ENGINEERING THE MECHANICAL PROPERTIES OF OCULAR TISSUES

Thesis by

Charles Sellers Nickerson

In Partial Fulfillment of the Requirements for the

degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2005

(Defended November 1, 2005)

© 2006

Charles S. Nickerson

All Rights Reserved

ACKNOWLEDGEMENTS

I wish that I had space to thank everyone who has supported me and contributed to this work directly and indirectly, but I will have to limit this to just a few of the people who have done the most. It would be unjust to thank anyone before my wife, Pamela, for her love, support, and patience. She and my children, Rosemary and Samuel, have kept me on the narrow path between high productivity and work-a-holism. I would also like to acknowledge the contribution of my parents for encouraging my creativity, feeding my ravenous appetite for new challenges, and teaching me how to work. My siblings, James, Dawn, Kenny, Lee, and Suzie have also made numerous indirect contributions. My late grandmother has also been a very important influence in all of my accomplishments because she always expected a superior performance and taught me the value thereof. My achievements are the product of divine providence, the sacrifice of my parents, and the influence of my family, and I hope that they recognize the significance of their contributions. Additionally, I wish to thank Pam's family for all of their support while we have been here. Our many friends have also provided much-needed moral support through the ups and downs along the way.

On a professional level, I would first like to acknowledge my advisor, Professor Julie Kornfield, for her tremendous intellectual contributions to this work, for giving me room to think and create, and for her kindness. I don't know which of those things has been most important but they combined to create a wonderful graduate experience for which I will forever be grateful. I must also thank Anne Hormann in the same breath. Anne keeps the group on track, which is no minor task, and does it with a fantastic attitude.

I would also like to express deep gratitude to Dr. John Park of Vitreoretinal Technologies, Inc. He made tremendous scientific contributions to this work and was a mentor for me throughout. Mr. Hampar Karageozian and Dr. Vicken Karageozian at Vitreoretinal Technologies provided key ideas that inspired this research; Vitreoretinal Technologies funded most of this work. Professor Vincent Monnier of Case Western Reserve University has also been a key collaborator in our cornea work and provided ongoing academic and financial support. I would also like to thank the Achievement Rewards for College Scientists (ARCS) Foundation for providing me with the additional financial support necessary to attend graduate school while raising a family.

Former Kornfield group members, Dr. Frederic Tessier and Dr. Giyoong Tae, laid the foundation for the cornea work and spent a great deal of time teaching me about analytical biochemistry, tissue handling, and rheology. Dr. Maria Lujan Auad and Dr. Mike Kempe gave me further guidance in the techniques of rheometry and interpretation of rheological data. Ame DeLeon also contributed to the vitreous work during her summer research program.

I wish to thank everyone involved with our collaborative work in Mexico. I enjoyed and benefited immensely from working with Prof. Hugo Quiroz-Mercado directly and with his students, Dra. Nayeli Ibarra, Dr. Daniel Moreno, and Dra. Griselda Alvarez-Rivera, at the Hospital “Dr. Luis Sánchez Bulnes” de la Asociación Para Evitar la Ceguera (APEC) en México. Dr. Jorge Rivera and Dr. Jose Luis Garrero also contributed to those studies. Their willingness to share their clinical expertise and teach me their techniques allowed me to gain an understanding of the practical aspects of ophthalmic research. Professor Alberto

Tecante at the National Autonomous University of Mexico (UNAM) graciously opened his rheology lab to me while in Mexico. His student, Dra. Mariana Ramirez, was also very kind to help me in every aspect of my visit.

I would like to thank the current members of the Kornfield group, Eric Pape, Derek Thurman, Lucia Fernandez-Ballester, Rafael Verduzco, Neal Scruggs, Mike Mackel, Ameri David, Ryan Turner, Zulie Kurji, Dr. Shuichi Kimata, and particularly Matthew Mattson. Matthew has been an invaluable sounding board for ideas and source of suggestions. I would also like to thank former group members not mentioned above, including Wei Shen, Rob Lammertink, and Erica Thompson.

There are several other members of the Caltech community who deserve thanks: Professor Zhen-Gang Wang and Jennifer Whitman for fruitful discussions regarding network tension, Dr. Scott Ross for help with the difficult problem of conducting nuclear magnetic resonance (NMR) analyses of cornea lysates, and Professor John Brady for discussions regarding flows near permeable boundaries and surface features. I also wish to thank my committee members, Professors Robert Grubbs, David Tirrell, and Linda Hsieh-Wilson, for their help throughout this process. Graduate school has been a wonderful experience and I wish to thank the Institute for admitting me and providing this nurturing and stimulating academic environment. Finally, I would like to thank my first Chemistry teacher, Mr. Bill McKinney. He was the first to explain to me what I consider to be the most universal truth of chemistry—that structure yields function.

ABSTRACT

The mechanical properties of the structural tissues of the eye (cornea, sclera, and vitreous) are critical for vision. Age and disease can cause changes in their physical properties and compromise visual acuity; in the extreme, such changes can lead to blindness. Thus, there is great interest in understanding the mechanical properties of ocular tissues and in developing appropriate therapeutic strategies.

The goal of this thesis is to discover and manipulate the molecular mechanisms that determine the bulk physical properties of the vitreous and the cornea. These tissues are both ordered biocomposites of fibrous collagen embedded in soft matrices of proteoglycans (PGs) and glycosaminoglycans (GAGs). The hydration state, mole fraction, and particularly the organization of these components determine the mechanical properties of the respective tissues. Whereas the mechanical strength of these tissues has traditionally been attributed to their collagenous components, we present evidence that the PGs and GAGs also make significant contributions. We also suggest hypotheses regarding the mechanisms by which the carbohydrate components contribute and how they can be utilized for therapeutic purposes.

In order to study the unique physical properties of the vitreous, novel instrumentation was developed. We describe the use of cleated surfaces on parallel disk tools to quantitatively measure the rheological properties of diverse slip-prone fluids and soft materials. Densely-packed protrusions (0.45mm x 0.45mm cross section x 0.6mm length, 0.9mm apart) penetrate the slip layer, preventing significant flow between cleats. This creates a no-slip boundary ~ 0.16mm below their tips, which serves as the sample gap boundary, in direct analogy to the parallel plate geometry. This “cleat” geometry suppresses slip without application of significant normal force, it imposes well-defined shear to enable absolute measurements, and is compatible with small sample volumes. The geometry was validated in steady and oscillatory shear using a series of materials not prone to slip (Newtonian oils and an entangled polymer melt). The advantage of cleated tools over other slip-prevention

methods was demonstrated using slip-prone materials, including an emulsion, a suspension, and porcine vitreous humor.

