagenase plus mimics (Fig. 6). The antibody detected double bands, one at the predicted molecular weight of 142 kDa and another at 165 kDa. Since both were detected by the antibody and were sensitive to purified collagenase, we considered both to be isoforms of collagen II (α1), as has been observed in previous studies.[35] In the WYR-, GAH-, or GAH+WYR-treated groups, the densitometric scanning of the collagen II (α1) bands showed no change compared with controls (Fig. 6A, lanes 6–8; Fig. 6B, green bars, $P > 0.05$), while treatment of vitreous with 0.025 mg/mL collagenase degraded most of the collagen II (α1; Fig. 6A, lane 2; Fig. 6B, red bar, $P < 0.001$). Addition of mimics approximately doubled the amount of soluble collagen compared to collagenase alone, but these changes were small relative to control conditions. This result indicated that the proteoglycan mimics could provide only slight protection against the enzymatic degradation of soluble collagens by collagenase.

Important to note in these experiments is that the majority of vitreous collagen is cross linked into fibrils and is not detectable using the Western blotting method described here. To assay changes in vitreous microstructure in collagenase- and mimics-treated conditions, we used quick-freeze DEEM, an imaging technique that provides submicroscopic resolution of the vitreous absent of fixation artifacts.

Vitreous Microstructure Density Increases After Mimic Application

Deep-etch electron microscopy images were obtained in control bovine vitreous and after treatment with collagenase, trypsin, or the collagen binding (WYR) mimic (Fig. 7). These images showed vitreous components in the form of fibers and globules with varying degrees of microstructural alignment. After trypsin treatment, the originally smoothly organized fibers were interrupted, fiber and globule density increased and decreased respectively, and fibers were substantially more aligned than in control vitreous. In collagenase-treated vitreous, fibers appeared thicker, signifying the potential collapse of the vitreous fiber network and fiber aggregation. In this case, fiber and globule density increased, as did overall fiber alignment. After treatment with the collagen II-binding proteoglycan mimic, WYR, the vitreous structure was more compact, with globules filling the space between the fibrils (Figs. 7D, D'). Despite increases in fiber and globule density relative to controls (similar to the collagenase-treated results), fibers were significantly more isotropic, with overall microstructural alignment similar to controls.

In summary, trypsin treatment significantly reduced globule density and the remaining fibrillar material was highly aligned. Collagenase showed similar trends, except there was no decrease in globule density and the aligned fibers appeared to be more collapsed and aggregated. Treatment with the mimic resulted in increased fiber and globule density and fiber alignment was unchanged relative to controls, a result opposite to that observed in the enzyme-treated experiments.

Discussion

In this study, we demonstrated that synthetic proteoglycan mimics designed to bind collagen and hyaluronan preserved the mechanical properties of vitreous gel treated with the enzymes trypsin and collagenase (Figs. 2, 4). Although the prior injection of the mimics could protect against vitreous liquefaction in trypsin-treated samples (Fig. 3), the mimics were unable to prevent collagenase-induced liquefaction (Fig. 5) and provided only marginal protection against the degradation of soluble collagen by collagenase (Fig. 6). Significant increases in the nonsoluble density of the vitreous microstructure following mimic treatment, however, supported a

