C h a p t e r 4

ENGINEERING THE VITREOUS HUMOR

4.1 Introduction

Having defined the mechanical properties of the vitreous and the functions of its two primary components in Chapter 3, we are now prepared to examine the nanoscale architecture of the network in search of points of attack for pharmacological vitreolysis. We will show that enzymatically cleaving the peptide bonds of collagen fibrils will destroy the network and liquefy the vitreous. However, this is not clinically relevant because of the toxicity of collagenase. Hyaluronidase has advanced to clinical trials for clearing blood clots in the vitreous.[1] Is it possible to induce liquefaction by cleaving the glycosidic linkages in HA? Better yet, is it possible to gently weaken or dissolve the vitreous by disrupting key noncovalent interactions within the network, such as hydrogen bonds? These questions are of significant fundamental and therapeutic interest – if answered, they will reveal the chemistry that binds the components of the vitreous together and thereby provide a road map for the rational design of pharmacological vitrectomy agents.

The ideal pharmacological vitrectomy agent would rapidly eliminate vitreoretinal adhesion and then liquefy the vitreous without harming the sensitive cells of the retina.[1] There are two possible chemical routes to these objectives: destroying components of the network (e.g., using collagenase) or causing the intact components to dissociate (e.g., using a denaturant). The wood-framed house metaphor is again useful in this context: a house can be leveled either by cutting the beams or by simply removing the nails. Most of the prior literature has focused on the former, while we will show that the latter is safer and also promising.

Proteases (most notably plasmin[2, 3], dispase[4], and collagenase[5]) have been proposed for vitreolysis, but none have proven to be clinically viable because many of the structural proteins of the vitreous (collagens, fibronectin, etc.) also support adjacent tissues, including the retina.[1] Proteases pose too great a danger because they diffuse to the edge of the vitreous during treatment and damage the retina irreversibly.[6] Hyaluronidase enzyme has also been proposed for pharmacological vitrectomy;[7] however, it has been proven unacceptable because it causes the vitreous network to shrink without inducing posterior vitreous detachment (PVD).[8] At points of vitreoretinal adhesion, shrinkage of the vitreous exerts traction on the retina. Traction at adhesion foci may induce macular holes, macular edema, vitreous hemorrhage, retinal tears, or even retinal detachment.[9] These dangers motivate the search for gentle pharmacological vitrectomy agents that denature network components rather than cleaving peptides. The possibility for retinal toxicity exists with gentle approaches as well, but *in vivo* rabbit and porcine studies and human clinical trials indicate that the effects of urea, for example, are minimal and transient.

Recent work by Oliveira, et al. has shown that posterior vitreous detachment can be induced in donor eyes by administration of RGD peptide.[10] Their supposition is that vitreoretinal adhesion is dominated by the noncovalent interactions of the RGD binding domain and that their short RGD fragment induces separation by competing for binding sites. Also, Vitreoretinal Technologies, Inc. has demonstrated that urea is a useful adjunct to surgical vitrectomy, improving surgical outcomes and reducing surgical time in a masked, double-blind human study.[11] Urea is a common biochemical reagent that does not cleave proteins; therefore, the beneficial effect is thought to come from its ability to disrupt hydrogen bonds. Both the RGD and the urea studies indicate that noncovalent interactions play a significant role in stabilizing the vitreous and vitreoretinal adhesion.

We present a systematic approach to examining the stability of the vitreous against an array of treatments designed to disrupt distinct types of molecular interactions: covalent bonds, hydrogen bonds, electrostatic effects, and hydrophobic attractions. We show that hydrogen bonds play a key role in stabilizing the vitreous and then proceed by examining the impact of injecting urea into an *in vitro* porcine eye model. Encouraging results from these *in vitro* studies and from clinical work by Vitreoretinal Technologies, Inc. prompted us to then administer small doses of urea to an *in vivo* porcine eye model to quantify its impact.

4.2 Materials and Methods

Chemical Stability of the Vitreous – Fresh porcine eyes (< 36 hours *post mortem*, stored at 5° C in physiological saline prior to arrival) were acquired through Sierra for Medical Science (Santa Fe Springs, CA). All pigs were 3 – 6 month old Chester Whites, weighing

50 – 100 kg and in good health at the time of slaughter. Eyes were gently dissected to remove the vitreous with minimal disruption. The intact vitreous specimens were then weighed (initial mass, typically M_o ~ 3 g) and place immediately in 10 ml of one of the treatment solutions listed below (Table 1) for 1 or 24 hours at 37° C. Treatments were chosen to probe the importance of specific types of intermolecular interactions (urea to disrupt hydrogen bonds, NaCl and $MgCl_2$ to screen electrostatic attractions/repulsion, etc.). After incubation, each vitreous specimen was removed from its treatment bath and weighed a second time (M_f) (Figure 1).

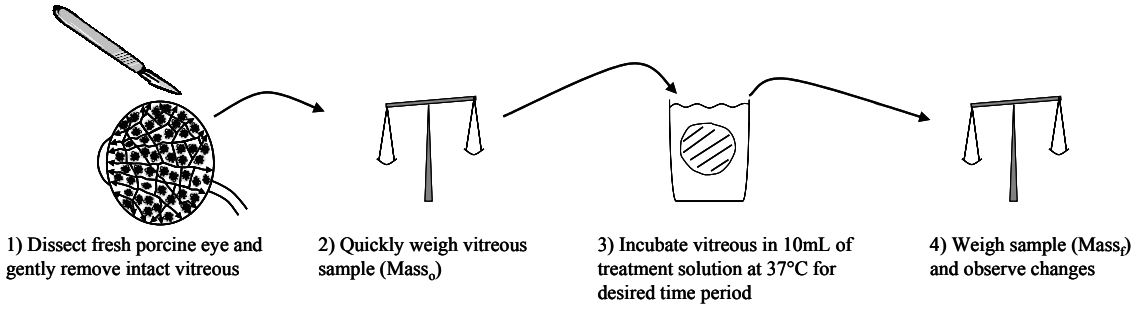

Figure 1. For chemical stability experiments each vitreous was: 1) gently removed from a fresh porcine eye (< 36 hours *post mortem*, stored at 5° C in physiological saline prior to dissection), 2) weighed (M_o), 3) placed in 10 ml of the appropriate treatment solution at 37° C for 1 to 24 hours, and 4) weighed again (M_f).

Treatment	Concentrations Examined [M] or pH	Targeted Intermolecular Interaction	Typical Lab Concentrations [M] or pH	Solubility Limit [M]
Enzymatic Cleavage	Hyaluronidase, Collagenase	Covalent bonds	N/A	N/A
Urea	0.5, 1, 2, 4, 5, 6, 8	Hydrogen bonding	4 – 8 for denaturation	8
Guanidinium HCl	6	Hydrogen bonding	4 – 6 for denaturation	6
Mid – high pH 10 mM phosphate buffer	(pH) = 7.4, 9.8, 12.0	Electrostatics and H-bond scheme (deprotonation)	7.45	N/A
Mid – low pH 10 mM citrate buffer	(pH) = 2.0, 2.6, 4.5, 6.0, 7.5, 8.1	Electrostatics and H-bond scheme (protonation)	7.45	N/A
NaCl	0, 0.05, 0.075, 0.1, 0.15, 0.25, 0.5, 1, 2, 5	Electrostatics (monovalent cation)	0.150 (physiologic)	5
$MgCl_2$	0.1, 0.02, 0.3, 0.4, 0.5, 0.6, 1	Electrostatics (divalent cation)	~ 0.001 (physiologic)	1
Triton® X–100	0.01, 0.016, 0.05, 0.1, 0.3	Hydrophobic Interactions	0.016 (1%)	None

Table 1. List of bath solutions in which vitreous specimens were placed for chemical stability tests. Each treatment was designed to probe the importance of a specific interaction: covalent, hydrogen bond, electrostatic, or hydrophobic.

Quantitative Analysis of the Mechanical Impact of Urea and pH – Urea and extreme pH changes were the only treatments capable of causing complete vitreous collapse; therefore their effects were studied further. Vitreous specimens were removed from fresh porcine eyes by gentle dissection and given 100 μl injections of one of the following urea treatment solutions: variable concentration (0, 3, 6, or 24% in 0.9% saline; pH = 6) or variable pH (2, 6, 8, or 12; concentration = 24%). Following injection, each specimen was placed in 10 ml of 0.9% saline for 24 hours at 37° C. After the incubation period, a central section of each vitreous was removed and its modulus was measured immediately on an ARES-RFS rheometer from TA Instruments, Inc. (New Castle, DE) using the same "cleat geometry" method (ω = 10 rad / sec, γ = 3%, T = 22° C) described in Chapter 2. After reaching a stable plateau modulus, a few treated and control specimens were subject to the "failure-stress" tests described in Chapter 3.

