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Structural changes of vitreous humor under enzymatic activity: an in situ rheological study

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Structural changes of vitreous humor under enzymatic activity: an *in situ* rheological study

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Bioengineering

by

Aysan Rangchian

2020

ABSTRACT OF THE DISSERTATION

Structural changes of vitreous humor under enzymatic activity: an *in situ* rheological study

by

Aysan Rangchian
Doctor of Philosophy in Bioengineering
University of California, Los Angeles, 2020
Professor Hossein Pirouz Kavehpour, Chair

The vitreous humor is a clear gel with complex fluid characteristics that occupies more than two thirds of the eye globe volume. Recently vitreous structure and the connection between its characteristics and vitreoretinal diseases have drawn significant attention. However, the fluid properties of the vitreous gel and its degeneration remain poorly understood. Reported experimental studies are aggressive for the structure of the vitreous and can affect the results drastically. Hence a less invasive method can provide results with minimized alterations to the structure of the vitreous. In addition, a method that does not require dissection of the vitreous provides the opportunity for time dependent studies.

A better understanding of the complex structure and behavior of the vitreous gel and their changes during aging and various diseases such as diabetes can lead to improvements in current treatments. This knowledge can also help with the development of new therapeutic options, both surgical and pharmaceutical, for a variety of vitreoretinal conditions. Moreover, studying the connection between the macromolecular structure of the vitreous and its fluid properties can shed light on the pathobiology of vitreoretinal diseases, which may lead to the advancement of preventive care.

Rheological methods are commonly used to study the properties of complex fluids. However, it is not possible to avoid damages to the delicate structure of the vitreous gel using traditional shear rheological methods. The purpose of this study is to use a novel *in situ* rheological technique to measure the viscoelastic properties of the vitreous humor gel. In

addition, we quantify the impact of enzyme activity on the vitreous humor structure over time to understand the caused changes in the mechanical characteristics of the vitreous. The viscoelastic behavior of vitreous gel is due to the presence of different biopolymers in its structure. In particular, fluid properties of the vitreous are directly related to the interaction of the fluid characteristics of collagen type II and hyaluronic acid networks. We studied the effects of collagenase type II on the vitreous in comparison with a control group using the *in situ* method. Furthermore, we analyzed the behavior of each component over time in both groups using mechanical analogies. We ran statistical analyses between the two groups to compare the changes in the collagen network characteristics over time. The results of analysis from individual components are in agreement with the results on the changes within the vitreous network in its entirety.

The dissertation of Aysan Rangchian is approved.

Jeffrey D. Eldredge

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2020

To my parents and my husband

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PUBLICATIONS

Journal Articles:

- **Aysan Rangchian**, Anibal Francone, Matthew Farajzadeh, Helia Hosseini, Kelly Connelly, Jean-Pierre Hubschman, and H. Pirouz Kavehpour. "Effects of Collagenase Type II on Vitreous Humor—An In Situ Rheological Study." Journal of Biomechanical Engineering 141, no. 8 (2019): 081007.

- Helia Hosseini, **Aysan Rangchian**, Mayumi L. Prins, Christopher C. Giza, Jeffrey W. Ruberti, and H. Pirouz Kavehpour. "Probing Flow-Induced Biomolecular Interactions with Micro-Extensional Rheology: Tau Protein Aggregation." Journal of Biomechanical Engineering 148, no. 3 (2020)

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- **Aysan Rangchian**, Anibal Andres Francone, Matthew Farajzadeh, Helia Hosseini, Kelly Connelly, Jean-Pierre Hubschman, and H. Pirouz Kavehpour. "Effects of enzymes on the fluid properties of vitreous." Investigative Ophthalmology & Visual Science 59, no. 9 (2018): 5712-5712.

- **Aysan Rangchian**, Nikki L. Shirazi, and H. Pirouz Kavehpour. "Heat transfer and phase change in an impinging droplet." In APS Meeting Abstracts. 2016.

CHAPTER 1

Introduction and motivation

Vitreous is the gel like fluid in the eye globe that has many roles ranging from protecting the retinal attachment [9, 10] and maintaining the oxygen level in the eye globe [11] to refracting the light through the eye [12]. Vitreous roles and its molecular structure are covered in depth later in this chapter. Rheological properties and molecular structure of the vitreous gel provide the means to accomplish its tasks within the eye globe. The viscoelastic properties of vitreous humor are due to the presence of multiple components in its structure. In addition to the presence of water and salt in the vitreous gel, the 0.1% remaining is mainly composed of proteins, primarily collagen type II, and a network of Hyaluronic Acid. Collagen is the most abundant protein in the body that is found in different parts of the body, from soft tissues and gels to stiff parts such as bones. It is worth mentioning, collagen type II exists mainly in the cartilage and the vitreous gel.

There have been many studies on the eye to understand its structure, functions and pathology of diseases and the connection between them [8, 13, 14, 10]. Scientists have investigated anatomy of the eye starting in the eighteenths century [10, 15]. Although, vitreous humor has been studied structurally, the accepted understanding about 30 years ago was that it does not have an essential role within the eye globe [16, 10, 17]. The complex structure of the vitreous was studied and later on the focus was on understanding the fundamental of its functions within the eye globe. This lead to development of experimental investigations on the vitreous properties from different stand points such as rheological properties. Vitreous gel has also been studied as an important factor of the pathology of many vitreoretinal diseases. Understanding of vitreous can be beneficial in many aspects, improving the current substitutes for the vitreous or developing new less invasive treatments. In this chapter,

we will take a closer look at the vitreous properties as well as their connections to various vitreoretinal diseases.

1.1 Vitreous history

This mysterious clear gel has been studied for many years. Various aspects of vitreous have been studied since the eighteenth century [17]; from biological structure to fluid properties. In addition, the vitreous diseases related to the changes in its liquidity was studied [18]. Many theories have been introduced since then from multiple directions of layers introduced by Demours [19] to the fibrillar composition suggested by Bowman [20].

The structural studies continued on the vitreous gel but just more than thirty years ago some scientists and ophthalmologist believed that the vitreous gel has a complex biological structure but not many effective roles within the eye other than filling the globe and stabilizing its structure [16]. However, more in depth studies and experiments revealed many valuable roles of the vitreous humor such as regulating the intraocular Oxygen level [11]. Substituting the vitreous gel with simple solutions such as saline is effective but it has side effects. This leads to the need of developing a new substitute during the vitrectomy surgery.

1.2 Vitreous biological structure

Vitreous humor is a gel-like fluid that fills a large portion of the eye globe between the lens and the retina. There are many studies that focus on the chemical and biological characteristics of the vitreous. The anatomy of the eye and the position of the vitreous humor within the eye globe are shown in Figure 1.1. It has been shown that more than 99.9% of the vitreous is made out of water and saline with less than 0.1% collagen (type II and IV) floating in the hyaluronan network. In this section we explain the importance of the understanding of the vitreous humor gel. Moreover, we review the previous studies reported on the biological structure of the vitreous humor and its individual components [21, 22, 23, 24, 25].

Understanding the biological structure of the vitreous humor is beneficial in many ways.

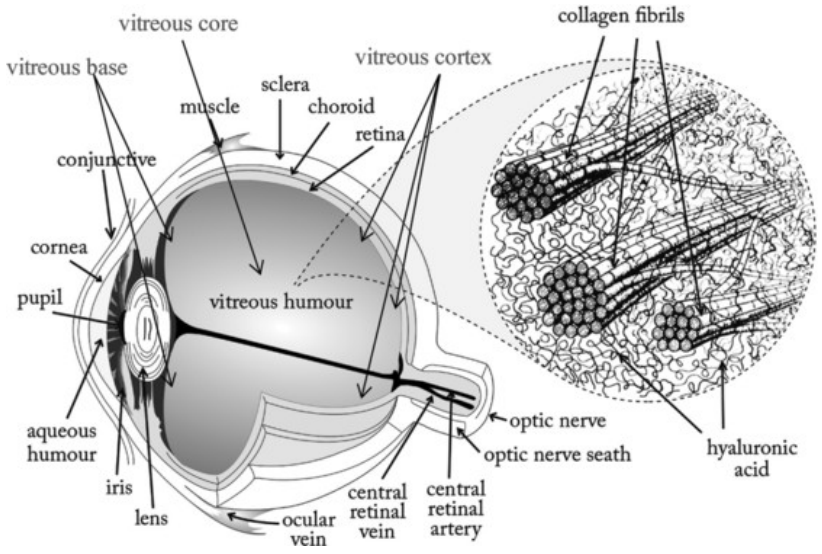


Figure 1.1: The vitreous humor structure and the network of biopolymers within. [1]

It can help to clarify the complex properties of the vitreous gel as one fluid, shed light on the roles of the vitreous within the eye globe, and introduce underlying causes of the changes in this gel. Moreover, the knowledge of the biological structure of the vitreous humor can also lead to development of substitutes with better qualities.