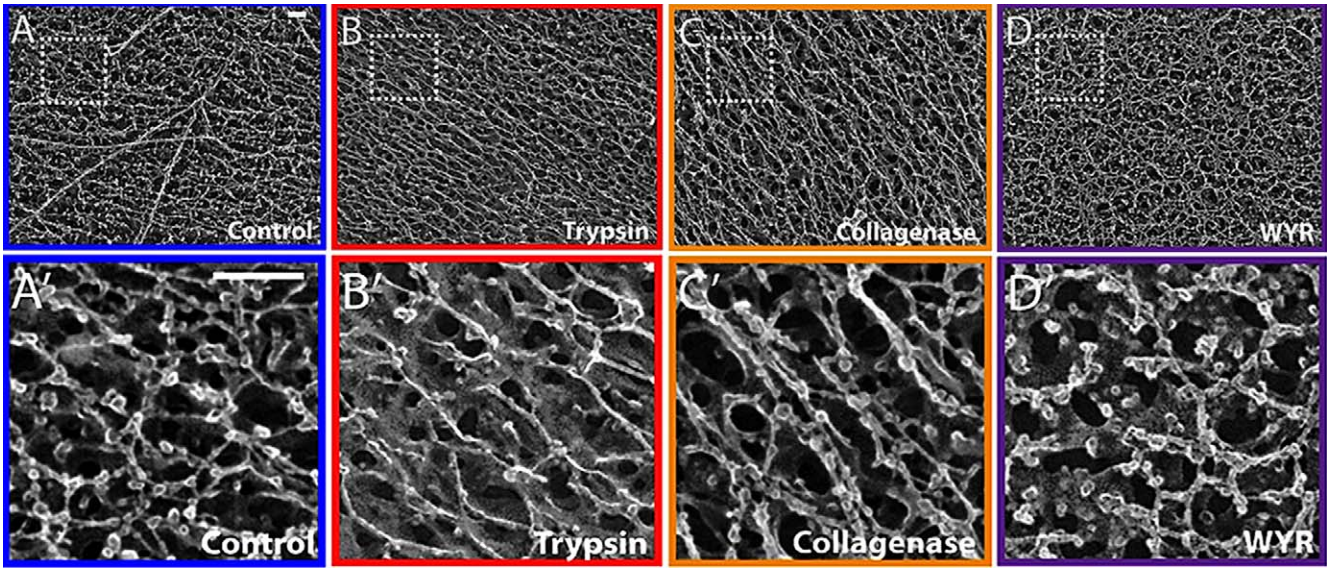

Figure 7. Representative DEEM images of untreated bovine vitreous and vitreous treated with collagenase, trypsin or the collagen II-binding proteoglycan mimic, WYR. *Dashed white boxes* in panels (**A–D**) indicate magnified regions shown in panels (**A'–D'**). (**A**, **A'**) Untreated vitreous: large, straight fibers are present on a background of smaller fibers. Small globules appear to be attached to the surface of the fibers and in the spaces between. (**B**, **B'**) Trypsin-treated vitreous: the large, straight fibers are not present. Smaller fibers are dense and aligned. Globule density is greatly reduced. (**C**, **C'**) Collagenase-treated vitreous: similar to (**B**, **B'**), the smaller, background fibers are aligned with no evidence of large fibers, but globules are present. Fibers are thicker suggesting some degree of collapse. (**D**, **D'**) WYR-treated vitreous: fibers appeared more compact and isotropic, with dense globules filling the space between (×25,000 magnification, *Scale bar*: 100 nm).