In Vivo Exploration of Vitreous Mechanics – Live animals used for this study were housed, treated, and examined by our collaborators in Professor Hugo Quiroz-Mercado's lab at the Hospital "Dr. Luis Sánchez Bulnes" de la Asociación Para Evitar la Ceguera (APEC) in Mexico City, in accordance with Association for Research in Vision and Ophthalmology (ARVO) standards for the ethical treatment of animals.[12] Landras mini pigs age 2-3 months were anesthetized, then 100 μl of 0.9% saline containing 0, 12, or 24% urea were injected into the vitreous of 11 of the 13 pigs. In each case, the left eye was injected with either 12 or 24% urea and the fellow (right) eye was injected with pure saline as a control. In the remaining 2 pigs, no injections were made in order to document differences between saline-treated "control" eyes and untreated eyes. Each solution was injected either 1 or 14

days prior to the animals being tested as outlined in Appendix B. On the assigned test day the animals were again anesthetized, and then the intraocular pressure (IOP) of each eye was measured. After IOP measurements, the animals were sacrificed by lethal injection with sodium pentobarbital and the eyes were examined for general health, retinal inflammation, and vitreous changes indicative of liquefaction, using an ophthalmoscope. After examinations were completed the eyes were enucleated, placed individually in physiological saline, and transported (~ 1 hour on wet ice) to Professor Alberto Tecante's lab at the National Autonomous University of Mexico (UNAM) for physical analysis. Within minutes after dissection, each vitreous was carefully weighed and loaded on a 15 mm diameter cleat tool on an ARES-RFS rheometer that was identical to the instrument used for *in vitro* analyses. The dynamic modulus (ω = 10 rad/s, γ = 3%, T = 22° C) was monitored until it reached steady state (~ 10 minutes). Thus, approximately 15 minutes elapsed between successive dissections and analyses. Results did not show any trend with elapsed time *post mortem.*

4.3 The Chemical Stability of the Vitreous

From a therapeutic perspective, though it is valuable to know the chemistry of the intermolecular interactions that are most significant for the mechanical stability of the vitreous network and to know the identity of the species involved, knowledge of either is sufficient for designing therapeutics to disrupt connectivity. For example, if a specific protein is identified as a critical crosslink, then a therapeutic that targets the binding sites of that peptide would be appropriate. Alternatively, if a particular type of bonding (e.g.,

hydrogen bonding) is critical for network stability, then a therapeutic designed to disrupt that interaction is appropriate, regardless of the identity of the particular proteins involved.

There are dozens of components of the vitreous, and the functions of many remain unknown (Chapter 1). A number of these components have been proposed as potential network stabilizers or crosslinks (Chapter 1) but, to our knowledge, there is no literature regarding how such a crosslink might be joined to collagen or HA. Incomplete information on the many potentially-significant species makes a "bottom-up" approach prohibitive, because it requires isolating and fully identifying all of the network components first, and then determining their functions within the network. For identifying a pharmacological vitrectomy agent, the bottom-up approach is inefficient because many of the components may not play significant structural roles.

We present a "top-down" approach to vitrectomy agent discovery organized by the main types of interactions that stabilize the nanostructure of biological tissues. We first determine the relative importance of specific covalent bonds (within collagen and HA), hydrogen bonds, electrostatic interactions, and hydrophobic interactions. These experiments yield direct suggestions for pharmacological vitrectomy agents by revealing the nature of the chemistry that must be disrupted. The most promising therapeutic approach identified in these experiments is explored further *in vitro* and *in vivo* in the following sections.

Covalent Bonding – Hyaluronidase cleaves the native high molecular-weight HA chains into smaller fragments that diffuse out of the vitreous more readily, causing up to a 60% reduction in vitreous mass (~ 10% reduction occurs without treatment) (Figure 2). This level of mass reduction is not seen after one hour because it takes several hours for the enzyme to cleave a significant portion of the chains into small pieces. The hyaluronidase effect is saturated with 100 units of enzyme: 1,000 units provided no additional mass loss. Thus, no quantity of hyaluronidase will cause global network collapse. As previously mentioned, hyaluronidase does not weaken vitreoretinal adhesion; therefore, HA-induced contraction could endanger the retina.

Collagenase enzyme treatment had a much more dramatic effect (data not shown): 1000 units of collagenase enzyme fully liquefied the vitreous, leaving no detectable residual solid in the bath solution. Collagenase was the only treatment to completely liquefy the vitreous. This result was anticipated based upon the structure of the vitreous and the literature cited above. As stated previously, however, enzymatic digestion of collagen is not a satisfactory pharmacological vitrectomy approach because it attacks all collagen-rich tissues and membranes.

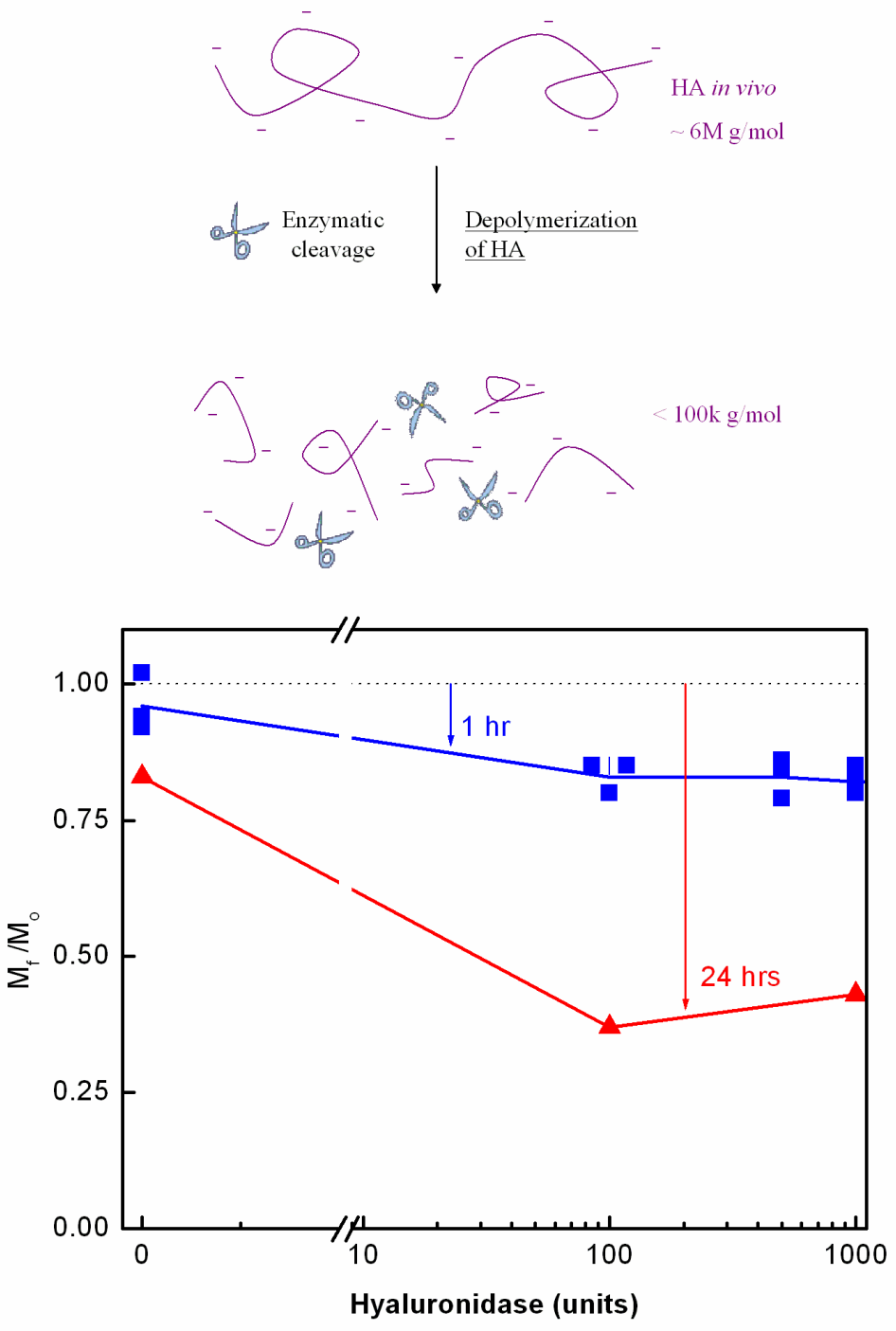

Figure 2. Hyaluronidase enzyme reduces the molecular weight of hyaluronic acid by random chain scission, which causes the vitreous to shrink but not collapse

Hydrogen Bonding (Urea and Guanidinium HCl) – Moderate concentrations of urea (1 – 4 M) cause the vitreous to lose ~ 50% of its mass after 24 hour but a sharp transition occurs at concentrations ≥ 5 M urea. At or above 5 M concentrations the vitreous totally and irreversibly collapses to a small opaque pellet of protein (> 98% of mass is lost). 6 M Guanidinium HCl yielded the same result as high concentrations of urea (data not shown).