It is easier to predict the behavior of vitreous humor when we find out the relationship between the individual components of the gel and their roles in the overall properties of the vitreous humor. In this section we summarize the characteristics of the main components of the vitreous humor and their roles in creating the unique properties of the vitreous gel.

1.2.1 Collagen structure

Collagen is the most abundant protein in the body [26] that has a structure with three chains of polypeptides (Figure 1.2). Twenty-eight types of collagen have been discovered in vertebrates [27, 28] based on the biochemical structure and compositions of their polypeptide chains [29]. Different structural classes of collagen are present in the body such as fibrils or networks [27]. Collagen type I is the most abundant form of existence in the human body and it can be found in many human organs such as skin, muscles, bones, and connective tissues [29]. Collagen type II is found mainly in the vitreous humor and the cartilage of the

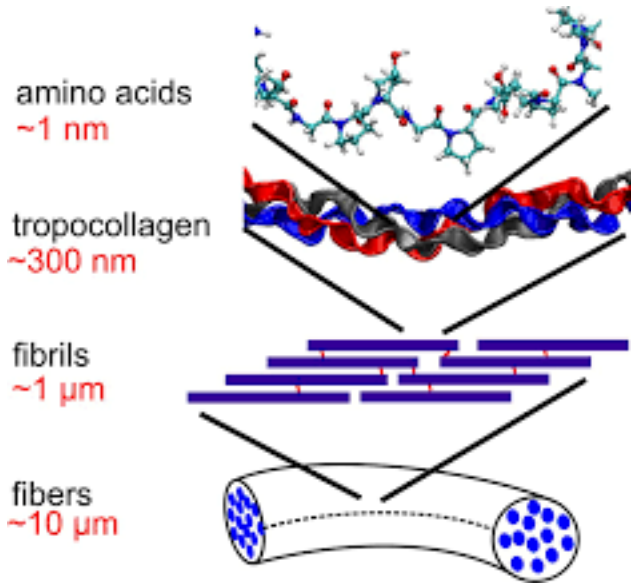


Figure 1.2: The hierarchy in the structure of collagen. [2]

knee.

1.2.2 Hyaluronic Acid structure

Hyaluronic acid or Hyaluronan is a naturally occurring polysaccharide that has significant roles in the extracellular matrix of the vitreous humor and cartilage in the human body [30]. The hyaluronic acid name was driven from the combination of a uronic acid found in the vitreous (hyaloid) [31]. Figure 1.3 shows the polymeric chain of the HA network. Since its discovery, there have been many studies on hyaluronic acid to understand its structure in detail and improve its applications. Hyaluronic acid is also used in many medical and ophthalmological applications [32, 33, 34] due to its great characteristics, particularly being biocompatible and biodegradable which makes it a very safe option [35]. Hyaluronic acid is currently used in various fields from drug delivery, carrying proteins and cancer treatment drugs, to cosmetic purposes as a filler [36]. The viscoelastic properties of this biocompatible polysaccharide makes it a great shock absorbent and carrier of different drug treatments [33, 34].

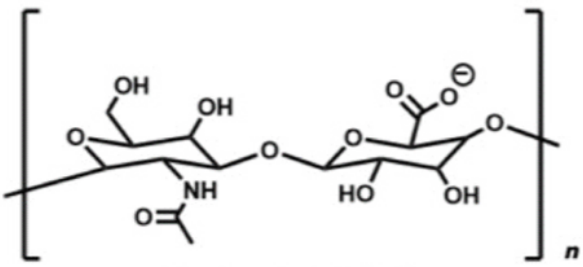


Figure 1.3: Polymeric structure of Hyaluronic Acid. [3]

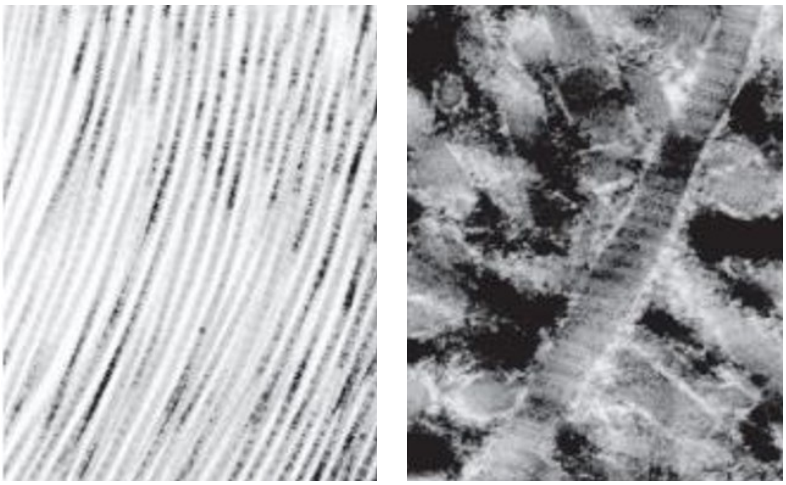
1.3 Fluid properties

Currently our understanding of the fluid properties of the vitreous is limited. The delicate network of the vitreous humor requires experiments with less damaged cause to its structure. It has been shown that there is a connection between the structure of the vitreous and its fluid properties. Also, that the fluid properties of different species are significantly different [37].

Aggressive or long period experiments can damage the structure of the network which results in altering the properties of the vitreous.[38]. Shear rheology methods have been used to study the properties of the vitreous gel have been studied [39, 24] as well as using different enzymes

1.3.1 Collagen properties

Out of all the collagen family, fluid properties are reported on collagen type I for its various applications [40, 41]. However, studies are limited on the fluid characteristics of collagen type II which is the main focus of this dissertation due to its significant role in the properties of the vitreous gel. There have been investigations using various methods on the rheological properties of the collagen type II as one of the components of the vitreous gel [24, 25]. Our biological understanding of the collagen type II structure is beneficial to predict some properties such as elasticity due to the network presence of the collagen type II. It is hypothesized that similar to the collagen type I, we should observe a viscoelastic behavior. The structural network of both collagen type I and type II are shown in Figure 1.4 investigate the complex



(a) (b)

Figure 1.4: Structural network of a) collagen type I in tendon, and b) collagen type II in cartilage. [4]

characteristics of the network of collagen type II within the vitreous humor.

1.3.2 Hyaluronic Acid properties

Hyaluronic acid plays a major role in many biomedical applications and understanding its behavior is significant to improve the use of this polysaccharide. As it was explained earlier, the biological structure of gels affects it the rheological properties, both of which are invaluable to discover the potential applications and guide us to improve the efficiency of usage. Figure 1.5 is an image of the microstrutcure of HA, where long chains are visible that affect the fluid properties of its network. Similar to other gels, hyaluronic acid has a viscoelastic behavior which has been studied extensively [42, 43]. This knowledge about one of the main components of the vitreous gel provides us with an opportunity to analyze the behavior of vitreous humor as one gel. We study the fluid properties of hyaluronic acid and its alterations over time within the vitreous humor.

Figure 1.5: Molecular structure of HA. [5]

1.4 Function and roles

Vitreous has many significant roles, developing the organ [13], providing the mechanical stability and protection of the structure [16, 17], optical role to provide a transparent medium for the light through the eye [39], and mediating the intraocular pressure and Oxygen [11].

Interruption in any of the roles of the vitreous can lead to various diseases, therefore a better understanding of the functions helps us to correctly find the cause of the disease and find ways to prevent it.

1.4.1 Developmental

The vitreous humor plays an important role in the development of the eye [44]. Coulombre showed the dependency of the size of the eye globe to the vitreous size [45] and further proved that the growth of the eye and the growth of individual cells relies on the growth of the vitreous [46]. It is also suggested that the vitreous growth leads to a tension force that prevents the folding of the neural retina [46]. It has been also shown that the retina controls the size of the eye globe by influencing the vitreous [47]. More recently, it was shown that

the injection of collagenase to the vitreous humor would induce enlargement of the size of the eye in chick embryos [48] which further clarified the importance of the vitreous humor in the development of the eye. It is believed that vitreous has more developmental functions and further studies are required to unravel more of its roles [10].

1.4.2 Protection

One of the most undoubted tasks of the vitreous is to provide the protection of the retina in case of a traumatic incident [10]. Hildling emphasized the behavior of the vitreous during both acceleration and deceleration phases of saccades movements, vitreous movement lags behind the eye wall, resulting in the markedly reduced acceleration [49] in the ox vitreous. Moreover, the role of vitreous to delay the movement of the eye in a sudden movement of the head has been shown [50] but the underlying reason was not clear. Later on, investigation of the viscoelastic properties of vitreous revealed that it is a great a shock absorbent during a head movement or injury [24].

1.4.3 Optical

The transparent vitreous gel allows the light to pass through with a high transmission rate [51] and reach the nerves on the retina to produce the image. In the 80s it was believed that transmission of the light is the primary function of the vitreous gel [52, 44]. The shaping of the eye to the exact measurements, which is the another role of the vitreous described earlier, is vital for its photo-receptive function [42]. The refraction index of the vitreous is 1.335 close to that of water [44] which considering the molecular structure of the vitreous it is expected. Low concentrations of the collagen network and HA network as well as their interaction within the vitreous are significant to keep the vitreous transparent.