The vitreous humor is a transparent gel comprised of a delicate, swollen double network of 10 – 20 nm collagen type II fibrils and charged GAG chains (hyaluronic acid). While extensive progress has been made in identifying the components and biochemistry of the vitreous, prior to the "cleat geometry" experimental limitations hampered quantitative determination of its mechanical properties. With cleated tools we overcame wall slip and avoided tissue compression during measurements of the dynamic moduli of fresh porcine and bovine vitreous. Shear moduli decreased five-fold from initial to steady-state values in the first hour after dissection. Steady-state values (Porcine: $G' = 2.6 \pm 0.9$ Pa and $G'' = 0.6 \pm 0.4$ Pa, $n = 9$; Bovine: $G' = 6.5 \pm 2.0$ Pa and $G'' = 2.0 \pm 0.6$ Pa, $n = 17$) are significantly greater than previously reported. The decrease in modulus after removal from the eye correlates with a decrease in mass: porcine vitreous expels ~5% of its mass within 5 minutes and continues to decay to a steady-state mass ~10% lower than its initial mass in the absence of external driving forces. The expelled fluid has a substantial hyaluronan concentration but a very low protein content. These results indicate that the vitreous network is under tension at its native volume, and its high initial modulus results from this state of tension. We hypothesize that hyaluronan plays a role in sustaining the "internal tension" by Donnan swelling.

The therapeutic goal in vitreous engineering is liquefaction: we seek pharmacological agents capable of gently separating the vitreous from the retina and destabilizing the network without damaging the adjacent tissues (retina and lens). We measured the stability of the vitreous against agents designed to target covalent bonds, hydrogen bonds, electrostatic attractions, and hydrophobic interactions using a simple weighing procedure. We found that in addition to covalent bonds, hydrogen bonds appear to play a particularly important role in stabilizing the vitreous network. This is in agreement with clinical observations that treating eyes with urea prior to vitrectomy provided a significant therapeutic benefit. We found that treating porcine vitreous with therapeutic doses of urea *in vitro* reduced the shear modulus by ~ 30%. Limited *in vivo* animal studies measured no

softening effect and indicated that the therapeutic benefit of urea may be a reduction of vitreoretinal adhesion.

The cornea is also composed of collagen fibrils embedded in a PG/GAG matrix. The cornea, however, contains far more collagen, PG, and GAG than vitreous, and its components are also more ordered: the collagen (type I) is in the form of 30 nm fibrils, precisely arranged lamellae and evenly spaced in a keratin sulfate-rich matrix. Our therapeutic goal in the cornea is to stabilize its nanostructure and mechanical properties against keratoconus, a degenerative disease in which the cornea softens and bows outward under the force of intraocular pressure.

We present coordinated biomechanical and biochemical analyses of corneal tissue that has been crosslinked using glycation. Non-enzymatic crosslinking alters the viscoelastic properties of protein-rich tissues, but a quantitative correlation between the formation of specific advanced glycation end products (AGEs) and physiologically relevant mechanical property changes has not previously been established. We report that corneas treated with 1% and 2% glyceraldehyde solutions produce a 300% and 600% rise in shear modulus, respectively, which strongly and linearly correlates with increased fluorescence and the formation of the AGEs argpyrimidine, lys-hydroxy-triosidine, and arg-hydroxy-triosidine (R^2= 0.999, 0.970, and 0.890 respectively). NMR studies are used to demonstrate that enzymatic digestion does not alter AGEs and has some advantages over acid hydrolysis. The level of mechanical reinforcement observed in these studies is probably sufficient to stabilize keratoconus corneas, based upon successful treatments with other crosslinking strategies.

Comparing quantitative correlations between modulus and AGE accumulation in corneas with analyses of collagen fibers isolated from mouse tail tendons suggests that glycation-induced corneal stiffening cannot be attributed solely to changes in collagen. We present a novel hypothesis that the mechanically-relevant AGE crosslinks are those that change the properties of the soft PG/GAG matrix and its coupling to the collagen fibrils, rather than the much more numerous AGEs that crosslink amino acids within fibrils.

TABLE OF CONTENTS

Acknowledgements iii
Abstract vi
Table of Contents x
List of Illustrations and Tables xii
Symbols and Abbreviations xv

Chapter I: Introduction
1.1 Background 1
1.2 The Vitreous Humor 4
1.3 The Cornea 11
1.4 Broader Implications 15
1.5 Organization of Thesis 16
Bibliography 17
Chapter II: The “Cleat” Geometry: A Novel Rheological Tool
2.1 Wall Slip – A Classical Problem with Implications in Biorheology ... 20
2.2 Materials and Methods 26
2.3 Quantitative Validation 29
2.4 Applications in Biorheology 37
Bibliography 43
Chapter III: The Vitreous Humor: Mechanics and Structure
3.1 Primary Structure and Composition 45
3.2 Materials and Methods 49
3.3 Mechanics of the Vitreous – the Key to Structure 52
3.4 Mass Loss Associated with Post Dissection Softening 60
3.5 Network Tension – the Contribution of Hyaluronic Acid 64
Bibliography 70
Chapter IV: Engineering the Vitreous Humor
4.1 Introduction 72
4.2 Materials and Methods 74
4.3 The Chemical Stability of the vitreous 78
4.4 Targeted Vitreous Engineering and Rheology 94
4.5 *In Vivo* Exploration of Vitreous Mechanics 99
Bibliography 107
Chapter V: Stiffening The Cornea: The Therapeutic Potential of Glycation
5.1 Introduction 109
5.2 Materials and Methods 111
5.3 Mechanical Properties of Glycated Corneas 116
5.4 Resistance to Proteolytic Degradation 118
5.5 Quantification of Specific AGEs 120
5.6 Solution and Solid-State NMR of Glycated Corneas 123

5.7 Fluorescence – Noninvasive Indicator of Glycation 126
Bibliography 130
Chapter VI: Understanding the Microstructural Changes that Cause Corneal Stiffening
6.1 Introduction 134
6.2 Materials and Methods 139
6.3 Advanced Glycation Endproducts in Mouse Tail Collagen............... 142
6.4 Mechanical Impact of Glycation on Mouse Tail Tendons................. 144
6.5 Proteoglycans May Play a Role in Tissue Stiffening......................... 152
Bibliography 157
Appendix A: IRB Approval Letter for use of Human Donor Tissue............. 160
Appendix B: Experimental Protocol for *In Vivo* Work 161
Index.. 163