We propose that in the vitreous the most important aspect of these well-known chaotropic agents is their ability to disrupt intermolecular hydrogen bonds. Specifically, intermolecular hydrogen bonds between network components and intrafibrillar hydrogen bonds within collagen type II. Hirano, et al. have also shown that urea is capable of disrupting hydrogen bonds that form between HA molecules in solution,[13] although the question of whether or not hydrogen bonds link HA molecules *in vivo* is still a topic of active debate.[14] Our emphasis on hydrogen bonds is generally consistent with the accepted view of chaotropic agents;[15] however, the precise mechanism by which they denature proteins remains unclear[15, 16] and appears to also involve hydrophobic effects.[17, 18] We have examined the effects of hydrophobic interactions separately using surfactant solutions (see *Hydrophobic Interactions* below). Contrast between the effects of the chaotropics and the effects of the surfactant allow us to distinguish between hydrophobic attractions and hydrogen bonding. Disrupting hydrophobic interactions may contribute to the effects of urea; however, we will demonstrate that they do not play a dominant role.

Regarding the kinetics of the effect: one hour is not sufficient time for urea or guanidinium to totally collapse the vitreous even at high concentrations, although significant size reduction was obvious after just a few hours. Given that urea and guanidinium are both small molecules capable of rapid diffusion and that H-bond disruption also proceeds rapidly (within minutes), the rate-limiting step in vitreous collapse appears to be the diffusion of large molecules out of the collagen network. This is consistent with the concept that H-bond disruption frees bound structural components but does not destroy them.

The proteinacious pellet that remains after vitreous collapse is composed primarily of collagen fibrils: they are stable against the chemical treatments listed in Table 1 hyaluronidase digestion but easily dissolved with collagenase. Collagen fibrils swell in high concentrations of urea due to the fact that the fibrils assemble initially by H-bonding.[19] However, the fibrils are reinforced with covalent crosslinks, and it is these crosslinks that maintain fibril integrity in high concentrations of urea.

An interesting qualitative observation is that the pellet retains the shape of the vitreous, but in miniature form. Analogous to a tiny balloon that has been deflated the vitreous pellet can, like a balloon, easily be stretched with forceps. Unlike a balloon, however, once the vitreous collapses it cannot be inflated again. Even when placed in saline or pure water over night, the pellet does not expand. Collapse appears to be total ejection of hydrophilic species; therefore, when the tissue is immersed in aqueous solutions, there is no longer a driving force for water to enter. To our knowledge, this is the first direct evidence that H-bonding is crucial to the architecture of the vitreous (Figure 3).

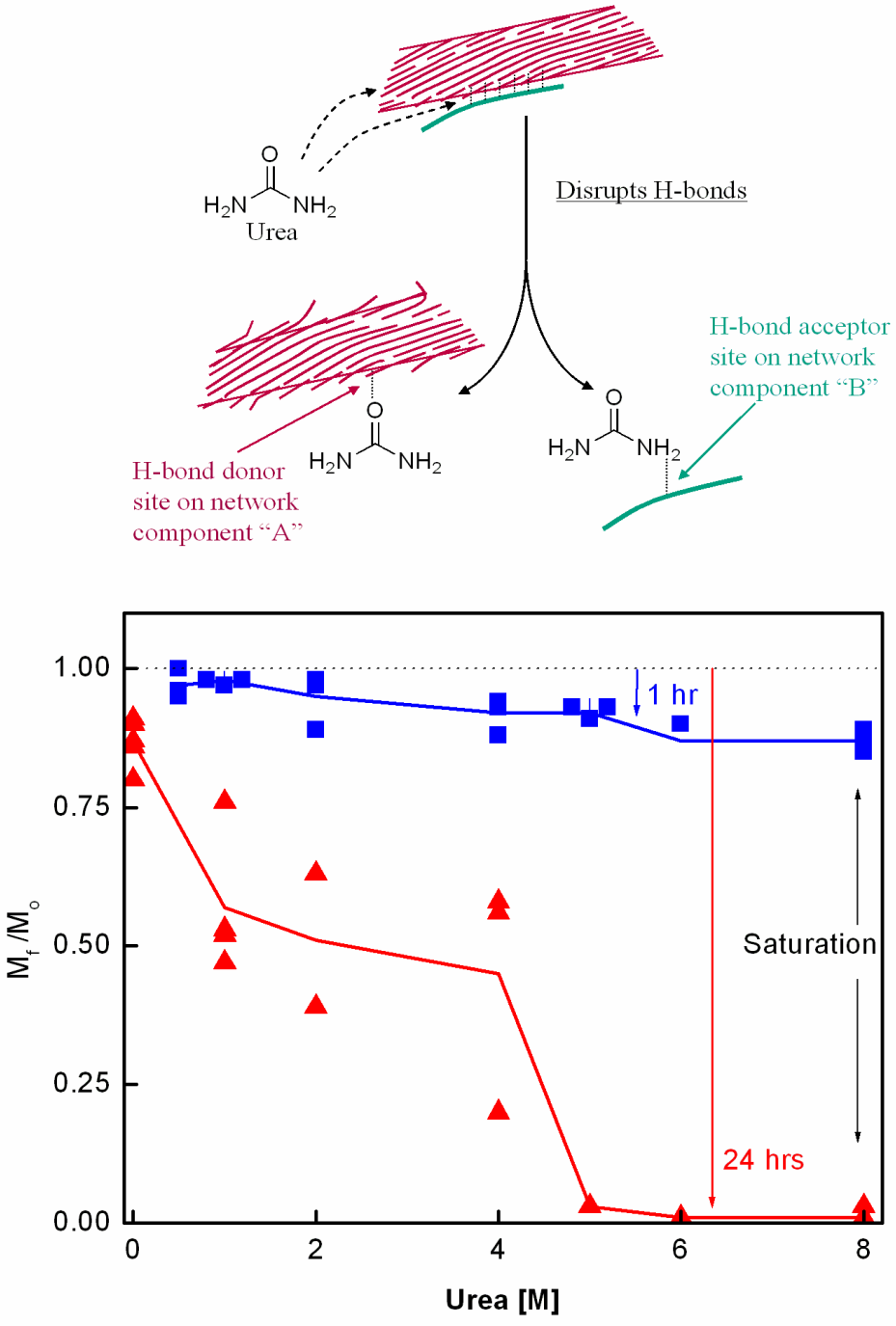

Figure 3. Incubation in high concentrations of urea causes totall vitreous collapse at long times but have little effect after one hour. There is also an intermediate concentration range [1 - 4 M] which causes a 50% collapse, while concentrations below 1 M have little effect even after 24 hours.

Electrostatics and H-bonding (effects of pH) – 10 mM citrate buffer rapidly causes catastrophic collapse of the vitreous below pH 4, such that after 1 hour 60% of the mass has been lost and after 24 hours only a small, opaque pellet of protein is left (> 97% mass loss). At neutral pH the structure of the vitreous is stable in 10 mM citrate and 10 mM phosphate buffers, but at very high pH (phosphate buffer) the structure of the vitreous is again destabilized.

It is unlikely that covalent bond scission is responsible for the monotonic, nearly-linear mass loss trend observed under moderately acidic conditions (pH 6.0 and 4.5). Harshly acidic solutions are capable of hydrolyzing glycosidic and peptide bonds;[20, 21] however proteins are normally heated in 6 N HCl to achieve peptide bond cleavage.[15] Hyaluronic acid has recently been shown to degrade in acidic buffer solutions,[14] but our hyaluronidase experiments show that glycosidic bond cleavage cannot cause total vitreous collapse. We attribute the impact of mildly acidic buffers to the protonation of the carboxylic acid moieties of aspartic acid, glutamic acid, and hyaluronic acid.

Protonating the carboxylic acid moieties listed above will impact the network by changing the charge profile of HA (pKa = 3.2) and some peptide residues and altering the hydrogen bonding scheme of the newly protonated species.[22] As pH falls, HA approaches its isoelectric point of 2.5 at which the number of negatively charged acid groups matches the number of positively charged amines. Very near the isoelectric point, HA has an attractive intramolecular potential corresponding well with the sharp drop in mass seen for the 1 hour incubations.[14, 22] Our observation of total vitreous collapse in this pH range is consistent with a view of HA contracting (Figure 4, schematic).

In addition to the drastic changes in HA, changes in pH may also induce significant changes in protein-protein binding. Specific interactions between collagen fibrils and other proteins and proteoglycans presumably rely on a combination of hydrogen bonding, hydrophobic effects, and electrostatics. As the amino acids that mediate these interactions become protonated, binding sites may lose functionality. One likely example is the carboxylic acid moiety of Asp and Glu: when these residues are protonated, the hydroxyl oxygen becomes a potential H-bond donor, loses its capacity for electrostatic interactions, and the amino acid becomes less hydrophilic.

Unlike acidic solutions, alkaline solutions have little effect on the vitreous until the pH is so high that peptide bond cleavage by deamidation[21] may be significant (Figure 4). Although HA does not undergo rapid chain scission at pH 12.5, solutions of HA show chain contraction and a drop in viscosity at such high pH.[14, 22] Therefore, a combination of protein degradation and HA conformational change may contribute to vitreous collapse under strongly alkaline conditions.