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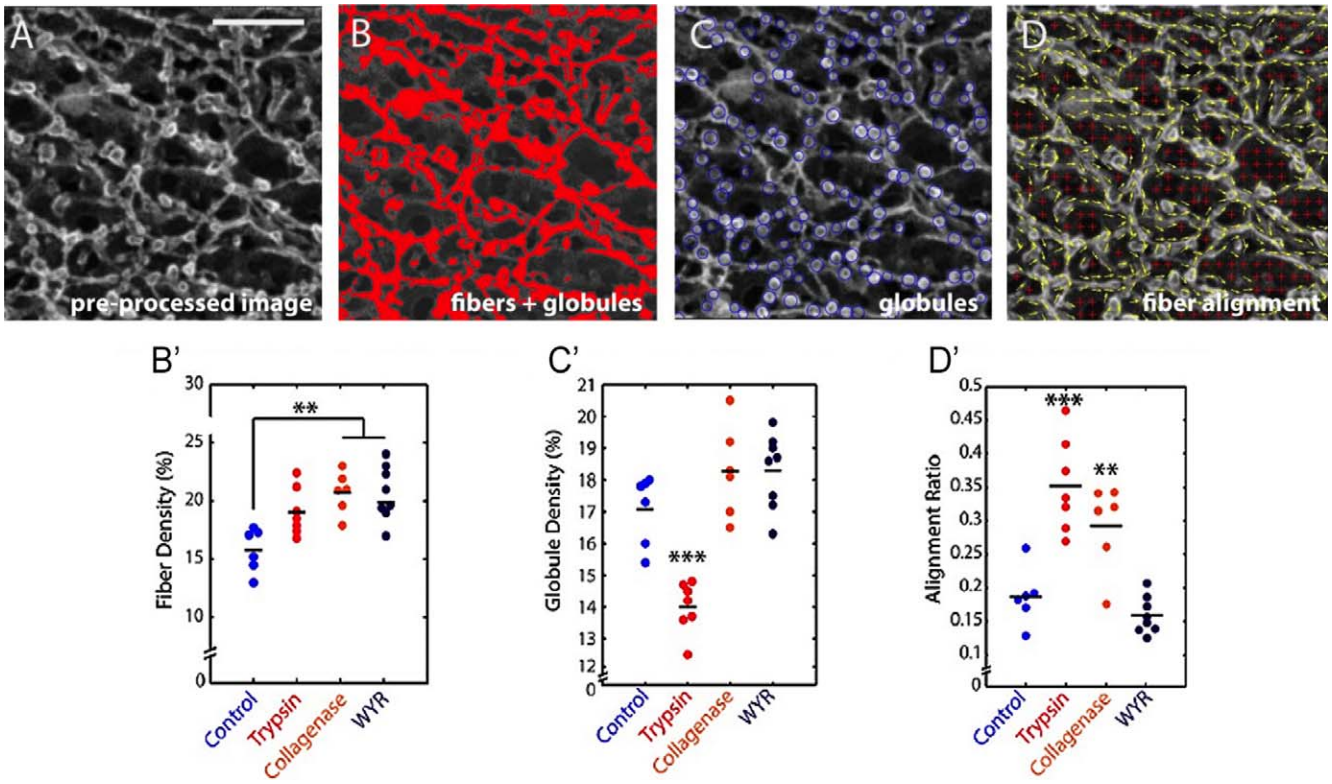

Figure 8. Deep-etch electron microscopy morphometrics. **(A)** Contrast-enhanced, smoothed image of bovine vitreous. **(B)** Fiber and globule density is estimated directly via thresholding lighter pixels (shown in *red*). **(C)** Globule density is measured using the Hough Transform and adaptive thresholding. *Blue circles* circumscribe detected globules (note that subtracting area measurements obtained in **(C)** from **(B)** gives fiber density alone). **(D)** Fiber alignment is calculated using the intensity gradient technique. *Yellow arrows* indicate direction of fiber alignment, while *red plus signs* indicate regions excluded from the analysis. **(B'–D')** Fiber density, globule density, and fiber alignment was quantified for control ($n = 6$), trypsin ($n = 7$), collagenase ($n = 6$), and WYR-treated vitreous ($n = 8$). **(B')** Fiber density is significantly increased in collagenase and WYR-treated samples relative to controls ($P < 0.01$). **(C')** Globule density is significantly reduced in trypsin-treated samples compared with all other groups ($P < 0.001$). **(D')** Fibers are significantly more aligned in trypsin ($P < 0.001$) and collagenase-treated vitreous ($P < 0.01$) relative to control and WYR-treated samples.

structural reinforcement mechanism for these molecules in the vitreous gel (Figs. 7, 8). From these data we postulate that the mimics function through binding to vitreous microstructure and stabilizing the complex fibrillar network while providing varying degrees of protection against liquefaction depending on the degradative target of the enzyme. It follows that the mimics could be valuable candidates in preserving vitreous microstructure in vivo through preserving vitreous viscoelasticity and limiting vitreous liquefaction and its harmful consequences.

The vitreous gel occupies most of the volume of the eye, serving as an important reservoir for many cytokines, growth factors and ascorbic acid, which consumes oxygen to protect the lens from cataract formation.[10,24,36] Unlike other extracellular matrices, the vitreous lacks resident fibroblasts or chondrocytes, meaning that, after birth, its structural components are relatively static compared with other tissues. Although some postnatal synthesis of ECM components likely occurs,[37–39] human vitreous begins to show evidence of degeneration as early as age 10.[2] The rate of degeneration can be accelerated by pathological conditions, such as high myopia, and genetic defects like Stickler, Marshall, or Wagner syndromes, which affect the structure of vitreous collagens (type II, type IX, type XI) and proteoglycans (versican), respectively. However, the great variation in the extent of vitreous liquefaction that occurs after age 50 has not been fully explained. Considering that vitreous degradation takes place continuously throughout life, slow but persistent enzymatic degradation could be involved in the development of age-related vitreous liquefaction.