LIST OF ILLUSTRATIONS AND TABLES

Page

Chapter 1

Figure 1 Normal Anatomy of the Eye........ 3

Figure 2 The Collagen-HA Network in the Vitreous........ 5

Figure 3 Micro and Nanostructure of the Cornea........ 11

Figure 4 Key Products and Intermediate of GA Glycation........ 13

Table 1 Known Components of the Vitreous........ 8

Chapter 2

Figure 1 Schematic of Wall Slip........ 21

Figure 2 Schematic of the Cleat Geometry........ 25

Figure 3 Viscosity of Newtonian Oils (Uncorrected)........ 30

Figure 4 Obtaining the Correction Factor δ........ 31

Figure 5 Viscosity of Newtonian Oils (Corrected)........ 32

Figure 6 Shear Modulus of PDMS Putty (Corrected)........ 33

Figure 7 Shear Modulus of Peanut Butter (Corrected)........ 36

Figure 8 Shear Modulus of Mayonnaise (Corrected)........ 37

Figure 9 Shear Modulus of Porcine Cornea (Corrected)........ 38

Figure 10 Shear Modulus of Porcine Vitreous (Corrected)........ 40

Table 1 Existing Approaches to Wall Slip Prevention........ 22

Chapter 3

Figure 1 The Collagen-HA Network in the Vitreous........ 46

Figure 2 Network Tension Release........ 48

Figure 3 Time Dependence of Bovine and Porcine Modulus........ 53

Figure 4 Comparison with Literature Values of Vitreous........ 54

Figure 5 Dynamic Strain Sweep of Porcine Vitreous 55

Figure 6 Dynamic Frequency Sweep of Porcine Vitreous........................ 56

Figure 7 Modulus of Human Donor Vitreous 57

Figure 8 Failure Behavior of Porcine Vitreous Under Steady Strain 59

Figure 9 Photographs Illustrating *ex oculo* Mass Loss............................. 61

Figure 10 Circular Dichroism of Vitreous Exudate 62

Figure 11 Measurements of Mass Loss 63

Chapter 4

Figure 1 Chemical Stability Procedure 75

Figure 2 Effect of Hyaluronidase Enzyme on Vitreous 81

Figure 3 Effect of Excess Urea on Vitreous 84

Figure 4 Effect of pH on Vitreous... 87

Figure 5 Effect of NaCl on Vitreous... 89

Figure 6 Effect of $MgCl_2$ on Vitreous... 91

Figure 7 Effect of Triton® X-100 on Vitreous... 93

Figure 8 Modulus Reduced by Urea Injections *In Vitro* 95

Figure 9 pH Effects on Modulus Reduction *In Vitro* 97

Figure 10 Failure Behavior is Not Affected by Urea Injections *In Vitro* . 99

Figure 11 Modulus is Not Affected by Urea Injections *In Vivo* 101

Figure 12 Vitreous Mass is Not Affected by Urea Injections *In Vivo* 103

Figure 13 IOP is Not Affected by Urea Injections *In Vivo* 105

Table 1 Chemical Stability Treatment Solutions 76

Chapter 5

Figure 1 Glyceraldehyde and Three of Its AGEs 111

Figure 2 Increase in Cornea Modulus... 117

Figure 3 Increased Resistance to Proteolytic Degradation 120

Figure 4 Rise in Three Glyceraldehyde AGEs 122

Figure 5 Modulus Rises with Accumulation of AGEs........................... 123

Figure 6 ^{13}C-NMR Spectra of Glycated Corneal Tissue 124

Figure 7 Fluorescence Rises with Accumulation of AGEs 127

Chapter 6

Figure 1 Micro/Nanostructure of the Stroma 135

Figure 2 Preconditioning Collagen Fibrils 141

Figure 3 CEL as a Function of MGO Treatment and Mouse Age 143

Figure 4 Stress-Strain Plot of Mouse Tail Tendons 145

Figure 5 Physical Changes That Accompany Glycation 148

SYMBOLS AND ABBREVIATIONS

AGE Advanced Glycation Endproduct

δ Penetration depth / Phase angle

G′ Storage Modulus

G″ Loss Modulus

GA Glyceraldehyde

GAG Glycosaminoglycan

G-3-phosphate Glyceraldehyde-3-phosphate

HA Hyaluronic acid

HPLC High Performance Liquid Chromatography

L_c Cleat length

MGO Methylglyoxal

MW Molecular Weight

NMR Nuclear Magnetic Resonance

PBS Phosphate-buffered Saline

PG Proteoglycan

PVD Posterior vitreous detachment

TBT Tendon breaking time

η Viscosity

η^* Complex Viscosity

σ Shear stress

γ Shear Strain

$\dot{\gamma}$ Strain Rate

Chapter 1

INTRODUCTION

1.1 Background.. 1
1.2 The Vitreous Humor.. 4
1.3 The Cornea 11
1.4 Broader Implications..15
1.5 Organization of Thesis 16
Bibliography 17

1.1 Background

The ability to create and maintain fixed spatial relationships between cells and organs is vitally important for higher organisms. Residing in fixed locations allows cells and tissues to work cooperatively through specialization and division of labor.[1] One illustration of the importance of precise physical properties and arrangements is mammalian vision, which relies on the precise geometry of the cornea, the mechanical strength of the sclera to support the retina, and the orbital ligaments to control the line of sight.

The mechanical properties of structural tissues such as these are derived from the nanoscale architecture and properties of their constituent molecules. Most structural tissues are biocomposites of fibrous proteins embedded in soft carbohydrate matrices. Collagen is the primary fibrous component; proteoglycans and glycosaminoglycans act as the matrix. The hydration state, mole fraction, and organization of these components vary between tissues and species, but the basic structure—high tensile-strength fibrils organized in soft matrices—is highly preserved. Rare genetic mutations that weaken collagen fibrils or

disrupt other aspects of this molecular pattern lead to devastating systemic diseases.[2-6] A number of more common diseases, such as arthritis and diabetes, are also associated with degeneration of collagenous tissues.

The debilitating nature and prevalence of heritable and degenerative disorders that affect connective-tissues has stimulated considerable biochemical and biomechanical research. Unfortunately, the molecular (biochemical) and biomechanical aspects of this important field have been investigated independently rather than in concert. We will present significant advancements that have come as a result of combining biochemical analyses with novel bulk characterization techniques.

Broadly stated, the goal of the present research is to discover and manipulate the molecular mechanisms that determine the bulk physical properties of the cornea and vitreous humor (Figure 1). This goal can be divided into three specific objectives:

1) To quantitatively determine the mechanical properties of connective tissues

2) To understand the molecular basis of these mechanical properties and their implications for disease and tissue engineering

3) To create therapeutic changes in the mechanical properties of the cornea and vitreous

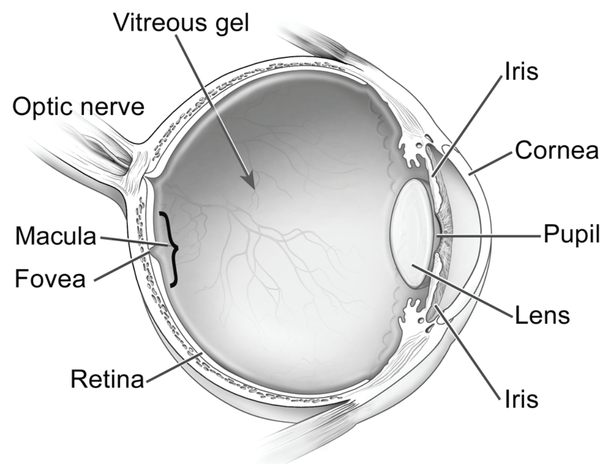

Figure 1. Diagram of the eye illustrating normal eye anatomy, including the vitreous humor (gel) and cornea. This figure reproduced by permission from the National Eye Institute, National Institutes of Health.