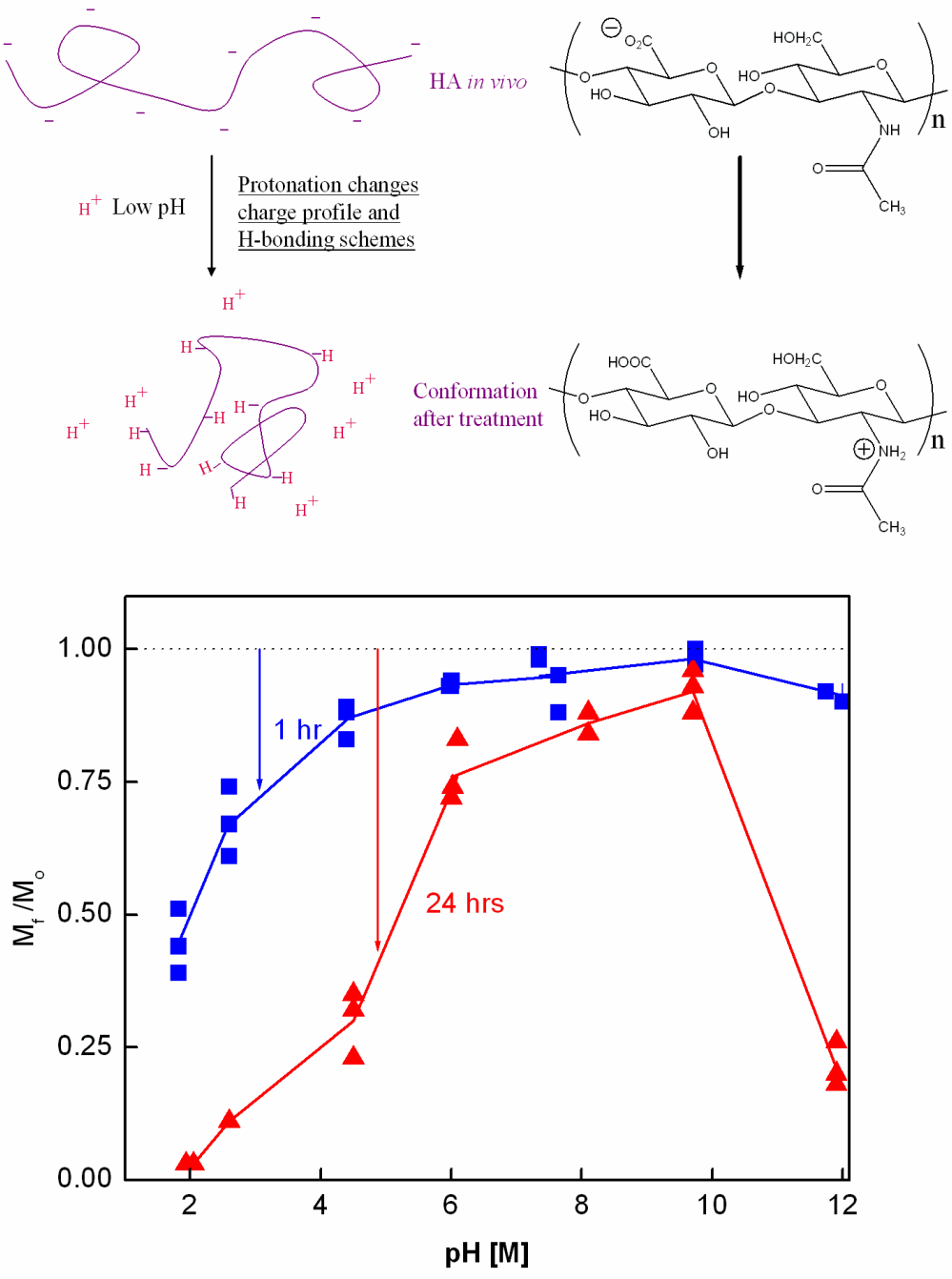

Figure 4. The stability of the vitreous is very sensitive to pH. The vitreous has maximum stability at physiological pH, as expected, but degenerates quickly below pH 6 and above 10.

Electrostatic Interactions Isolated (*NaCl and $MgCl_2$)* – Increasing salt concentration screens electrostatic interactions and shifts the Donnan equilibrium of polyelectrolytes toward higher polymer concentration. Addition of a monovalent salt, here sodium chloride, produces a moderate, dose-dependent mass loss for 24 hour incubations, but essentially no effect after 1 hour (Figure 5). The effect of added salt is relatively mild: even at the solubility limit of NaCl (~ 5 M at pH ~ 7), the vitreous retains half its weight after 24 hours. Protein-protein interactions may not be significantly disrupted, since the polyelectrolyte effect of HA can explain the observed mass loss.

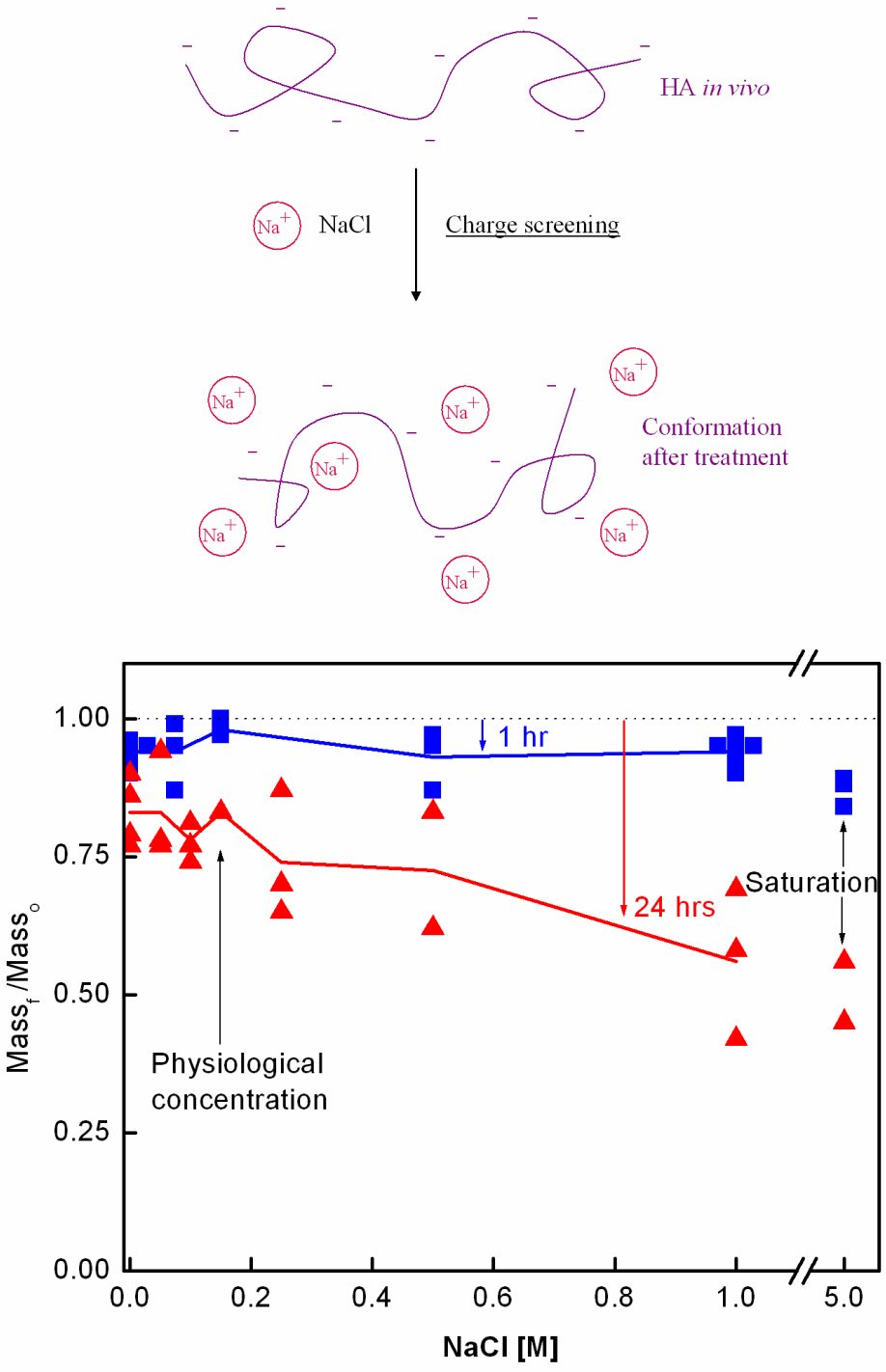

Figure 5. Vitreous soaked in NaCl loses mass in a dose- and time-dependent fashion. Salts screen bound charge, decreasing electrostatic repulsion and attractions.

It is well known that divalent cations cause polyanions to contract. In the case of the vitreous, adding $MgCl_2$ does produce a greater mass loss than NaCl, consistent with the hypothesis that HA contraction plays a major role (Figure 6).