1.4.4 Oxygen mediating

The oxygen solubility and its handling in vitreous substitutes have been studied for a long time [53]. Recent studies reveal that there is a gradient of the oxygen content from the back

of the lens, which is not an ideal location to have high concentration of oxygen, to higher oxygen content near the center of the vitreous humor [11]. The oxygen level is highest near the retinal vasculature and is consumed by vitreous humor, best when it is in its gel state, and other intraocular tissues in the eye [11]. In addition, follow up data from patients two years after Pars Plana Vitrectomy show that 80% of patients develop cataract problems [54]. The exact process of the cataract formation after PPV is still unclear but it is hypothesized that the vitreous substitutes can not play the role of vitreous humor in the regulation of the oxygen content with the gel.

1.5 Method of characterization

In this section we cover rheological and visualization methods to understand complex biofluid and their characteristics. Rheological methods are used to investigate the behavior of fluid. The cause of certain behavior is the underlying molecular structure of the network of fluid, we here describe the imaging techniques that can help to better understand certain behavior and the diagnostic imaging that can improve our understanding of the properties of vitreous.

1.5.1 Rheology

Understanding the characteristics of biofluids in the human body are significant from many aspects: shedding light on the subsequent changes as the body ages, clarifying the pathology of diseases, guiding us to improve treatments, and many more applications. Rheology is a method to quantify the properties of fluids. Rheology is mainly divided to bulk rheology and microrheology. Here we describe both methods and their advantages and disadvantages.

1.5.1.1 Bulk rheology

The fluid is studied as a whole in bulk rheology. The characteristics are due to the entire network of the gel. Bulk rheology provides information about the characteristics of the gel as one fluid and its reactions under different circumstances. Bulk rheology is mainly divided

to two groups based on the direction of the application of the force on the gel. Shear and extensional forces can be applied on the fluid. We explain the shear rheology in detail in Chapter 2. Shear rheology has been used to study dissected vitreous gel [55] and the changes in its structure after different enzymatic injections [25, 56]. In the shear rheology method, the vitreous is dissected outside the eye globe. In the dissection the vitreoretinal connection is damaged and after the test, the sample can not be used for future experiments. Therefore, it is not possible to run time dependent studies using a shear rheology method.

1.5.1.2 Microrheology

Microrheology is a method that provides data on the microscopic properties of material by tracing the thermal motion of the tracer particles in the substance [57]. Localized properties of fluid measured by microrheology methods lead to significant information about the behavior of the sample however, this method lacks the certainty compared to bulk rheology methods [58]. Moreover, it is important to have complete understanding of characteristics for complex fluid. Microrheological methods have been used to measure the local properties of the vitreous humor [59, 60] and to investigate the effect of intravitreal injections on the dissected bovine vitreous [61]. Using these methods, the focus is on a small portion of the vitreous as opposed to the entire structure of the gel.

1.5.2 Visualization

The physical appearance of the micro structure of complex fluid provide significant information about the characteristics of the sample. In polymeric networks, the length of the polymer chains, their bonds and the interaction with other networks can help us better understand the behavior of the samples. In addition, ophthalmologist use diagnostic imaging techniques to evaluate the state of the patient's eye. This information can provide the opportunity to relate certain diseases to the changes in the structure of the vitreous and to develop a trend for the symptoms.

1.5.2.1 Microscopic images

Different techniques are used on biopolymers depending on their structural configurations. Vitreous and its components have been studied using different microscopic methods [62, 63]. However, imaging the transparent vitreous gel has many complications. Imaging is useful but further experimental testing is required to enhance our understanding of the vitreous gel.

1.5.2.2 Ophthalmological visualization

It is very significant to understand the condition of the eye using visualization methods. Similarly, patients with vitreoretinal conditions can benefit from imaging of the vitreous. It allows the ophthalmologist to have a better understanding of the stage of the condition [64]. These observations are vital to the patients, however, these symptoms are usually after the changes occur in the vitreous hence it is not possible to understand the underlying cause of the condition.

1.6 Pathology of vitreoretinal diseases

Vitreous is directly connected to the retina, and any changes in this attachment can lead to many diseases [65]. In particular, changes in the fluid characteristics of vitreous can affect the force that is exerted on the retina and cause an uneven force to be applied on the retina. This force pulls on the retina and can lead to posterior vitreous detachment (PVD). This condition can become worse over time in addition to the aging of the eye and lead to more serious conditions such as vitreomacular traction, macular hole [25], and retinal detachment or retinal tear [66]. Studies show the direct relationship between of the pathology vitreoretinal diseases and changes in the structure of the vitreous gel, due to various reasons such as aging [65, 67]. We have studied this connection and its underlying factors in more depth in Chapter 3 and 4. As it was mentioned in Chapter 1 Section 1.2 and 1.3, the structure and fluid properties of the vitreous humor are mainly driven by the characteristics

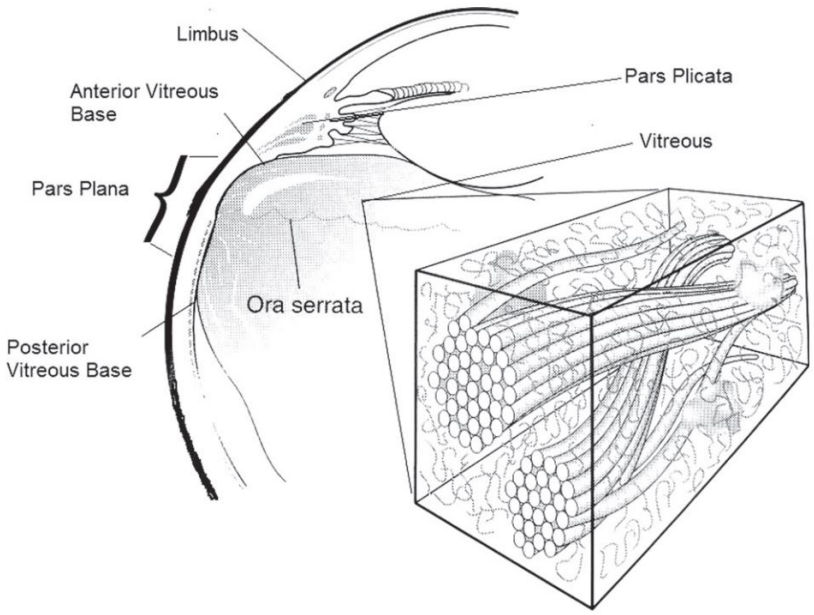


Figure 1.6: The structure of the eye and the pars plana location. [6]

of two networks of biopolymers. Any alterations in the structure of the collagen and/or Hyaluronic Acid networks lead to variations in the characteristics of the vitreous gel.

As described earlier, the rheological experiments of biopolymers reveal significant information about the mechanical and fluid characteristics of that network and its roles. Therefore, better understanding of the fluid characteristics of the vitreous humor provides invaluable information on the pathology of vitreoretinal diseases. Studying fluid properties of the vitreous and quantifying its changes due to various reasons can help us to have a better understanding of the pathology of vitreoretinal diseases [55] and to improve the treatment options. Some patients with severe vitreoretinal diseases or symptomatic PVD would require a pars plana vitrectomy surgery (PPV). During this surgery the vitreous humor is accessed and removed through an incision on pars plana. Pars plana is "flat portion" in Latin, and is shown on the anatomy of the eye globe in Figure 1.6.

1.7 Motivation

In this dissertation, our motivation is to shed light on the fluid properties of the vitreous over time using our *in situ* novel technique that does not require any dissection of the vitreous. We show the bench-marking of our technique as well as its other applications in studying the rheological properties of other unknown biofluids. We analyze the behavior of the vitreous humor as one gel over time as well as distinguishing the properties of the two main networks of biopolymer within the gel. In addition, we investigate the alterations of the vitreous humor and its two main components after the injection of active enzyme as opposed to a control group over time. This is the first study to report the rheological characteristics of the vitreous using an *in situ* method which provides the opportunity of preserving the eye globe and the vitreous is intact. Moreover, we value this study as a possible method of characterization of the potential intravitreal pharmaceutical approaches. Figure 1.7 summarises the benefits of the knowledge of biomechanics of the vitreous gel.