Our approach to these objectives is to combine analytical chemistry, rheology, and polymer physics with *in vivo* animal studies and the clinical experience of collaborators from industry and medicine. Biochemical and biomechanical investigations were conducted in parallel with drug discovery and clinical research, providing feedback between clinical and laboratory work. Clinical research identified potential therapeutics and evaluations of efficacy, while laboratory research addressed fundamental questions regarding the basis of the mechanical properties of collagenous tissues and how they can be engineered. The success of this approach in exploring potential therapeutics for the vitreous humor and cornea demonstrates the utility of an integrated approach to understanding and engineering connective tissues in general.

1.2 The Vitreous Humor

The vitreous is a transparent, collagenous gel that fills the posterior chamber of the eye. It is more than 98% water, avascular, and nearly acellular; thus, the vitreous was historically considered an inert space-filler.[7] However, over the past few decades it has become clear that the vitreous plays an essential structural role in the development, maintenance, and pathologies of vision. Sebag has summarized the functions of the vitreous as developmental—mediating proper growth of the eye; optical—maintaining a clear path to the retina; mechanical—supporting the various ocular tissues during physical activity; and metabolic—providing a repository of various small molecules for the retina.[8] Proper performance of these functions depends upon the unique physical properties of the vitreous.

The vitreous is thought to derive its physical properties from its hydrated double network of collagen type II fibrils and high molecular-weight, polyanionic hyaluronan macromolecules (Figure 2).[8-10] Heterotypic collagen fibrils (10 – 20 nm diameter) are composed of a small, collagen type V/XI core surrounded by collagen type II. Human vitreous hyaluronan (HA) is polydisperse with an average molecular weight that is estimated to be ~ 5,000,000.[9] Prior literature indicates that the vitreous completely liquefies when digested with collagenase enzyme, whereas it only shrinks when digested with hyaluronidase.[8, 9, 11] On this basis it has been presumed that the network of collagen fibrils provides mechanical strength, and the swollen HA macromolecules simply fill the space between fibrils to prevent aggregation. In Chapter 3 we will discuss the collagen-HA double network in greater depth and present rheological and biochemical evidence that hyaluronan *does* contribute

profoundly to the elastic character of the vitreous. This realization changes the way we view the network, particularly in the context of vitreous degeneration and engineering.

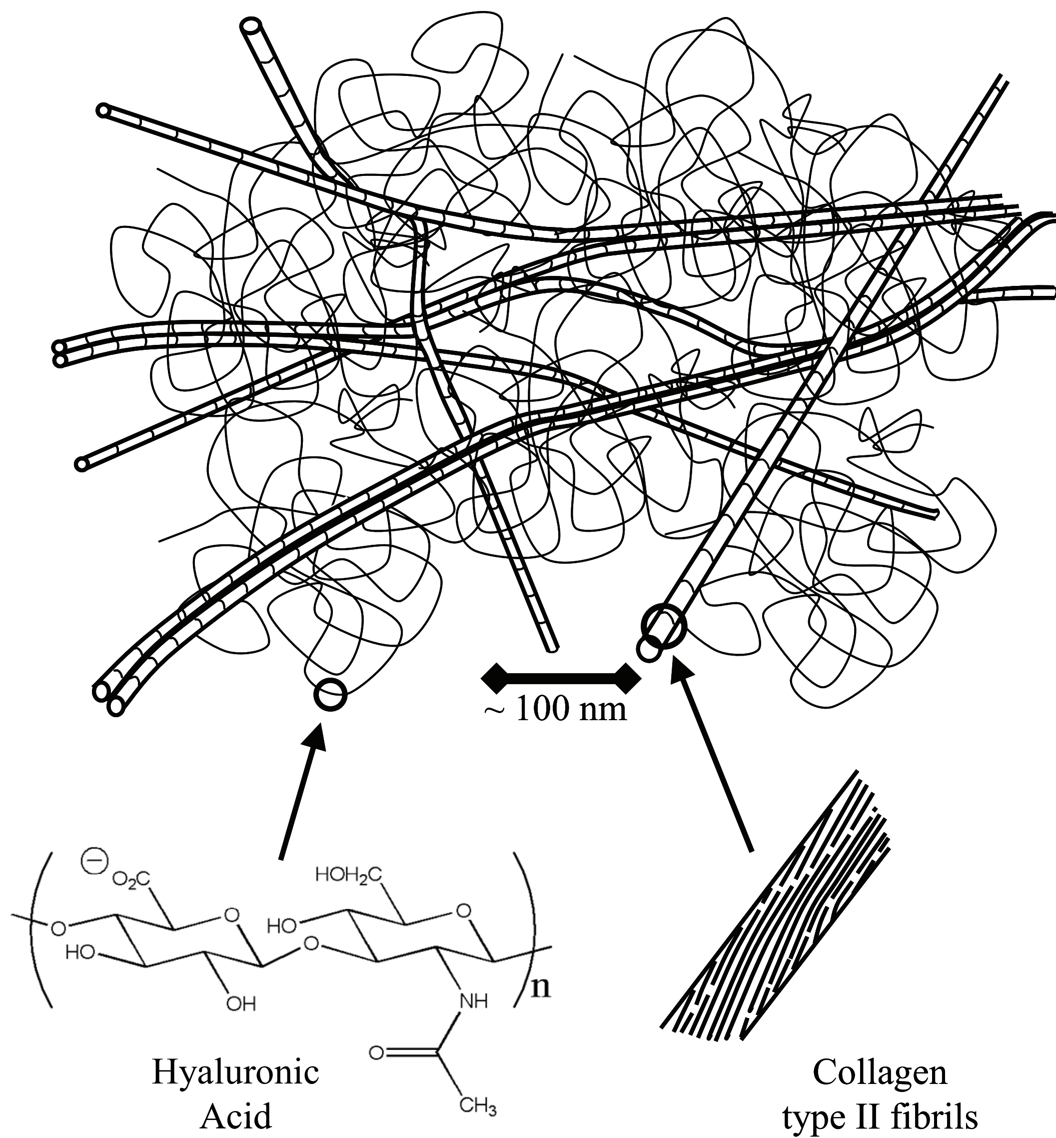

Figure 2. Schematic depiction of the network structure of the vitreous. The vitreous is composed of a highly-swollen double network of collagen type II fibrils (~ 15 nm in diameter) and hyaluronic acid (~ 5M MW).