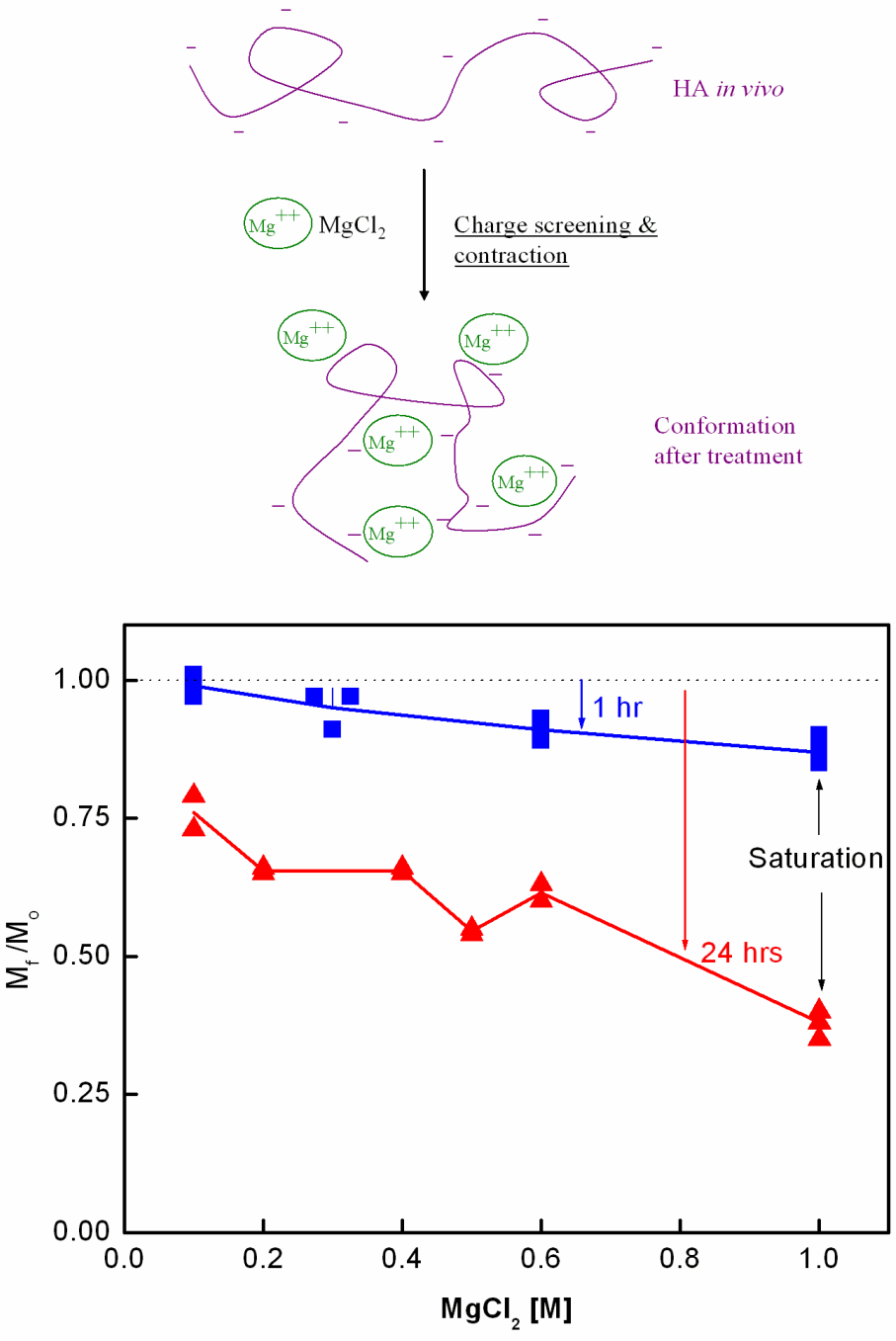

Figure 6. Vitreous soaked in $MgCl_2$ loses mass in a dose- and time-dependent fashion. The mass loss is significantly more than for NaCl solutions of much higher concentrations, apparently an effect of higher valency of the cation on HA.

Hydrophobic Interactions (*Triton® X-100)* – Triton® X-100 induces moderate weight loss at very high concentrations, but the effect is the smallest of all the treatments measured. Triton® X-100 is a non-ionic surfactant typically used to solubilize membrane proteins at 1-5 mM concentrations. We see that, while very large concentrations of Triton® tend to destabilize the network, standard concentrations have only a moderate effect even after 24 hours (Figure 7). Hydrophobic interactions apparently do little to stabilize the structure of the vitreous.

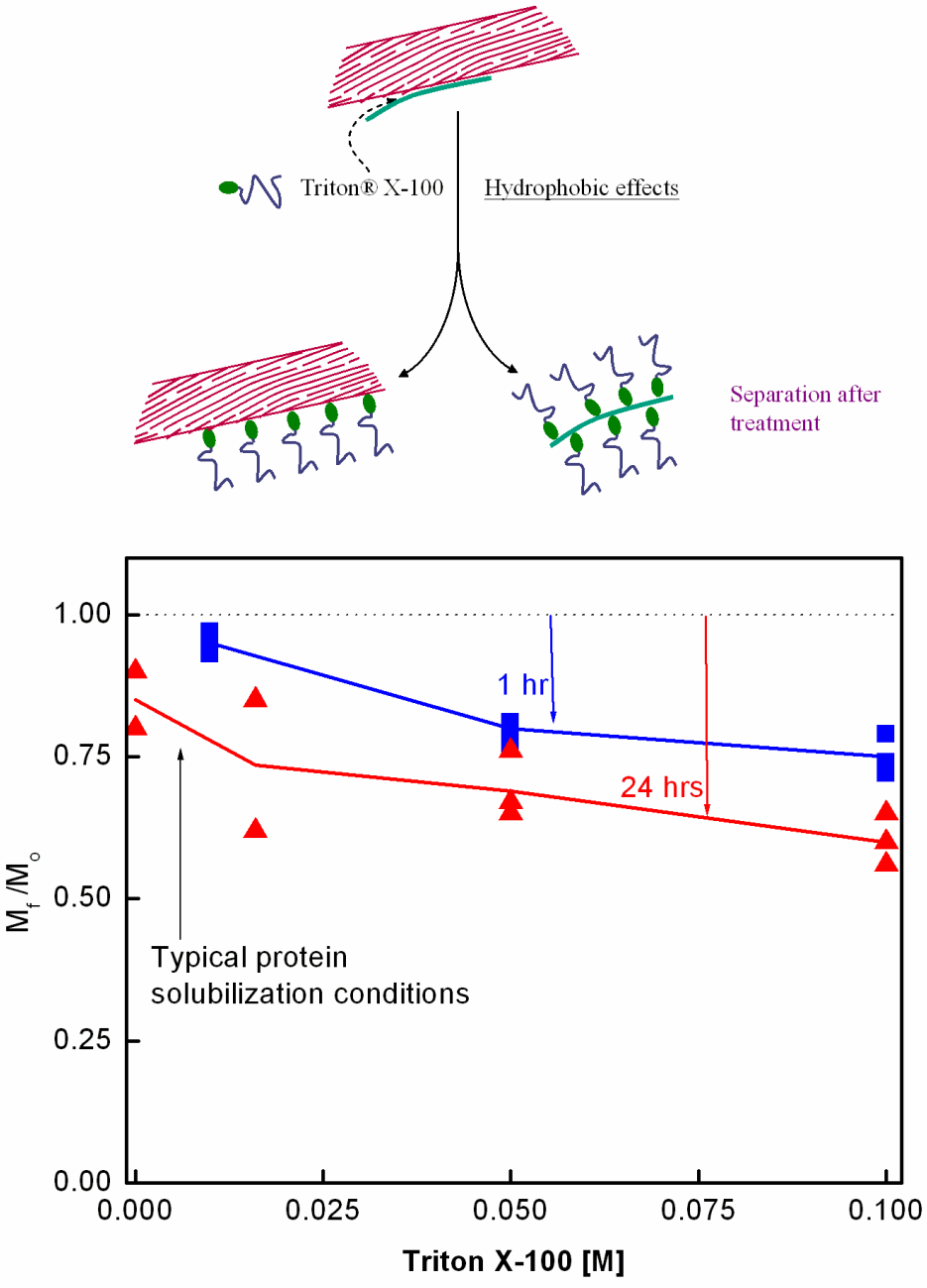

Figure 7. Concentrations of Triton® X-100 normally used in laboratory settings had almost no effect on the stability of the vitreous. Moderate, dose-dependence mass loss is apparent at higher concentrations.