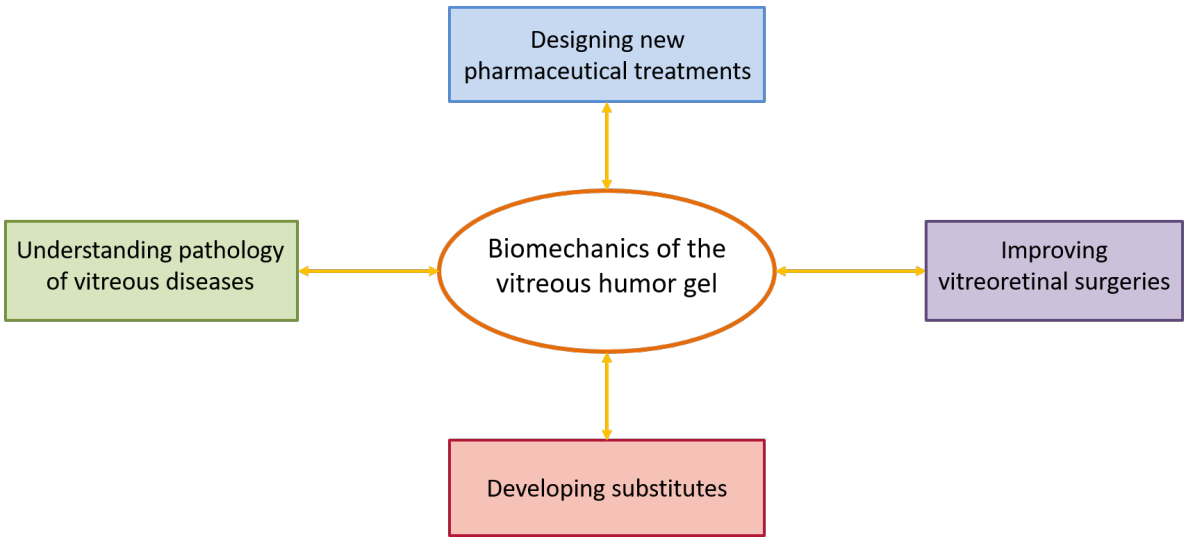


Figure 1.7: The impacts of this study range from improving the current understanding of the pathology of vitreoretinal diseases to finding and improving new pharmaceutical and current surgical treatments. Also, a better knowledge of the vitreous is helpful to improve its substitutes.

1.8 Background of this study

The fluid properties of the vitreous humor have been studied in our lab for more than a decade. We have investigated the shear rheological properties of the vitreous [24], the effects of vitrectomy cut rates on the chopped vitreous [55], and developed a novel *in situ* rheological technique to measure the characteristics of the vitreous [7]. The previous work and progress of this research in this study is shown in Figure 1.8.

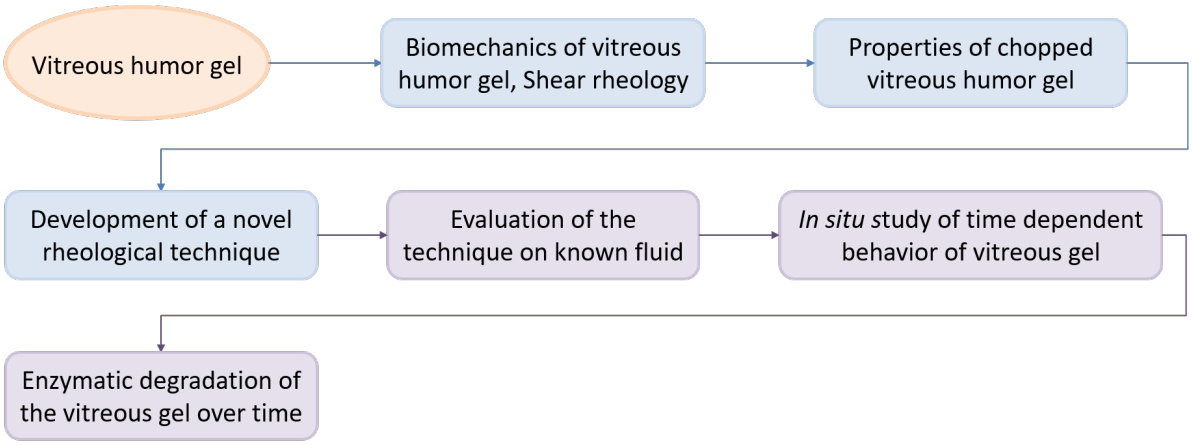


Figure 1.8: The previous work on this study is shown with blue boxes and the purple boxes show the work shown in this dissertation.

CHAPTER 2

Experimental methodology

This chapter covers a summary of the methodology of this study as well as the steps in the experimental procedure such as preparation of the samples and enzymes, the injection process, and the data collection. We also discuss the previous studies on this research in our group and the invention of the novel probe technique. Moreover, we show the data from the experiments carried out to benchmark this probe using Newtonian and non-Newtonian fluid with known rheological properties.

Changes in the mechanical behavior of the vitreous occur due to various reasons including aging and diseases such as diabetic, which may lead to many vitreoretinal diseases. The degeneration process of the vitreous has been studied theoretically as well as experimentally on the dissected vitreous samples; however, *in situ* experimental procedures with minimal disturbance to the vitreous inside the eye globe to validate the existing hypotheses are limited. Here we show the progress of rheological experiments in our group from the shear rheological studies to *in situ* experiments and time dependent studies on the vitreous.

2.1 Sample Preparation

There are differences reported between the vitreous gel of different species. The overall structure of the human vitreous gel is similar to bovine and porcine vitreous [37]. In this dissertation, we have used porcine vitreous to investigate the time dependent behavior of the vitreous as well as its changes after active enzyme injections. We have also studied bovine and rabbit vitreous samples in comparison with the porcine vitreous. Due to the size difference of the rabbit and bovine eye, we chose the porcine samples for the time dependent

studies.

2.1.1 Porcine vitreous

Fresh porcine eyes were received on the day of the experiment in an insulated box with dry ice. Excessive tissue is cleaned from the ocular globe to provide clear access for the incision. Eyes are numbered and stored separates from one another in a container to be individually hydrated with Phosphate Buffered Saline solution. When the rheometer setup is ready, we make a small triangle incisions on the eye to start the experiment. The procedure of the experiment is explained in depth in Chapter 3.

2.1.2 Collagenase type II

Lypholized powder of collagenase type II (C1764 146 SIGMA, Type II-S, 0.5–5.0 FALGPA, Sigma-Aldrich) was diluted with PBS with Calcium and Magnesium (D1283 Sigma Aldrich) to achieve a 0.7 mg/mL concentration. We store the diluted collagenase in separate 1 mL vials with a closed lid in the freezer right after the preparation. We thaw the collagenase solution before preparing the eyes for the experiment. After the initial rheological experiment, the micro syringe is used to slowly inject 50 μL of the enzyme through the incision. The needle should be exerted to the center of the vitreous. The needle was marked for precision between different samples. It is assumed that the sizes of different porcine eyes do not vary significantly.

2.2 Rheology

The fluid properties of vitreous provide a better understanding of its structure and the pathology of vitreoretinal diseases. Rheological procedures are one of the methods to shed light on the complex gels such as vitreous humor. Rheology is divided to two groups based on the region of interest: 1. bulk rheology (or macrorheology), and 2. microrheology.

In bulk rheological experiments the sample is studied as a whole. External force is applied

on the sample and the caused strain is measured. There are various method to apply the force on the sample fluid such as creep flow, frequency sweep, and continuous ramp. In traditional rheology methods where an external force is applied, the structure of biofluid may be damaged, in particular for biofluids where the networks are very fragile. In addition, generally the required volume of the sample is in the ml range which is not practical or very expensive for many biofluids.

Microrheology method is used to focus on the smaller segments of fluid. In these experiments, the Brownian movement of a tracer is studied within the sample which provides information on the localized properties of a fluid [68]. In this method, external disturbance is not applied on the fluid which can be beneficial in many bio applications[69, 70, 71], but is not useful to study the behavior of a gel as a whole.

Our novel probe technique enables us to study small volume samples with the advantage on focusing on the bulk rheology properties. It also provides us with the opportunity to run *in-situ*

2.2.1 Shear rheology

The fluid properties of vitreous gel were studied using shear rheology using frequency sweep and creep flows. As mentioned before, in a creep flow a constant torque is applied on the fluid and the caused strain is measured and creep compliance is calculated. The creep compliance is inversely related to the elasticity of the fluid. In the shear rheology of the vitreous humor, a dissection is required to extract the gel [24, 25].