With age the collagen-HA network degrades and loses mechanical integrity: pockets of fluid (lacunae) form near the retina as the components of the vitreous network aggregate and pull away from the retina.[8] Posterior vitreous detachment (PVD) is normally inhomogeneous, leaving points of adhesion that cause localized traction on the retina. Incomplete PVD and the resultant vitreoretinal traction are thought to play a role in a number of diseases, including macular holes, macular edema, vitreous hemorrhage, retinal tears, and retinal detachment.[8] The only treatment currently available for alleviating vitreoretinal traction is surgical removal of the vitreous (vitrectomy).[12] Motivated by the need for a less invasive and traumatic treatment, efforts have been made to find "pharmacological vitrectomy agents" capable of inducing PVD and liquefying or significantly softening the vitreous, thereby alleviating traction without surgery.[13] Proposed therapeutics from the literature will be discussed in detail in Chapter 4, but they generally consist of enzymes designed to cleave the proteins responsible for the mechanical integrity of the vitreous. Little attention has been given to the possibility of targeting noncovalent intermolecular interactions. We present results that indicate that disruption of hydrogen bonds strongly destabilizes the vitreous network, whereas disruption of electrostatic or hydrophobic interactions has a much weaker effect.

In addition to collagen type II and HA, 15 "minor" proteins and proteoglycans have been identified in the vitreous (Table 1). These components are minor in terms of mass, but may be crucial for the structure and stability of the vitreous, much as nails are a "minor" component of a wood-framed house. A number of these components, including link protein, fibronectin, and vitronectin, are known to connect proteins with polysaccharides in

other tissues. They may perform a similar function in the vitreous, stabilizing the collagen-HA network and linking it to other structures in the eye; however, little is known about the role of minor components in the molecular architecture of the vitreous network.

Given the importance of the viscoelastic properties of the vitreous to its function and to pathology, it is striking that there is no consensus on the value of its modulus in the prior literature. This is due in part to a lack of sufficient experimental methods for quantitatively measuring the mechanical properties of the vitreous and how they change as a result of various treatments.[14] To address this need we developed a novel rheological tool that enabled us to make the first quantitative measurements of the mechanical properties of the vitreous. We discovered that the modulus of the vitreous is significantly higher *in situ* than after removal from the eye. Further exploration of this discovery led us to a novel hypothesis regarding the mechanical properties of the vitreous: that HA increases the modulus of the vitreous by swelling the collagen network to a state of tension.

The novel tool also allowed us to measure modulus changes that resulted from treating the vitreous with a particular proposed pharmacological vitrectomy agent—urea. Clinical observations that urea may facilitate vitreous removal[15, 16] led us to investigate its influence on the mechanical properties of the vitreous *in vitro* and *in vivo*. Slit lamp observations of urea-treated vitreous, together with reduced surgical time during vitrectomy, suggested to the clinicians that urea "liquefied" the vitreous. By quantitatively characterizing the modulus of the vitreous, our work showed that treatment did not liquefy vitreous *in vitro* or *in vivo.* By working side-by-side with a team of eye surgeons working under the direction

of Professor Hugo Quiroz-Mercado at the Hospital "Dr. Luis Sánchez Bulnes" de la APEC in Mexico, we were able to reconcile clinical observations with rheological measurements. The clinical benefit was more likely the result of reduced vitreoretinal adhesion and phase separation as the collagen network contracted away from the retina to relieve tension. We also explored the effects of other agents on vitreous and found that hydrogen bonding plays a more significant role in stabilizing the vitreous network than electrostatic or hydrophobic effects. Taken together, these results provide a basis for rational design of future pharmacological vitrectomy agents.

Component	**Concentration** Human/Pig [μg/ml]	**Location**	**Proposed functions**
Water	>980,000[9]/ same	Throughout	Maintains vitreous mechanical properties and facilitates transport[7]
Salts (NaCl, KCl, $CaCl_2$, and $MgCl_2$)	~9,000[9]/same	Throughout	Global charge balance, Donnan swelling; vitreous is isotonic with blood and most other tissues[7]
Total Protein	800[7]/700[17]	Throughout	—
Total polysaccharide	240[18]/~250[17]	Throughout	—
Collagen type II	~225[9]/150[17]	Throughout as heterotypic fibrils	Resist elongation of the eye and provide structural framework for the vitreous body[7]
Hyaluronic acid	65-400[9]/165[17]	Throughout	Resist compression of the eye, hydrate tissue, space collagen fibrils[7]

Albumin	293[19]/	Throughout	Soluble protein, no known structural role
Link protein	0.6(bovine)[20]	Unknown	1:1 with versican; it may be there to link versican to HA[20]
Collagen V/XI	~30[9]/ Unknown	Throughout	Form the core of heterotypic collagen II fibrils[9]
Collagen IX	<30[9]/ Unknown	Throughout	Decorate surface of heterotypic collagen II fibrils, prevent fibril aggregation, possibly link fibrils to noncollagenous components[9]
Collagen VI[21]	Unknown	Concentrated on the zonular fibers	Bind collagen fibrils to HA and other species (has been shown to bind von Willebrand factor, collagen II fibrils, decorin and HA)[22, 23]
Collagen XVIII[9]	Unknown	Vitreoretinal interface	Vitreoretinal adhesion; has been co-localized with opticin at vitreoretinal interface; contains endostatin as a non-collagenous domain[9]
Cartilage oligomeric matrix protein (COMP)[24]	Unknown	Unknown	Unknown, but also found in cartilage and tendon; contains von Willebrand factor domains (see collagen VI)[25]
Microfibril-associated glycoprotein-1 (MAGP1)[26]	Unknown	Unknown	Decorate exterior of zonular fibers[26]
Opticin[9]	Unknown	Vitreous base and lamina cribrosa	Acts in conjunction with collagen XVIII to mediate vitreoretinal adhesion[9]
Fibrillin	Minor but probably > [coll VI][9]	Attached to lens capsule	Structural fibrils for lens capsule anchoring & articulation[9]
Fibronectin	6[9]/ >76(bovine)[27]	Throughout	Mediate binding between collagen and polysaccharides[25]

Vitronectin	4[28]/ Unknown	Unknown	Mediate collagen-polysaccharide binding; sensitive to denaturation[25]
Versican	60[29]/ 22(bovine)[20]	Unknown	1 per 150 moles of HA; possible link between HA and collagen and has been show to dissociate (if it was associated) in 4M guanidinium HCL; HA binding has been demonstrated[17, 25]
VIT1[30]	Unknown	Unknown	May have structural role[30]
Laminin/ Collagen type IV[31]	Unknown	Inner limiting membrane surrounding vitreous	While not components of the vitreous proper, they may participate in peripheral vitreous adhesion

Table 1. Known components of the vitreous humor listed with available information regarding concentration (μg / mL), distribution, and proposed function.