Several proteases have been postulated to contribute to age-related vitreous liquefaction. It has been suggested that type IX collagen chondroitin sulfate chains, hyaluronan and opticin are involved in separating or connecting collagen fibrils. The age-related loss of the chondroitin sulfate side-chain of type IX collagen has been suggested to be one cause of age-related vitreous collapse.[15,16] Brown et al.[40] found that MMP-2 could directly degrade type IX collagen in its intact helical form in vitreous. Recently, Los et al.[17] detected trypsin-1 and trypsin-2 in human vitreous, supplementing their findings that MMPs alone could not explain all collagen degradation products in human vitreous. They found trypsin isoforms produced type II collagen degradation products of similar sizes to those present in the vitreous, which is in agreement with their observation of fragmented collagen fibrils in the vicinity of liquefied spaces. Our data show that chondroitin sulfate proteoglycans designed to bind to collagen and hyaluronan preserve vitreous stiffness and that the hyaluronan-binding mimic prevents trypsin-induced vitreous liquefaction (Figs. 2, 3). Hence, proteoglycan mimics may be suitable candidates to protect against endogenous trypsin-induced vitreous degeneration and liquefaction in humans.

In experiments in which vitreous was treated with GAH and trypsin for 1 hour in reversed order, we noted that both the

GAH+trypsin and trypsin+GAH groups preserved vitreous stiffness. However, only the GAH+trypsin group preserved vitreous liquefaction at the same level as controls (Fig. 3). The fact that the trypsin+GAH group preserved vitreous stiffness to the same degree as GAH+trypsin raised the possibility that the proteoglycan mimics could restore the physical properties of the vitreous after it was degraded. Although vitreous liquefaction was not restored, vitreous stiffness may be maintained by replacing the proteoglycans that were digested by the 1-hour treatment with trypsin.

It is known that trypsin is able to digest proteoglycans and many other proteins that, as our results showed, could interrupt the protein network and soften the vitreous gel. Apart from the previous view that the collagen triple helix is resistant to cleavage by most proteases except collagenases, there is a report that trypsin-2 could directly degrade the triple helix of type II collagen in cartilage and rheumatoid arthritis synovitis tissue.[41] We assume the actual degradation process is relatively slow and inefficient in eyes, due to the low trypsin concentration in vitreous and the presence of several serine protease inhibitors. However, it is likely that vitreous degradation is a continuous, multienzyme process, and that enzyme activities may vary in different individuals, possibly explaining the variability in the extent of vitreous degeneration with age.

The proteoglycan mimic that inhibited ECM degradation in models of OA is an aggrecan-like molecule. The mimics used in this study were designed to be insensitive to proteolytic cleavage, so they should be resistant to trypsin and other vitreous enzymes. These mimics were able to reduce fragmentation of key ECM components, inhibit the catabolic cascade seen during OA and improve the mechanical properties of the tissue. They also reduced inflammation.[42,43] Those properties also support proteoglycan mimics as attractive candidates for prevention of vitreous degradation.

In a previous study, we used enzymes (collagenase, hyaluronidase, and trypsin) and mechanical testing to show that type II collagen, hyaluronan and proteoglycans play complementary roles in maintaining the structural properties of the vitreous.[1] These findings supported earlier results from Nickerson et al.[31] who proposed a 'network tension hypothesis' where hyaluronan-induced swelling stiffens the vitreous by stretching the collagen fibrils. Bishop et al.[44] found that removal of hyaluronan (by digestion with Streptomyces hyaluronan lyase) only caused reduction in gel wet weight, and a small percentage of the total hyaluronan (6%) could maintain the vitreous in a fully distended state. Pirie et al.[45] found that total removal of collagen fibrils (e.g., by collagenase digestion) converted the vitreous from a gel into a viscous liquid. These results suggested that collagen plays a major role as the vitreous scaffold and hyaluronan works together with it in maintaining vitreous collagen fibril spacing and normal gel structure. In a previous experiment (data not shown), we found dose-dependent vitreous liquefaction in response to increasing concentrations of collagenase. As the concentration of collagenase increased, gel stiffness decreased. These data provided the basis for our current experiments, as the concentration of collagenase was chosen to partially liquefy the vitreous.