The schematic of the shear rheometer and the effect on the sample are shown in Figure 2.1. As a constant external torque (T) is applied, this refers to the creep flow. We focused on one element of the sample to show the fluid relationship between the applied force and the caused alteration. The direction of the applied shear (τ_{21}) and the caused velocity profile, $v_1(x_2)$, are shown in this figure. In addition, the caused strain on the element is shown by γ_{21} which is the change of the displacement in the x_1 direction which is the direction of $v_1(x_2)$ over the change in the change in the x_2 direction. The element is shown in the bottom of

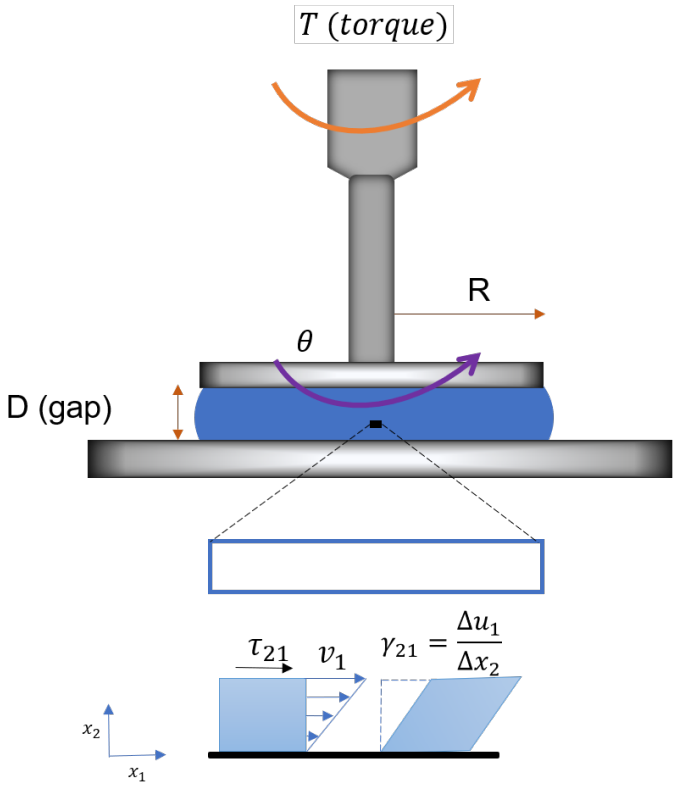


Figure 2.1: Schematic of the shear rheology. An external torque (T) is applied which caused the sample to have a rotational displacement (θ). The bottom schematic shows the effect of the applied torque on a small region of the sample.

the figure when τ_{21} is applied and its consequence.

2.3 *In situ* rheology

As mentioned earlier, shear rheology needs the sample to be completely in a transferable form as a fluid, for instance the vitreous needs to be removed from the eye globe to be examined. In addition, the smallest volume that can be measured using a 20 mm cone and plate geometry is in the order of 30 μL [72]. All the reported experimental studies on the rheological properties of vitreous are using shear rheological [73, 39, 24, 10, 74] or microrheological [37, 75, 76] methods.

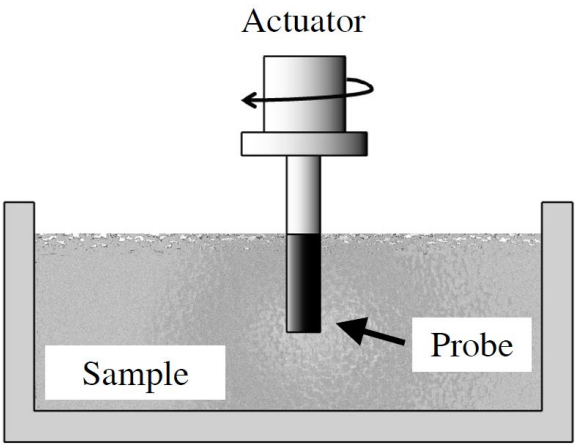


Figure 2.2: The probe like geometry is used in a container to run the validation experiments using known fluid. [7]

2.3.1 Proof of concept

We have tested different samples of known properties in both categories of Newtonian and viscoelastic behavior. In the shear rheology experiment, we used a 20 mm diameter parallel plate with the truncation gap of 57 μm as our baseline for comparison. The same samples were used with the *in situ* method where the sample is in a vial and the probe is exerted fully inside the fluid (with at least 1 mm clearance from the edge of the container) to be covered with the sample. The schematic of the experimental setup is shown in Figure 2.2.

The parallel plate and the probe are tested using creep flow. Creep curves for two different concentration of Xanthan gum are compared and shown in Figure 2.3. Each curve is the calculated average of three experiments.

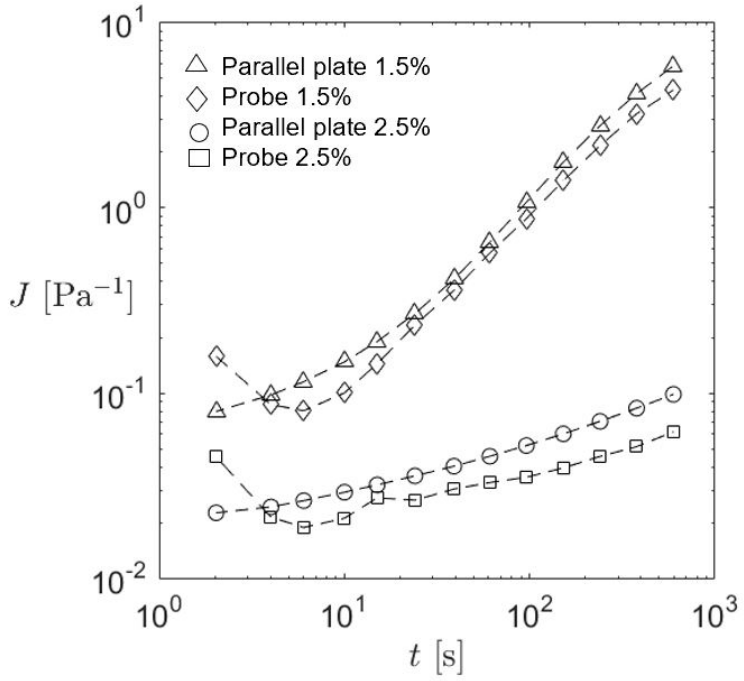


Figure 2.3: Creep curves from parallel plate and probe measurements for 1.5% and 2.5% Xanthan gum solutions. Markers represent average values calculated at selected time points from three replicates, with intermediate dashed lines linking markers. Due to rheometer software limitations the first data point is measured at 1 second, and not at shorter times which are common for creep tests. The response at early times is not reliable due to inertial effects and machine limitations. [7]

2.3.2 Vitreous samples

The samples are tested using the shear rheological probe [55] and the *in situ* technique under the creep flow. Figure 2.4 presents the schematic of the *in situ* experimental setup. We make an triangle incision on the pars plana of the porcine eye and enter the probe inside the vitreous towards the center. The incision should be large enough to avoid the contact of the probe to any tissue, as this can significantly alter the results. The details of the time dependent study and the injection protocols are covered in chapters 3 and 4.

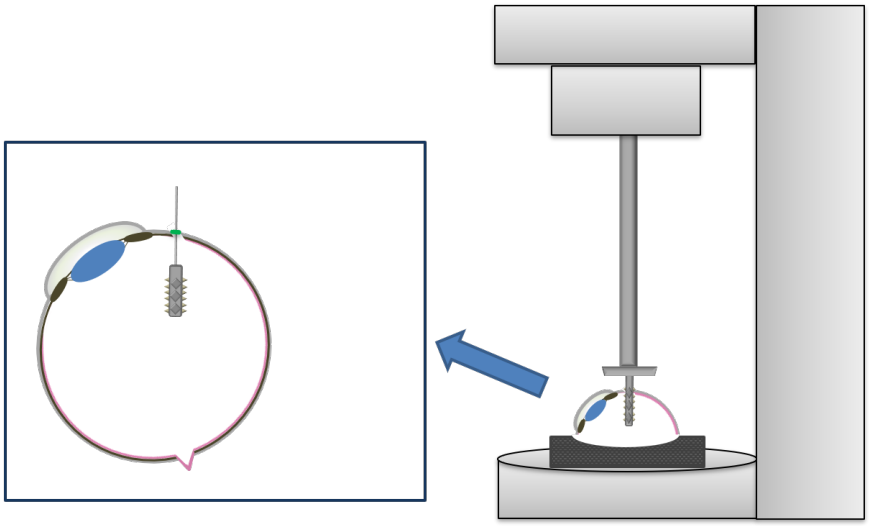


Figure 2.4: The schematic of the setup of *in situ* experiment with the eye secured in the mounting block. The probe is inserted fully in the eye through the incision.

2.4 Mechanical analogies

The fluid properties of complex fluid can be very complicated, particularly if there are more than one network of biopolymers present in one fluid. Mechanical analogies are commonly used to model the behavior of various biopolymeric gels [8]. Figure 2.5 shows the shear creep and creep recovery for a viscoelastic liquid sample where with a constant stress applied (i.e., creep), eventually the sample shows liquid like behavior with a increase with constant rate in the strain of the sample. An example for this behavior would be an uncrosslinked polymeric network with no permanent attachments [77].