1.3 The Cornea

Like the vitreous, the cornea is composed of collagen fibrils embedded in a proteoglycan (PG) and glycosaminoglycan (GAG) matrix; however, unlike the vitreous, the cornea has a highly-ordered structure. The major structural element of the cornea (~ 90% of its thickness) is the stroma, which is composed of approximately 200 lamellae of oriented collagen type I fibrils embedded in a hydrated PG/GAG[12] (Figure 3). The precise arrangement of collagen fibrils allows the cornea to retain optical clarity in spite of the relatively high density of collagen fibrils (30 nm diameter) required to retain the shape of the cornea.

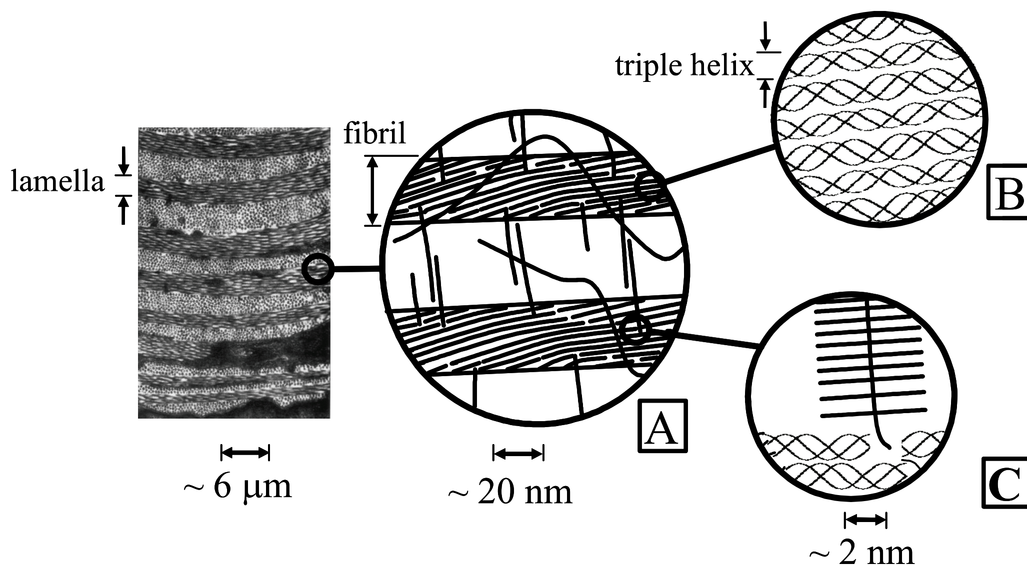

Figure 3. Stroma microstructure. [A] Represents the rigid collagen type I fibrils and smaller strands of proteoglycan that compose the lamellae of the corneal stroma. [B] Shows an enlargement of part of one of the fibrils, displaying the collagen triple helices aligned within a fibril. [C] Depicts the protein core of a proteoglycan non-covalently associated with the surface of a collagen fibril and decorated with polysaccharide chains. Micrograph was used by permission from Prof. K. Kadler, U. Manchester.

Whereas a primary therapeutic objective in the vitreous is softening and inducing PVD to alleviate vitreoretinal traction, a major, unmet clinical need in the cornea is enhancing its mechanical stability to prevent the progression of keratoconus. Keratoconus ("cone-shaped cornea") is a condition in which the cornea softens and slowly begins to protrude outward under the force of intraocular pressure.[12] It affects roughly 1 in 2,000 people, normally beginning in the teens or early twenties, and causes progressive loss of visual acuity, eventually leading to blindness.[32] In early stages, keratoconus is treated by application of hard contact lenses that correct vision and help maintain the shape of the cornea. If keratoconus progresses further, cornea transplantation is the only known treatment. The expense and difficulty of obtaining transplant tissue and the invasive nature of the surgery motivate our efforts to find a chemical treatment for keratoconus.

Collaborators at ISTA Pharmaceuticals, Inc. (Irvine, CA) developed a non-toxic, glycation-based crosslinking strategy to stabilize the cornea against keratoconus using glyceraldehyde (GA). Glyceraldehyde reacts with primary amines to form several known advanced glycation endproducts (AGEs), including two crosslinks and three AGEs that are also formed in reactions with methylglyoxal (MGO), another species investigated in this work (Figure 4). We have demonstrated that therapeutic (nontoxic) doses of glyceraldehyde are capable of significantly increasing the shear modulus of porcine corneas. Equivalent increases in modulus, achieved through alternative crosslinking strategies, have been shown to stabilize keratoconus eyes in clinical trials.[33, 34]