4.4 Targeted Vitreous Engineering and Rheology

Urea and low pH were the only nonenzymatic bath treatments to fully collapse the vitreous; therefore, we proceeded by exploring the therapeutic potential of low-pH urea solutions in an *in vitro* porcine eye model. As mentioned previously, Vitreoretinal Technologies, Inc. has observed a clinical benefit to intravitreal urea injections prior to vitrectomy in the form of reduced surgical time and improved retinal reattachment.[11, 23, 24] In safety studies conducted prior to clinical trials, they observed that injecting 100 μl of up to 12% urea into the vitreous of rabbit eyes showed no adverse reactions and that urea concentrations up to 24% elicited only mild, transient retinal inflammation. In collaboration with Vitreoretinal Technologies, we have found that injections of 24% urea elicited no adverse response in porcine eyes. Using these findings as guidelines for therapeutic doses, we have examined experimental injections to a maximum of 100 μl of 24% urea, although it is likely that higher concentrations would be safe for clinical use. Adult human eyes have an internal volume of ~ 5 ml;[25] therefore, a 100 μl bolus of urea is rapidly diluted ~ 1/50. The volume of a human vitreous is approximately x1.5 larger than porcine and x3 larger than rabbit vitreous. Thus, the effective retinal exposure in an adult human (~ 0.5% for a 24% injection) is significantly lower than that of a rabbit (~ 1.5% for the same injection).

When we injected porcine vitreous specimens with 100 μl of 6% urea or less (pH 5 - 6), and incubated in a 10ml saline bath at 37˚ C for 24 hours, no softening was observed (Figure 8). However, increasing the concentration to 24% caused a discernible drop in modulus and narrowing of the relative standard deviation. The decrease in deviation is not symmetric: the relatively-consistent minimum modulus remains unchanged, while the

maximum moduli are significantly reduced. The change in *average* modulus is ~ 35% (from 9.3 to 6.1 Pa); in contrast, the relative modulus above the base line (G'_{bl}) decreases ~ 60%, from G'_{avg} - G'_{bl} = 3.8 to 1.6 Pa, based on G'_{bl} = 4.5 Pa computed from minimum modulus values in Figures 8 and 9.

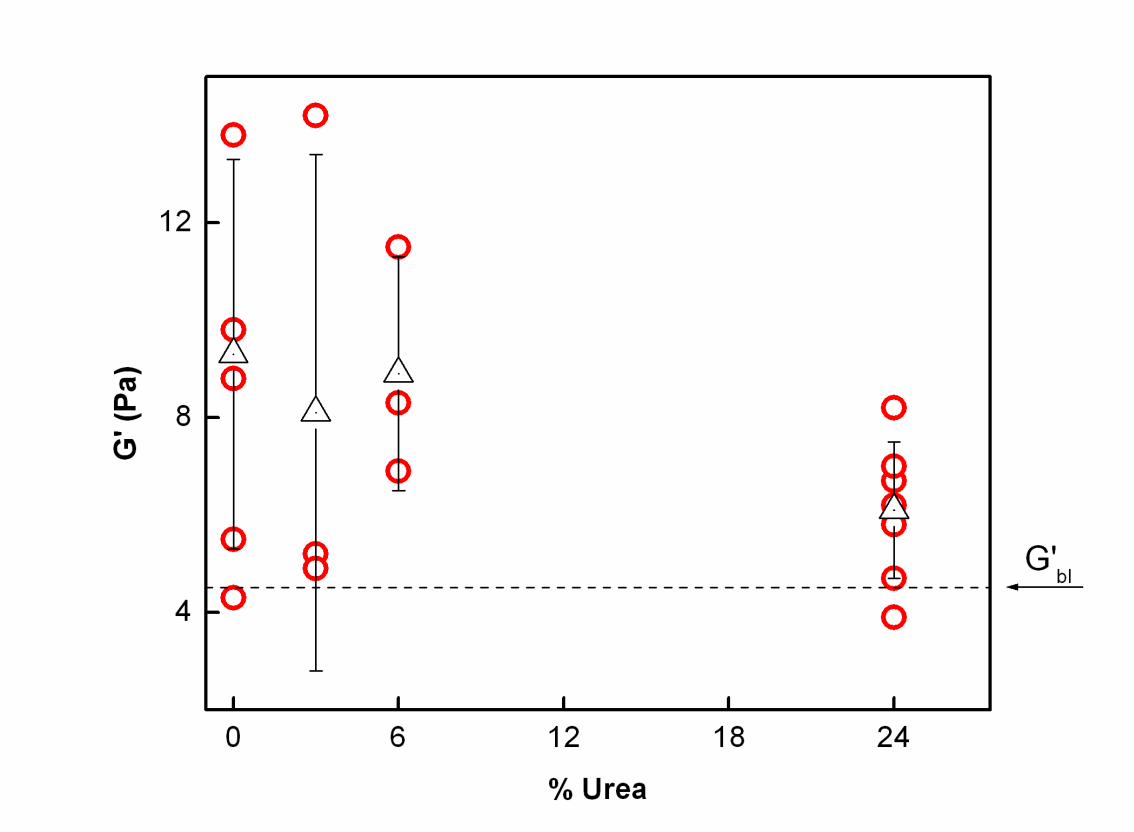

Figure 8. Fresh porcine vitreous specimens were injected with 100 µl of a urea solution (0, 3, 6, or 24% urea in 0.9% saline) and placed in 10 ml of saline to incubate at 37° C. Each circle represents the initial storage modulus of a porcine vitreous immediately after it was removed from the incubator. G'_{bl} (dashed line) indicates the apparent minimum or baseline modulus of porcine vitreous.

The high relative standard deviation (~ 50% for saline-treated controls) appears to be due to sample-to-sample variability. Porcine eyes are not delivered as matched pairs and sample-

sample difference are some times obvious even under visual inspection. Due to the delicate nature of the network, the age of the animal (3 – 6 months), previous head trauma, dietary factors, and even genetic differences may alter its structure. These effects would be compounded by any additional variability in handling (enucleation, transportation, and dissection).

We speculate that G'_{bl} represents the properties of a vitreous network in which only covalent network junctions are intact, i.e., the underlying, crosslinked collagen network. Higher modulus values may reflect added structure and connectivity due to physical associations among proteins that may increase the rigidity of the collagen network or noncovalent interactions that link the network to HA. In that case, the variability in modulus would reflect the differing degrees of residual noncollagenous structure in each particular sample. This interpretation of sample variability is consistent with rheological measurements of human vitreous that showed great consistency between matched pairs but drastic differences between individuals (see Chapter 3).

Decreasing the pH of the urea solution below neutral also has a marked softening effect (Figure 9). In these experiments the treatment solution pH was varied (2, 6, 8, and 12) at a fixed concentration of 24%. Due to the dilution of the injected solution and the buffering capacity of the vitreous, these changes in the pH of the injected solution only mildly alter the pH of the vitreous (Figure 9, top axis). Vitreous has a powerful buffering effect due to the buffering capacity of the proteins and the carboxylic acid moieties of HA. Thus, it is surprising to find even a moderate pH dependence. Nevertheless, high pH urea solutions are less effective than neutral or acidic ones in reducing the mean modulus and sample-to-

sample variability. One possible explanation is the influence of acids on urea. Urea has been show to sequester protons from acid solutions by forming a cation.[26, 27] It has been argued that the added proton prevents urea degradation and dimerization in aqueous solutions, which may influence the treatment.

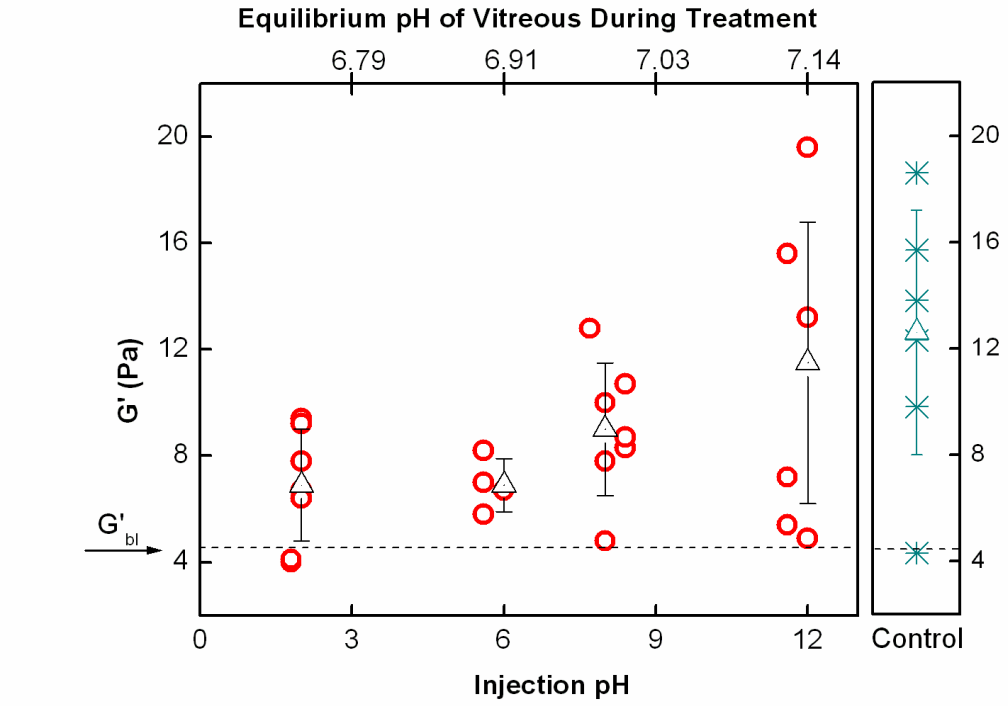

Figure 9. Fresh porcine vitreous specimens were injected with 100 µl of 24% urea solution (pH 2, 6, 8, or 12) and placed in 10 ml of saline to incubate at 37° C. Open circles represent the initial storage modulus of individual porcine vitreous immediately after it is removed from the incubator. Stars represent the modulus of controls injected with 100 µl of isotonic saline at neutral pH. G'_{bl} (dashed line) indicates the apparent minimum or baseline modulus of porcine vitreous. Top axis indicates the actual pH of the vitreous measured after incubation. Note that the pH of the tissue (top axis here) varies over a narrow range relative to the soak tests described in Section 4.2.