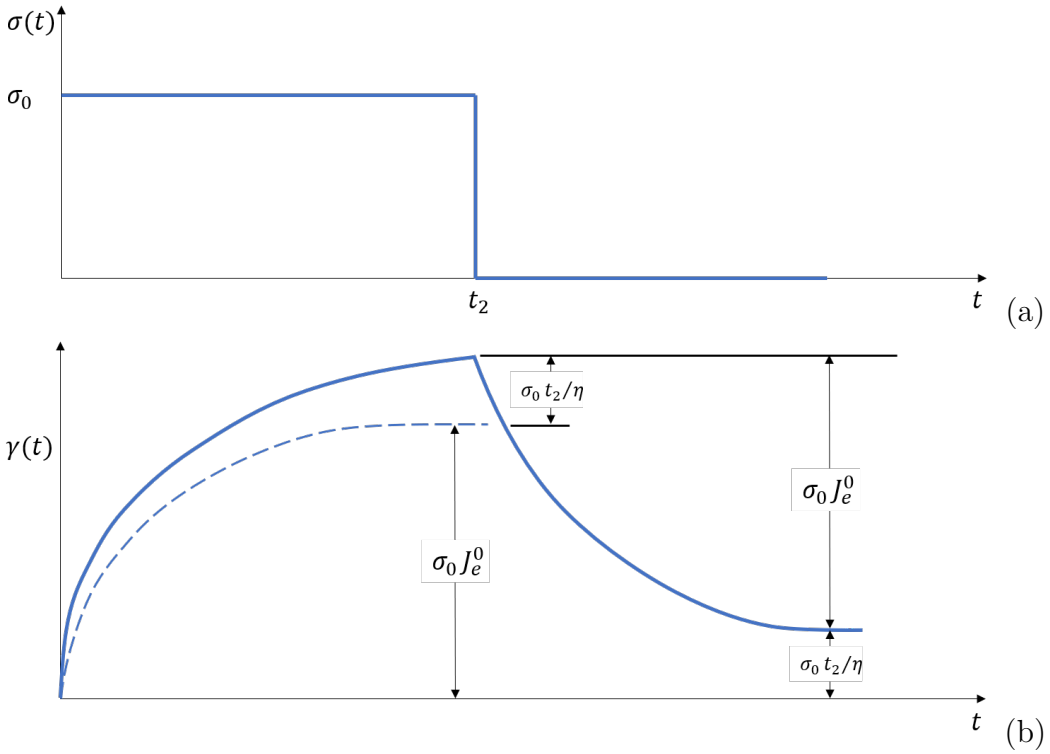


Figure 2.5: General curves for a) stress and b) strain of creep test for a viscoelastic liquid polymer gel. Replicated from [8]

Another common behavior seen in the polymeric networks is the solid like behavior where after a constant stress is applied, the fluid reaches an equilibrium compliance where the caused strain does not change (Figure 2.6).

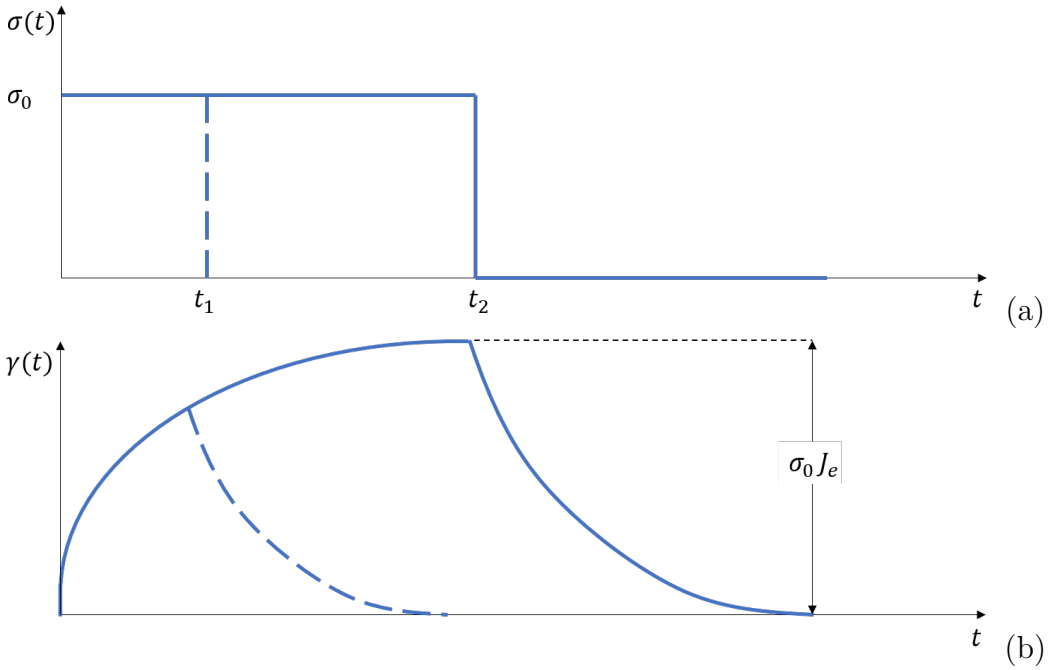


Figure 2.6: General curves for a)stress and b)strain of creep test for a viscoelastic solid polymer sample. Replicated from [8]

2.5 Enzymatic digestion

In this section we introduce the method of characterization in this study. The characteristics of each one of the main components of the vitreous are studied over time. The schematic in Figure 2.7 demonstrates the alterations over time after the collagenase type II injection.

We examined thirty-eight porcine eyes using in situ rheological creep tests to measure the mechanical properties of the vitreous humor of the eyes prior to, 1 hour and 24 hours after the intravitreal injection. Eyes in one group were injected with collagenase type II solution and eyes in the control group were injected with phosphate buffered saline solution (PBS) with calcium and magnesium chloride. Prior to the injection, viscosity and creep compliance intercept values between both groups were not statistically different. At 1 hour and 24 hours after the injection, vitreous properties in the eyes from the first group showed a statistically significant increase in the J intercept values (representing the inverse of elasticity) compared to the control group. In addition, 1 hour and 24 hours after the injection, the viscosity of vitreous in the eyes from the first group was lower than the values from the control group.

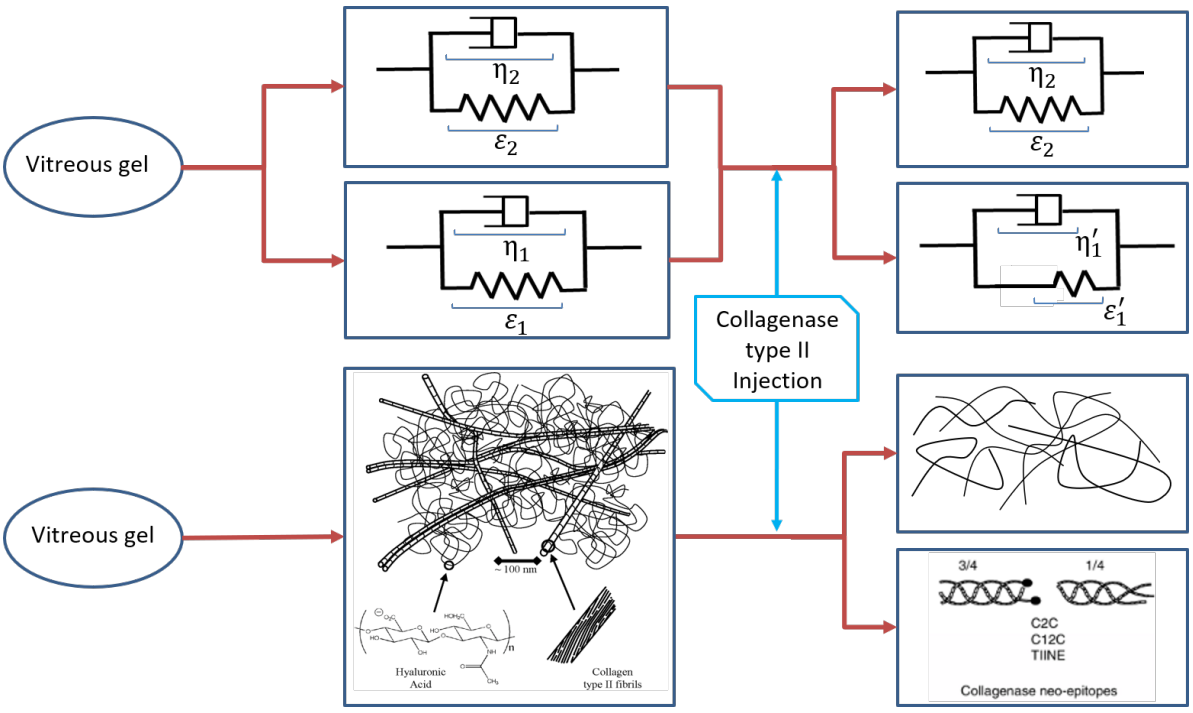


Figure 2.7: Schematic of the two main networks of biopolymers in the vitreous humor gel. The collagenase injection is used in one group to study the caused changes on the vitreous gel properties.

These findings are a foundation for future studies on the effectiveness of intravitreal drugs that modify the mechanical properties of the vitreous humor.

2.6 Fitting

In this section we cover the fitting criteria for the creep curves. An example of a typical creep curve using the *in situ* technique is shown in Figure 2.8. A linear fit is applied after the steady state is achieved. The J-axis intercept of the fitted line provides us with the value of the creep compliance intercept (i.e., elasticity of the gel), and the inverse of the slope of this line is the viscosity of the vitreous.