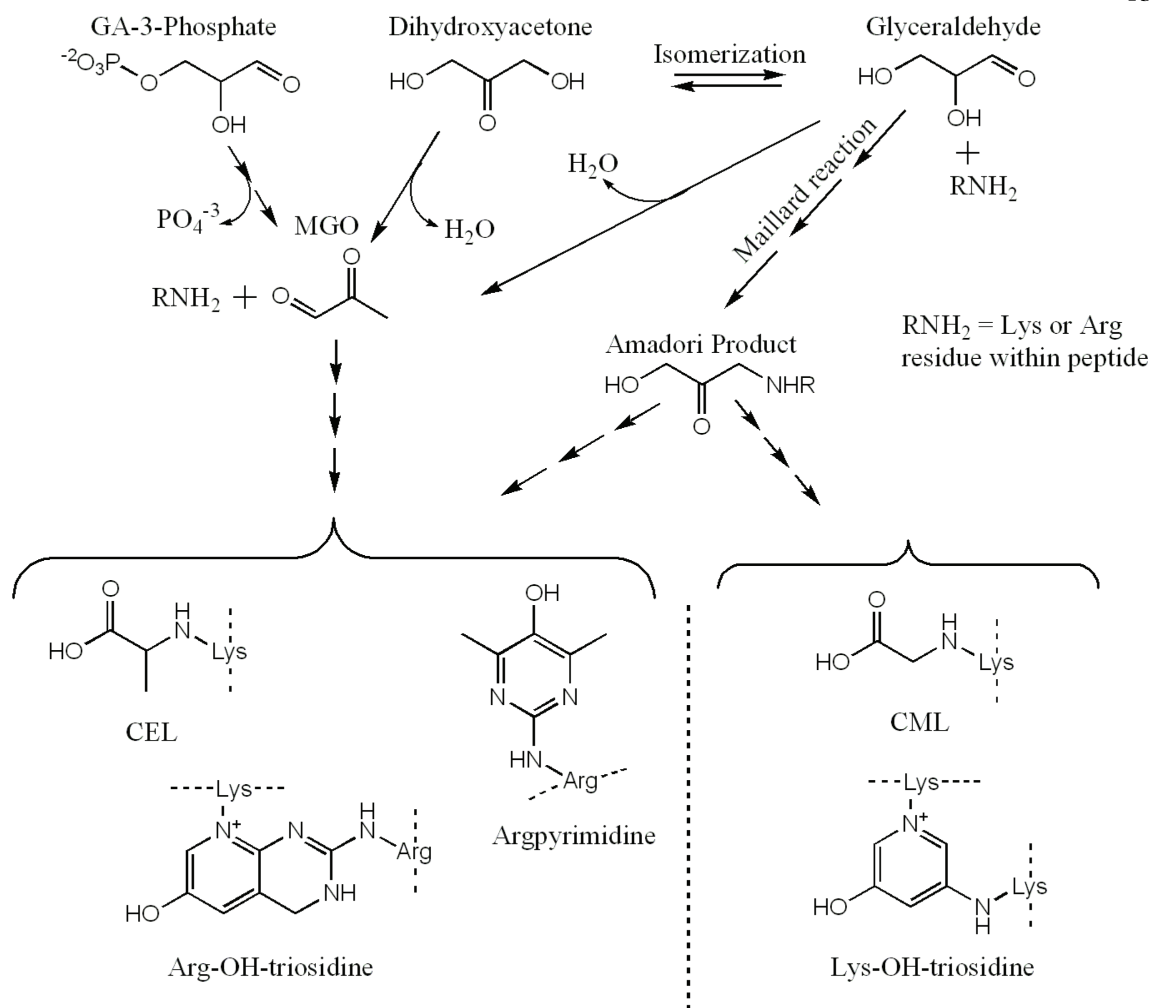

Figure 4. Glyceraldehyde, glyceraldehyde-3-phosphate (GA-3-phosphate), and methylglyoxal (MGO) all lead to similar AGEs, including argpyrimidine, arg-OH-triosidine, lys-OH-triosidine, and carboxyethyl lysine. RNH_2 indicates a primary amine on the side chain of an Arg or Lys residue within a peptide.

Biochemical analyses of GA-treated corneas revealed an additional protective effect of GA treatment: they are far less susceptible to proteolytic degradation (Chapter 5). This is particularly significant in light of the current hypothesis that keratoconus-induced softening comes as a result of overactive proteases in the cornea.[35] The enzyme protective effect also indicates that GA may be a suitable treatment for corneal ulcers, which have also been

linked to increased proteolytic activity and treated with crosslinking strategies.[36] The effect of GA on corneal ulcers has not yet been addressed.

Glycation-induced changes in enzyme resistance and modulus also correlate with increased fluorescence and AGE accumulation. We were able to isolate and quantify specific AGEs from glycated corneas and demonstrate that modulus increases linearly with accumulation of each of them, including argpyrimidine, a pendent adduct. Thus, it appears that crosslinking and noncrosslinking AGEs rise together and that various individual AGEs could serve as a surrogate to track tissue stiffening, whether or not the individual surrogate AGE is a crosslink. It may be possible to use an equivalent empirical relation to noninvasively measure (e.g., by fluorescence) the degree of tissue stiffening in clinical practice.

Quantitative correlations between the chemical and mechanical impact of glycation on corneal tissue also yield new insight into the molecular mechanisms of AGE-related tissue stiffening. The literature holds that glycation stiffens collagenous tissues by changing the properties of the constituent collagen fibrils;[37, 38] however, our results demonstrate that glycation-induced corneal stiffening cannot be attributed solely to changes in the properties of the collagen fibrils. We present a novel hypothesis that the mechanically relevant AGE crosslinks are those that change the properties of the soft PG/GAG matrix and its coupling to the collagen fibrils, rather than the much more numerous AGEs that crosslink amino acids within fibrils.

New insights into the increase in modulus associated with AGEs may also be broadly relevant to aging, diabetes, and tissue engineering research. The mechanisms by which glycation stiffens tissues *in vitro* may be relevant to certain pathologies of aging and diabetes. When properly understood, glycation has the potential to be turned from a pathologic process to a therapeutic strategy. The cornea is a good example, but it is merely a case-in-point. This strategy can be applied to a number of areas, from wound healing to bioadhesion to improving the mechanical properties of protein-based and polyamide synthetic tissues. Imparting strength to weakened connective tissue through glycation may provide an alternative to tissue transplants in diseases such as keratoconus.

1.4 Broader Implications

A unifying theme that emerges from both the vitreous and cornea work is that collagenous tissues depend integrally on the contributions of their carbohydrate components for mechanical strength. We hope that future efforts to engineer the mechanical properties of collagenous tissues will recognize the important mechanical role of carbohydrate components and apply this knowledge in the design of therapeutics.

The overarching goal of this thesis is to bridge the gap between the chemical, biomechanical, and clinical aspects of tissue engineering. Working closely with physicians to focus on these three aspects in parallel has allowed developments from the lab to rapidly influence therapeutic formulations for clinical trial (e.g., optimal pH of urea treatment), and feedback on the *in vivo* relevance of *in vitro* discoveries allowed us to rapidly verify the significance of new findings. We hope that the success we have had in elucidating the

molecular interactions that play a significant role in biomechanics will provide a model for productive cross-field collaborations.

1.5 Organization of Thesis

There were no rheological methods suitable for quantitative characterization of the vitreous prior to this work. Chapter 2 presents the novel "cleat geometry" developed specifically for this purpose.

Chapters 3 and 4 address the properties and network structure of the vitreous. In Chapter 3 the mechanical properties of the vitreous are defined. A novel hypothesis regarding a direct contribution of hyaluronic acid to the mechanical stiffness of the vitreous is also presented. In Chapter 4 the stability of the vitreous network in various chemical environments is examined as a basis for selecting potential pharmacological vitrectomy agents. Hydrogen bonding is shown to play a key role in stabilizing the vitreous network and urea is examined as a potential therapeutic for softening the vitreous.

Chapters 5 and 6 address glycation in the cornea. In Chapter 5 the chemical and mechanical impact of glycating corneal tissue with glyceraldehyde is examined. In Chapter 6 mechanical measurements of glycated collagen fibers from mouse tail tendons are used to demonstrate that the enhanced mechanical strength of glycated collagenous tissues cannot be attributed solely to the stiffening of collagen fibrils – the surrounding matrix (presumably proteoglycans) must also play a role.