In these studies, we found that the collagen II–binding mimic, WYR, preserved the stiffness of collagenase-treated vitreous (Fig. 4) but did not protect against liquefaction (Fig. 5), and provided only slight protection against the degradation of soluble collagen (Fig. 6). Lack of protection against liquefaction but preservation of vitreous structure suggests that irreversible damage occurred to the vitreous, but also that this structural damage was compensated for via alternative mechanisms. In particular, increased microstructural density in DEEM images in WYR-treated vitreous compared with controls supports a biomechanical reinforcing role for the mimics (Figs. 7, 8). We hypothesize that despite not protecting against collagenase-induced liquefaction as well as the degradation of soluble collagen, the mimics functioned by binding to the complex network of insoluble vitreous, which preserved vitreous stiffness and increased microstructural density.

This result was in contrast to our trypsin studies where we found that the hyaluronan-binding proteoglycan mimic protected against vitreous liquefaction. We suspect that, as endogenous proteoglycans that separate collagen fibers are degraded by trypsin, excess mimics are able to replace them and function in a similar manner (e.g., preserve stiffness, prevent liquefaction) due to their overall similarity in composition. However, in the case of collagen fiber degradation, the mimics likely cannot recapitulate the original fiber architecture, which results in vitreous liquefaction.

Deep-etch electron microscopy imaging provided additional support for this interpretation. Heuser and colleagues[46] invented this technique and first used it to catch synaptic vesicles in the act of exocytosis at the postsynaptic membrane of the neuromuscular junction. We used this method because it is able to preserve the original morphology of delicate biological structures that would otherwise be destroyed by fixation. This method provided a three-dimensional view of the associations between components of the vitreous and the changes in fibril orientation, fibril density and protein globules following treatment with proteolytic enzymes.

Trypsin treatment significantly reduced the presence of circular globules in the DEEM images (Figs. 7B, B', 8C'), while globule density increased in collagenase- and mimic-treated vitreous. Fibers were strongly anisotropic and agglomerated in collagenase images suggesting that structural collapse contributed to the increase in globule density (Figs. 7C, C', 8D'). However, in mimic-treated vitreous, globule (and fiber) density increased independent of gross fiber alignment and structural remodeling (Figs. 7D, D', 8B'–D').

These results point to proteoglycans being identified as circular globules due to disappearance after trypsin treatments, with mimics manifesting as a combination of globule and fibrous domains. Attempts at identifying the fibrillar network more conclusively were unsuccessful, as hyaluronidase treatment only partially degraded this network (not shown). Hence, although quick-freeze DEEM is ideal for preventing fixation artifacts, the inability to definitively identify each of the microstructural components within the images is a limitation to the current technique. Studies aimed at comparing fixed and unfixed vitreous using DEEM, as well as using immunogold antibodies to definitively identify microstructural components in fixed samples are ongoing.

In summary, we evaluated three chondroitin sulfate proteoglycan mimics, demonstrating their potential ability to maintain the physical properties of enzymatically degraded bovine vitreous. By reducing the fragmentation of some of vitreous components and preserving them from further liquefaction, we expect that these compounds could retard further vitreous degradation and preserve the physical properties of aged vitreous. Future experiments will test the perdurance of mimics in preserving vitreous stiffness and preventing liquefaction using the in vitro testing method (free of bacterial contamination in extended cultures) developed in this study. Application to live animal studies will allow mimic toxicity, longevity, stability, and efficacy to be monitored in studies aimed at preventing vitreous degradation and liquefaction. Vitreous stabilization or restoration (as opposed to replacement) warrants further attention as a pharmaceutical target to delay or prevent age-related vitreous liquefaction and associated ocular diseases.

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