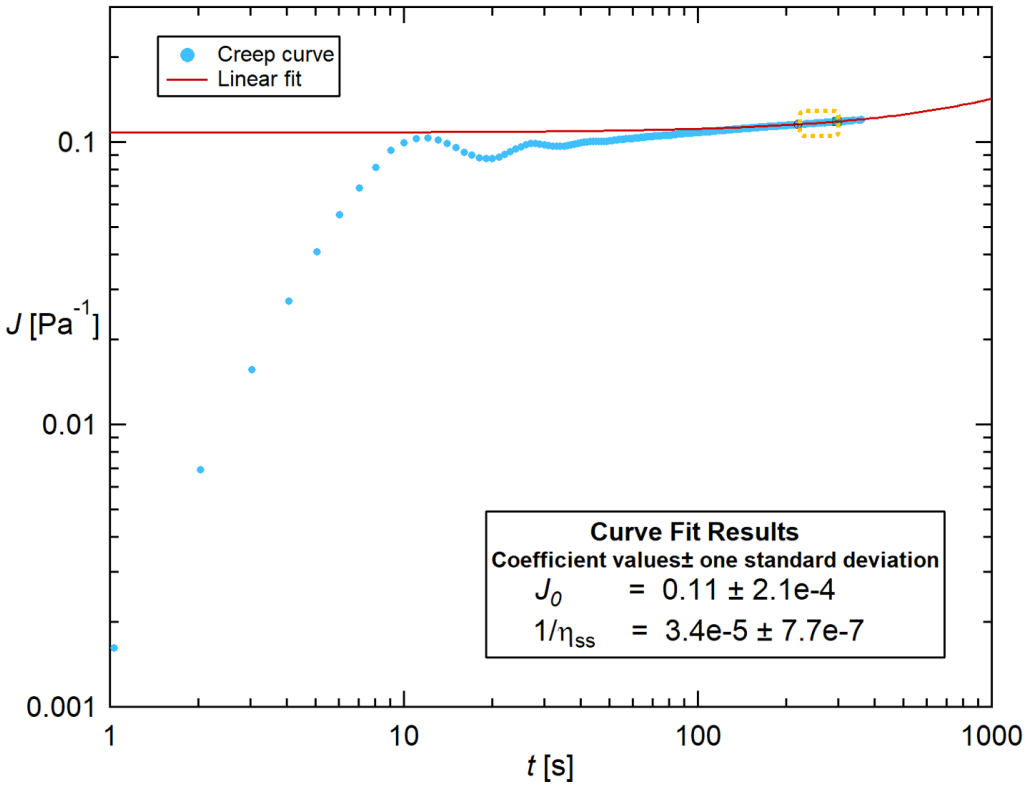


Figure 2.8: The region of interest is shown with the yellow dotted box. A linear fit is used on the shown region of the creep curve for each eye to find the steady state viscosity and the creep compliance intercept.

2.7 Acknowledgement

Sections of this chapter was published previously in our lab [7]. The author would like to thank the authors for the material.

CHAPTER 3

Molecular structure

The purpose of this chapter is to quantify the impact of enzyme activity on the vitreous humor structure over time to understand the mechanical characteristics of the vitreous humor gel. Changes in the mechanical behavior of the vitreous occur due to various reasons. Aging is one of the main reasons which can lead to vitreoretinal diseases. The degeneration process of the vitreous has been studied; however, *in situ* experimental procedures to validate the existing hypotheses are limited. We examined thirty-eight porcine eyes using *in situ* rheological creep tests to measure the mechanical properties of the vitreous humor of the eyes prior to, 1 hour and 24 hours after the intravitreal injection. Eyes in one group were injected with collagenase type II solution and eyes in the control group were injected with Phosphate Buffered Saline solution with calcium and magnesium chloride. In this chapter, we explain the method of our test in depth as well as analyzing the collected data. We report the first time dependent behavior of the vitreous gel and its alterations after active enzyme injection. As it was noted earlier, two main rheological properties are the viscosity and elasticity of any viscoelastic fluid. In this chapter we show both of the mentioned properties of the vitreous humor.

Prior to the injection, viscosity and creep compliance intercept values between both groups showed no statistically significant difference. At 1 hour and 24 hours after the injection, vitreous properties in the eyes from the first group showed a statistically significant increase in the J intercept values (representing the inverse of the elasticity) compared to the control group. In addition, 1 hour and 24 hours after the injection, vitreous viscosity was lower in the eyes from the first group than in the eyes from the control group. These findings provide a foundation for future studies on measuring the effectiveness of intravitreal drugs

that modify the mechanical properties of the vitreous humor.

3.1 Introduction

Vitreous is the gel-like fluid inside the ocular globe of the eye. Vitreous is important in various physiological processes such as embryological development, optical transmission and mechanical support [39, 16, 15]. Viscoelastic characteristics of the vitreous gel has a significant role in many of its functions in the eye [78]. For example, the gel provides an excellent protection for the ocular tissues [16, 24, 13, 25], and maintains the lens transparency by providing a gradient of Oxygen concentration (i.e., lower concentration towards the center of the vitreous) [79, 80, 81, 10, 11].

Many vitreoretinal diseases are related to the changes in the viscoelastic properties of the vitreous. Therefore, changes in the network structure of the vitreous (e.g., age related degeneration) or removal of it (i.e., vitrectomy) [82, 83, 14] can cause oxidative and structural damages to the center of the lens resulting in opacification [81]. In the study of the movement and structure of the vitreous, Hildling emphasized the behavior of the vitreous during both the acceleration and the deceleration phases of saccadic movements; vitreous movement lags behind the eye wall, resulting in markedly reduced acceleration [49]. It is suggested that the adhesion of the vitreous internal structure to the sclera [49] and its viscoelastic properties [44, 25] cause the lag in the movement. Movement of the eye can also provide information about the significant compartment variations in the vitreous network [84].

Pathogenesis of many vitreoretinal diseases are related to the changes in the fluid properties of the vitreous. Therefore, many authors study the vitreous dynamic by focusing on the movement of the vitreous *in vivo* through various imaging techniques [84, 85, 86], where the velocity field of the vitreous was used to model the viscosity and elasticity of the vitreous gel. The macromolecular structure of the vitreous is nearly 99.9% water and salts (by weight percent, wt%), and is less than 0.1 wt% heterotypic collagen fibrils [23, 24].

The network of heterotypic collagen fibers (II, V/XI and IX) and proteoglycans are embedded within a network of hyaluronan [23, 63]. A number of recent studies have suggested

that the viscoelastic features of the vitreous are associated with its macromolecular structure [55, 25]. Similar to many viscoelastic biomaterial and gels [57], vitreous humor dissipates and stores energy under applied stress. The exact relationship between vitreous macromolecular structure and its mechanical properties remains unknown [25]. Given that the viscoelastic properties of the vitreous are mostly unknown, a more direct measurement method is required to quantify these properties. It has been shown previously that rheological experiments can reliably measure the mechanical characteristics of fluid, in particular the vitreous [16, 24, 15, 7].

Rheology is the study of flow behaviors, and rheological experiments provide specific information on the characteristics of fluid [87]. Previous rheological studies have used creep flow [24, 25, 23, 63, 57, 7] and oscillatory flow [39, 24, 25] to quantify vitreous humor properties. Studies recommend that many vitreoretinal diseases such as Posterior Vitreous Detachment (PVD) occur as a result of changes in the rheology of the vitreous gel [24, 25, 65]. However, the role of the vitreous in a variety of vitreoretinal diseases is still not well understood [88]. Better understanding of the rheological properties and behavior of the vitreous gel may lead to improved or new therapeutic approaches. The novel technique developed by our group enabled us to measure rheological properties of porcine vitreous *in situ* [7]. In this method, no dissection is required and the vitreous network is preserved. A cylindrical probe enters the eye globe through a small incision. Previously, the porcine and bovine eyes were tested using this method [7].

The objective of this study is to determine the effect of the collagenase type II on the structure of the vitreous humor. The change of structure is monitored through *in situ* measurement of the viscoelastic properties of the vitreous fluid at different times. The measured properties are then compared to that of a control experiment where the eyes are injected with PBS.

3.2 Methodology of the *in situ* technique

In this section we discuss details of the experimental setup to measure the rheological properties of the vitreous as in its entirety. We also cover the mechanical analogies used in our model as well as the fitting criteria. The results of the statistical analysis are also discussed extensively.

3.2.1 Experimental preparation

Thirty-eight porcine eyes were assigned to either the study group (n=27, group 1) or the control group (n=11, group 2). During the entire procedure, the eyes were kept hydrated in a container and were surrounded by diluted Phosphate Buffered Saline solution (PBS) with Calcium and Magnesium Chloride (D1283 Sigma-Aldrich), to prevent evaporation of the water content of the vitreous during the experiments. The specimens were transported on ice to the lab less than 6 hours after enucleation and were prepared for the experiment immediately at room temperature. Experiments were done using a stress-controlled shear rheometer (TA instruments, AR-2000) using our patented probe [7]. Using surgical tools, a triangle shaped incision with a base of around 5 mm and height of 2 mm, was made through the sclera on the pars plana to allow the rheometer's probe (0.86 mm in radius) to enter into the vitreous cavity, the schematic of the eye during the experiment is shown in Figure 3.1.