BIBLIOGRAPHY

1. Scott JE. Extracellular-Matrix, Supramolecular Organization and Shape. *Journal of Anatomy*. Oct 1995;187:259-269.
2. Berg C, Geipel A, Noack F, et al. Prenatal diagnosis of Bruck syndrome. *Prenatal Diagnosis*. Jul 2005;25(7):535-538.
3. Baxter BT. Heritable diseases of the blood vessels. *Cardiovascular Pathology*. Jul-Aug 2005;14(4):185-188.
4. Chien S, Muiesan P. Spontaneous liver rupture in Ehlers-Danlos syndrome type IV. *Journal Of The Royal Society Of Medicine*. Jul 2005;98(7):320-322.
5. Glorieux FH. Caffey disease: an unlikely collagenopathy. *Journal Of Clinical Investigation*. May 2005;115(5):1142-1144.
6. Evereklioglu C, Madenci E, Bayazit YA, Yilmaz K, Balat A, Bekir NA. Central corneal thickness is lower in osteogenesis imperfecta and negatively correlates-vith the presence of blue sclera. *Ophthalmic And Physiological Optics*. Nov 2002;22(6):511-515.
7. Fatt I, Weissman BA. *Physiology of the Eye*. Second ed. Boston: Butterworth-Heinemann; 1992.
8. Sebag J. *The Vitreous - Structure, Function, and Pathobiology*. New York: Springer-Verlag Inc.; 1989.
9. Bishop PN. Structural macromolecules and supramolecular organisation of the vitreous gel. *Progress in Retinal and Eye Research*. May 2000;19(3):323-344.
10. Sebag J, Balazs E. Morphology and ultrastructure of human vitreous fibers. *Investigative Ophthalmology and Visual Science*. 1989;30:1867-1871.
11. Bos KJ, Holmes DF, Meadows RS, Kadler KE, McLeod D, Bishop PN. Collagen fibril organisation in mammalian vitreous by freeze etch/rotary shadowing electron microscopy. *Micron*. Apr 2001;32(3):301-306.
12. Oyster CW. *The Human Eye: Structure and Function*. Sunderland, MA: Sinauer Associates, Inc.; 1999.
13. Sebag J. Is pharmacologic vitreolysis brewing? *Retina*. Feb 2002;22(1):1-3.
14. Nickerson CS, Kornfield JA. A "cleat" geometry for suppressing wall slip. *Journal of Rheology*. 2005;49(4):865-874.
15. Karageozian HL. Determine the safety and efficacy of Vitreosolve administered intravitreally to induce a complete posterior vitreous detachment (PVD) in non proliferative diabetic retinopathy human subjects. *Investigative Ophthalmology & Visual Science*. 2005;46.
16. Ochoa-Contreras D, Romero-Castro RM, Rivera-Sempertegui JO, Karageozian V, Karageozian H, Quiroz-Mercado H. Anatomical and visual outcome of retinal detachment surgery in children with intravitreal carbamide previous a vitrectomy surgical procedure. *Investigative Ophthalmology & Visual Science*. May 2003;44:U94-U94.
17. Noulas AV, Theocharis AD, Feretis E, Papageorgakopoulou N, Karamanos NK, Theocharis DA. Pig vitreous gel: macromolecular composition with

particular reference to hyaluronan-binding proteoglycans. *Biochimie.* Apr 2002;84(4):295-302.

18. Gloor BPMD. The vitreous. In: Adler FH, Hart WM, eds. *Adler's Physiology of the Eye*. Ninth ed: Mosby-Year Book, Inc.; 1992:255-276.

19. Clausen R, Weller M, Wiedemann P, Heimann K, Hilgers RD, Zilles K. An Immunochemical Quantitative-Analysis Of The Protein Pattern In Physiological And Pathological Vitreous. *Graefes Archive For Clinical And Experimental Ophthalmology.* 1991;229(2):186-190.

20. Reardon A, Heinegard D, McLeod D, Sheehan J, Bishop P. The large chondroitin sulphate proteoglycan versican in mammalian vitreous. *MATRIX BIOLOGY.* 1998;17(5):325-333.

21. Bishop P, Ayad S, Reardon A, McLeod D, Sheehan J, Kielty C. Type VI collagen is present in human and bovine vitreous. *Graefes Archive For Clinical And Experimental Ophthalmology.* Nov 1996;234(11):710-713.

22. Bidanset DJ, Guidry C, Rosenberg LC, Choi HU, Timpl R, Hook M. Binding Of The Proteoglycan Decorin To Collagen Type-VI. *Journal Of Biological Chemistry.* Mar 15 1992;267(8):5250-5256.

23. Kielty CM, Whittaker SP, Grant ME, Shuttleworth CA. Type-Vi Collagen Microfibrils - Evidence For A Structural Association With Hyaluronan. *Journal Of Cell Biology.* Aug 1992;118(4):979-990.

24. Nguyen BQ, Fife RS. Vitreous Contains A Cartilage-Related Protein. *Experimental Eye Research.* Sep 1986;43(3):375-382.

25. Ayad S, Boot-Handford RP, Humphries MJ, Kadler KE, Shuttleworth CA. *The Extracellular Matrix FactsBook*. second ed. London: Academic Press Limited; 1998.

26. Henderson M, Polewski R, Fanning JC, Gibson MA. Microfibril-associated glycoprotein-1 (MAGP-1) is specifically located on the beads of the beaded-filament structure for fibrillin-containing microfibrils as visualized by the rotary shadowing technique. *Journal Of Histochemistry & Cytochemistry.* Dec 1996;44(12):1389-1397.

27. Menasche M, Dagonet F, Ferrari P, Labat-Robert J. Fibronectin in the vitreous body - distribution and possible functional role. *Pathologie Biologie.* May 2001;49(4):290-297.

28. Esser P, Bresgen M, Weller M, Heimann K, Wiedemann P. The Significance Of Vitronectin In Proliferative Diabetic-Retinopathy. *Graefes Archive For Clinical And Experimental Ophthalmology.* Aug 1994;232(8):477-481.

29. Theocharis AD, Papageorgakopoulou N, Feretis E, Theocharis DA. Occurrence and structural characterization of versican-like proteoglycan in human vitreous. *Biochimie.* Dec 2002;84(12):1237-1243.

30. Mayne R, Liu J, Ren ZX, Mayne PM, Cook T. Genomic structure and chromosomal location of a novel extracellular matrix protein from mammalian vitreous. *Investigative Ophthalmology & Visual Science.* Mar 15 1999;40(4):S12-S12.

31. Dunker S, Kleinert R, Faulborn J. Immunohistological staining of the vitreous. *Ophthalmologe.* Jan 1998;95(1):8-12.

32. NKCF. National Keratoconus Foundation. http://www.nkcf.org/.

33. Spoerl E, Seiler T. Techniques for stiffening the cornea. *J Refract Surg.* Nov-Dec 1999;15(6):711-713.

34. Wollensak G, Sporl E, Seiler T. Treatment of keratoconus by collagen cross linking. *Ophthalmologe.* Jan 2003;100(1):44-49.

35. Kao WWY, Vergnes JP, Ebert J, Sundarraj CV, Brown SI. Increased Collagenase And Gelatinase Activities In Keratoconus. *Biochemical And Biophysical Research Communications.* 1982;107(3):929-936.

36. Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. *Current Eye Research.* Jul 2004;29(1):35-40.

37. Duquette JJ, Grigg P, Hoffman AH. The effect of diabetes on the viscoelastic properties of rat knee ligaments. *J Biomech Eng.* Nov 1996;118(4):557-564.

38. Wollensak G, Spoerl E. Collagen crosslinking of human and porcine sclera. *Journal Of Cataract And Refractive Surgery.* Mar 2004;30(3):689-695.