While the pH and concentration effects are significant, the modulus reduction in both cases was less dramatic than expected based upon the documented clinical benefits.[23, 24] There are several possible explanations for this discrepancy. First, it is possible that the primary clinical benefit of urea is the induction of PVD rather than a drastic effect on bulk mechanics (this will be discussed further in Section 4.5). A second possibility is that urea destabilizes the vitreous indirectly by increasing the susceptibility of network components to enzymatic degradation. In most vitrectomy cases, the barrier protecting the vitreous from blood and proteases has been compromised. Urea is known to affect the structure of collagen fibrils and permanently alter the ultrastructure of collagenous tissues.[28-30] The structural changes may occur equally *in vivo* and *in vitro*, however *in vivo* proteolytic degradation of the weakened network may have a significant influence over time. It is also possible that the moderate softening effects observed in the lab do reflect the *in vivo* changes in treated vitreous that lead to clinical benefits.

To explore this final possibility, failure stress and strain values from urea-treated vitreous were compared with saline controls and with values from fresh tissues presented in Chapter 3 (Figure 10). As stated in Chapter 3, high stresses are relevant because they develop during vitrectomy as the vitreous is aspirated through the vitrectomy tool tip (an aperture the size of a 22-gauge needle, I.D. ~ 0.4 mm). The failure of vitreous under high stress was measured by shearing the vitreous between cleated plates for 150 seconds at a rate of $\dot{\gamma}$ = 0.1 s^{-1}. Urea treatment did not affect the average failure stress or strain (~ 25 Pa and 450%, respectively). This is consistent with our inference that urea removes network reinforcements but does not have a strong effect on the underlying network; bulk failure

involves straining the network to such an extent that the majority of the bonds are broken. High-strain tests push past the loss of noncovalent interactions and on to a regime dominated by covalent bonds.

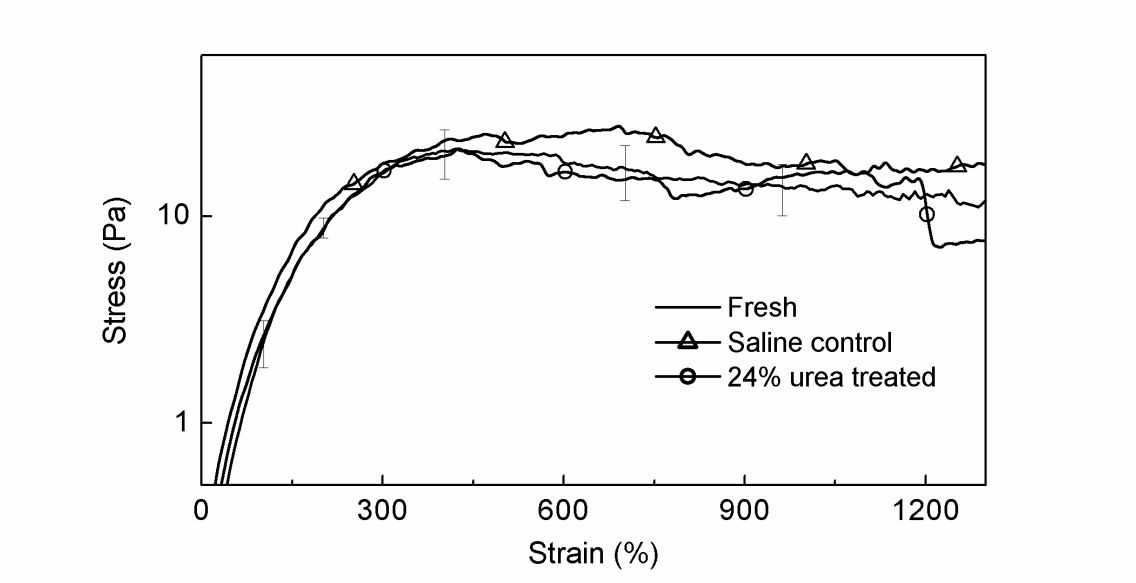

Figure 10. Vitreous treated with 24% urea and incubated for 24 hours fails with the same average stress and strain as saline controls and fresh vitreous ($\dot{\gamma}$ = 0.1 s^{-1}).

4.5 *In Vivo* Exploration of Vitreous Mechanics

Based on the effects of urea treatment *in vitro*, rheological methods were applied to an *in vivo* porcine study with five objectives:

- To quantify vitreous "liquefaction" observations reported by surgeons by measuring the fraction of the vitreous body that is liquid vs. gel and the storage modulus changes induced by treatment with urea

- To have one observer monitor every stage of the therapeutic process, from live medical evaluation through dissection and rheological analysis, to correlate qualitative clinical indicators of liquefaction with rheological measurements
- To reconcile retinal surgeon's reports of vitreous liquefaction *in vivo* with the modest rheological changes observed *in vitro*
- To look for potential intraocular pressure (IOP) changes resulting from treatment
- To look for inflammation or adverse effects on the retina that might accompany treatment with 24% urea

The *in vivo* study clearly demonstrates that 100 μl injections of 12% and 24% urea do not liquefy young, healthy porcine vitreous (Figure 11). Nevertheless, the surgeons *reported* seeing liquefaction in these same eyes. By performing observations side-by-side with surgeons, we learned that the clinical evaluation of liquefaction consists of visualizing the motion of the posterior aspect of vitreous (*post mortem*) using a slit lamp microscope. A vitreous was considered "liquefied" if oscillations of the visible irregularities in the posterior vitreous were out of phase with an oscillatory perturbation induced by shaking the eye with forceps. While out-of-phase motion does indicate inhomogeneity in the vitreous, it also suggests that the vitreous retains elastic character; liquids flow and do not return to their initial positions like the points on the vitreous used by the surgeons to indicate "liquefaction." Therefore, the *in vivo* study reconciled the *in vitro* results with clinical observations and fundamentally changed the way the therapeutic effect of urea is viewed.

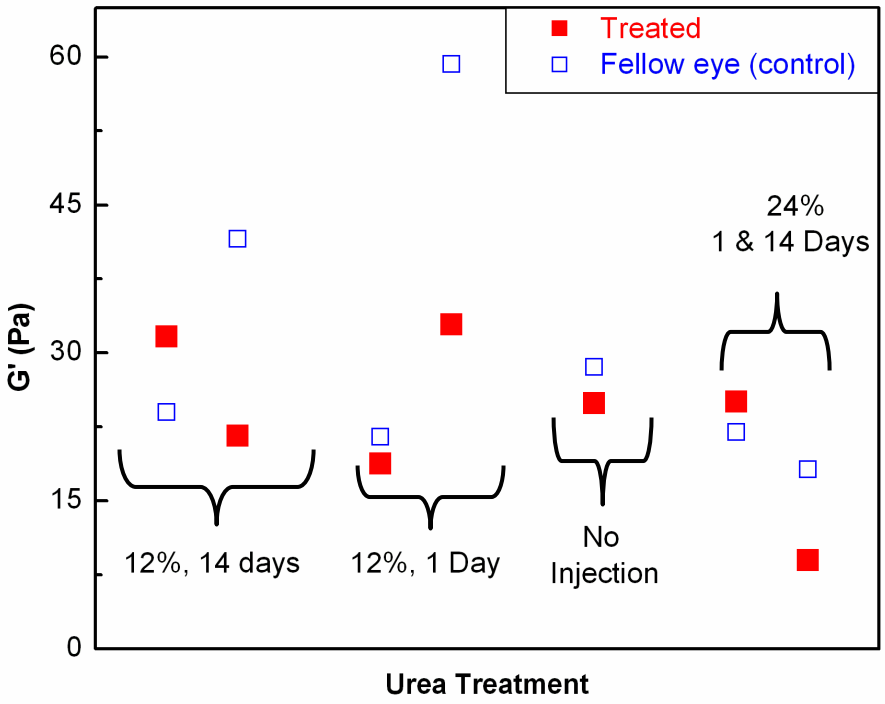

Figure 11. The initial storage modulus of fresh vitreous samples from matched pairs of treated and control eyes is not affected regardless of the treatment. All experiments were conducted at 10 rad/s with 3% strain to avoid damaging the tissue.

Using the slit lamp method described above, out of phase vitreous motion was not observed in either of the eyes treated with 24% urea when examined immediately *post mortem*. It was, however, observed in the vitreous of 2 of the 12%, 14 day treated eyes and 2 of the saline control eyes. The surgeons confirmed that the "vitreous motion" we observed in these specimens was the same as had previously been observed and that these vitreous specimens were liquefied. The eyes were then enucleated and transported individually in phosphate buffered saline (PBS) on ice to the rheological lab of our collaborator, Professor Alberto Tecante at the UNAM, where I performed the mechanical characterization.

The vitreous is not significantly softened *in vivo* by any of the treatments (Figure 11), including eyes that appeared to have "liquefied" by visual examination. Among the four eyes (two treated/fellow pairs) in the 12% urea/14 day group, the standard deviation was 65%, so the observation that the average modulus of the treated eyes was 18% lower than that of the fellow eyes is not significant. Indeed, the modulus of one of the eyes treated with 24% urea for 14 days had a modulus 13% greater than its fellow eye (again, not statistically significant). The 1-day data points in Figure 11 represent measurements on posterior vitreous only, while the full vitreous body was measured in the 14-day experiments. Even analyzing cortical vs. basal vitreous had no apparent effect on modulus.

Further evidence that treatment did not liquefy the vitreous is that treatment did not reduce the gel mass of the vitreous (Figure 12). If any degree of liquefaction had occurred at the vitreoretinal interface, the mass of urea-treated vitreous specimens should have been consistently and measurably reduced compared with their fellow eyes. No such mass reduction was seen regardless of treatment concentration or time. The average mass difference between vitreous bodies of treated and fellow eyes (1 ± 93 mg, n = 10; mass difference ± standard deviation, sample size) was much smaller than the standard deviation of the weight difference.

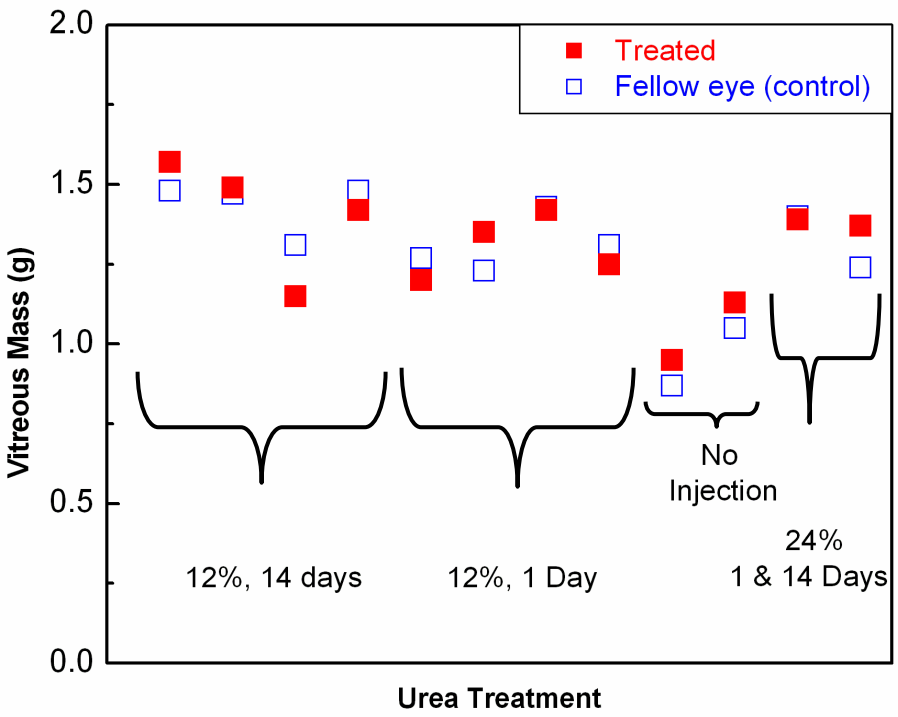

Figure 12. The vitreous of treated eyes do not weigh less than their fellow (control) eye, regardless of the treatment solution or treatment time. This clearly indicates that treatment does not induce vitreous liquefaction.

Apparently, the clinical definition of "liquefaction," prior to quantitative mechanical analysis, actually referred to posterior vitreous detachment rather than significant changes to the bulk properties of the gel. The "specs" visualized at the posterior aspect of the vitreous are probably portions of the peripheral vitreous; release of the posterior vitreous from the retina via detachment allows the protein fibrils to collapse locally and form scattering centers, the same process that produces "floaters" that interfere with human vision. Indeed, we observed during dissection that vitreous was more easily separated from

the retina in urea-treated eyes. The most difficult aspect of vitrectomy is separating the vitreous from the retina. This may explain why surgeons find urea treatment to be helpful and report the perception of liquefaction. We propose that the primary clinical advantage gained from urea treatment is posterior vitreous detachment rather than significant vitreous liquefaction.

In addition to liquefaction studies, the impact of treatment on the general health of the eyes was evaluated by retinal surgeons. Prior to sacrifice, the animals were anesthetized and the IOP of each eye was measured using a Schiotz tonometer. The IOPs of all eyes were normal (Figure 13). The average IOP of treated and control eyes were 15.6 ± 2.0 mm Hg (n=10) and 16.1 ± 2.0 mm Hg (n=10), respectively. Pair wise analysis also shows no significant effect of urea on IOP. The average pair wise IOP difference between treated and control eyes is 4% ± 16%.

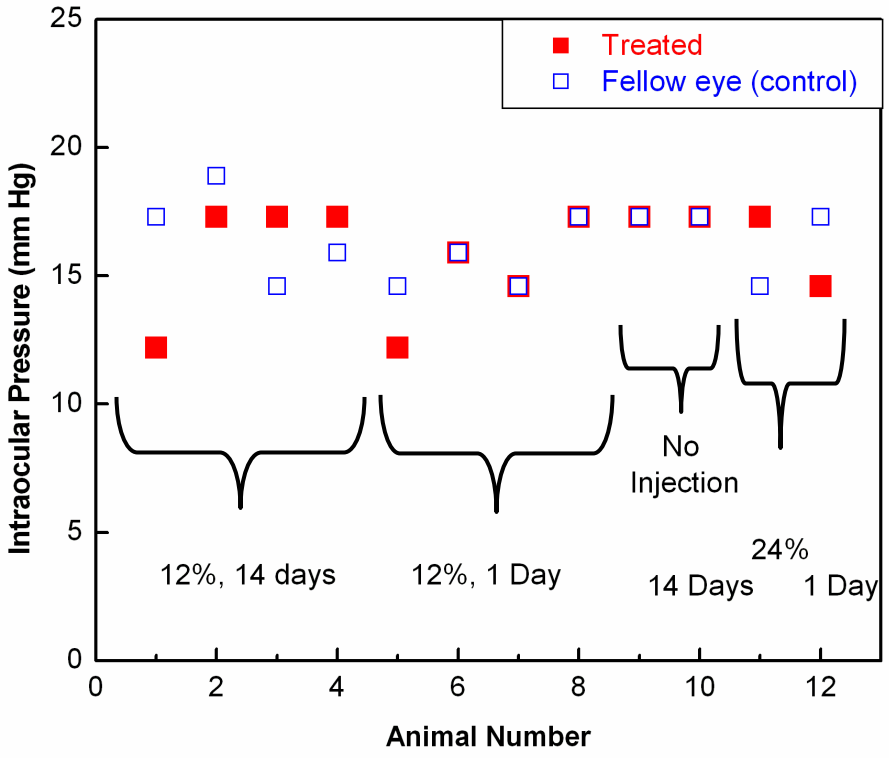

Figure 13. Intraocular pressure, measured with a Schiotz tonometer using a 5.5 g weight, appears to be independent of treatment solution or time. Similar results are obtained with the same instrument and a 10 g weight.

Evaluation by ophthalmoscope demonstrated no adverse reaction or inflammation of the retinas. The tolerance of porcine eyes to an increased dose of urea, relative to rabbit eyes, may be a result of the larger intraocular volume. Cataracts were observed in 3 eyes (2 in treated eyes, 1 in an untreated eye) but they appeared to be unrelated to treatment; the type of cataract observed is common in this species. All eyes were otherwise in good health.

In summary, we have shown that high concentrations of urea and low pH can cause total vitreous collapse *in vitro*. In the case of urea, this is apparently achieved without breaking covalent bonds. Low levels of urea appear to disrupt some component(s) of the vitreous

network without having a significant impact on the strength of the underlying collagen framework. In the course of investigating the therapeutic potential of urea *in vivo* and *in vitro*, we have shown that the clinical benefits of urea for vitrectomy do not rely on changes in bulk mechanical properties and have found evidence that urea destabilizes vitreoretinal adhesion, which could explain its surgical benefit in vitrectomy. We have also shown that therapeutic doses of urea do not negatively impact the health of the porcine retina.

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