3.2.2 Rheological experiment

Each eye was placed on a 3D printed block (Figure 3.2. a & b) and the probe was inserted through the incision (Figure 3.2. c). Lyophilized powder of Collagenase type II (C1764 SIGMA, Type II-S, 0.5-5.0 FALGPA, Sigma-Aldrich) was diluted to a concentration of 0.7 mg/mL in PBS with Calcium and Magnesium Chloride. The eyes were injected (through the incision on the vitreous cavity) with 50 μL of collagenase in the first group and 50 μL PBS in the second group. The eyes were tested by a 6 minute long rotational creep flow test with a constant torque of M=1.0 μNm before the injection (T_0), 1 hour (T_1) and 24

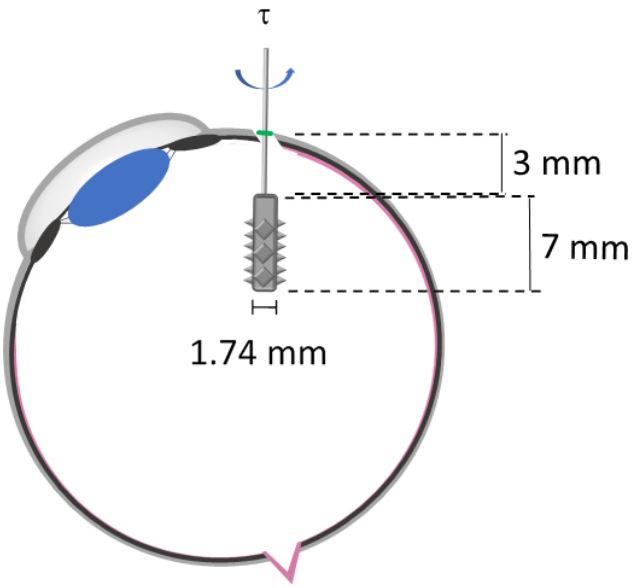


Figure 3.1: Schematic of the eye during the *in situ* rheological test probe towards center. The roughened end of the probe has a 0.86 mm radius. We insert 10 mm of the probe into the eye. This ensures that the roughened part is fully in the vitreous and the angle is towards the center of the vitreous.

hours (T_{24}) after the injection. The probe was inserted close to the injection site to carry out the measurements. Collagenase and PBS solutions, both water-based solutions, are injected slowly in small volumes using a micropipette. This ensures that the injected fluid mixes with the vitreous main solvent which is water (more than 99%). There are no large size molecules in the injected fluid; therefore, the rheology of the vitreous is not affected. Eyes were stored at 4°C after the 1 hour tests and removed about 15 minutes before the T_{24} creep test to equilibrate to the room temperature of 20°C. This procedure was consistent for all the eyes in the experiment.

3.2.3 Creep test

In a creep test, a constant torque is applied (Figure 3.3.a) and the angular displacement of the flow is recorded [77]. In a theoretical creep curve, viscosity is derived from the slope of the fitted line on the curve at the steady state region (i.e., liquid state), shown in Figure 3.3.b in Equation 3.1:

$$\mu = \tau/\dot{\gamma} \tag{3.1}$$

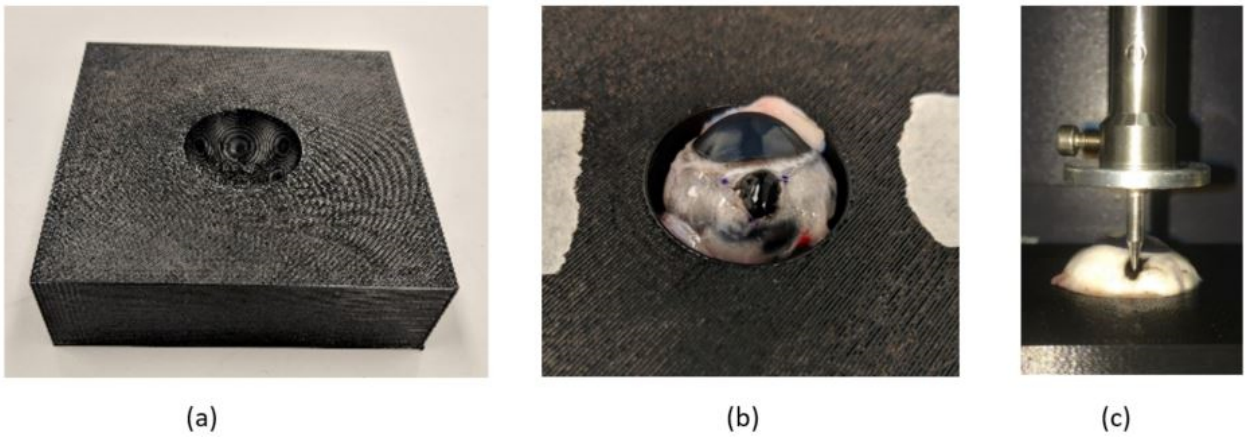


Figure 3.2: a) The mounting block with a half sphere cut out (13 mm radius) which was 3D printed to keep the eye in place and prevent unnecessary movements, b) The location of the incision is consistent for all of the specimens, the eye is secured in the 3D printed mounting block and the block is taped to the surface of the rheometer, c) The rough surface of the probe is fully inserted in a porcine eye with minimal interaction with retina or sclera.

where $\dot{\gamma}$ shows the rate of strain caused by the shear stress, τ, applied on the fluid.

Creep compliance is an indicator of the instantaneous elasticity of the fluid and is defined as:

$$J = \gamma/\tau \tag{3.2}$$

where γ is the strain and τ is the shear stress applied on the sample. The intercept of the fitted line on the creep curve with the y-axis is referred to as the creep compliance intercept, $J_s(0)$ [1/Pa], that is used as an elasticity parameter and in this study is one of the important features to describe the viscoelastic behavior of vitreous. More details can be found in the previous rheological studies using the creep test [24, 25, 7].

3.2.4 Data analysis

Igor Pro software is used to find the fitted line in the steady state region of the fluid from which viscosity and creep compliance values are extracted and reported in the result section. An unpaired t-test is performed on the viscosity and creep compliance intercept values when comparing the two groups. However, a paired t-test is carried out when comparing the results of different time points from one group. P-values are reported and $P<0.05$ indicates

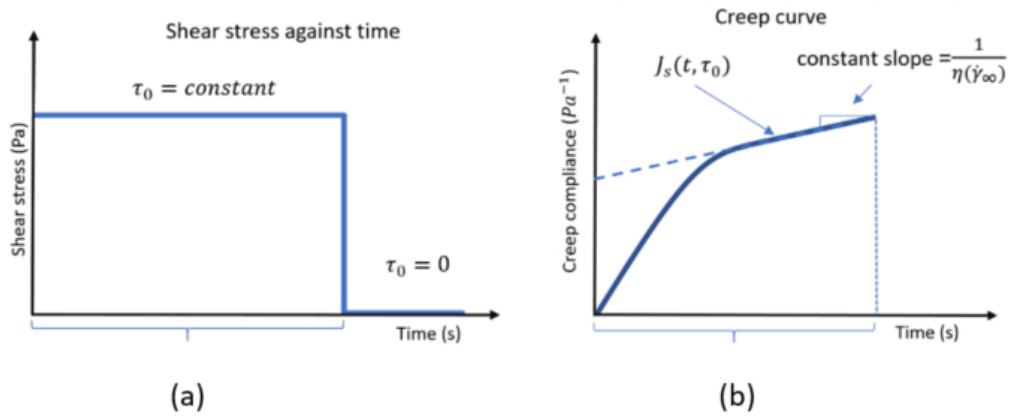


Figure 3.3: a) Graphical representation of a creep test where a constant shear stress (τ) is applied to the sample for a constant period of time (t), during the experiment, b) Representation of a typical theoretical creep curve, which shows the creep compliance over time. J_s at t=0 s shows the intercept of the steady state region of the sample with the y axis indicating the elasticity of the sample. In addition, the slope of the steady state part of the graph is inverse of the viscosity (μ) of the sample in the steady state.

the significant statistical differences between any of the two groups in the comparison. R-studio software is used to calculate the p-values and plot the results.

3.3 Creep curves for vitreous as one component

Creep curves are the reported output of each creep test in this study, similar to the previous *in situ* study of the vitreous [7]. Comparison between a typical creep curve for one eye from each group is shown in Figure 3.4 before, and 24 hours after the intravitreal injection. The linear segment of the creep curve indicates the steady state region of the flow. The change in the slope of the linear segment of the creep curve for group 1 from T_0 to T_{24} is significant. Group 2 creep curves change only minimally over the same period of time. The mean and standard deviation values are reported in Table 3.1 for each group at each time point.

3.3.1 Creep compliance intercept

The creep compliance intercept values (J_s) of both groups are compared at every time point (group 1 vs group 2) as well as within each group over time (Figure 3.5). The details of the statistical analysis between the two groups are shown in Table 3.2. There is no statistically

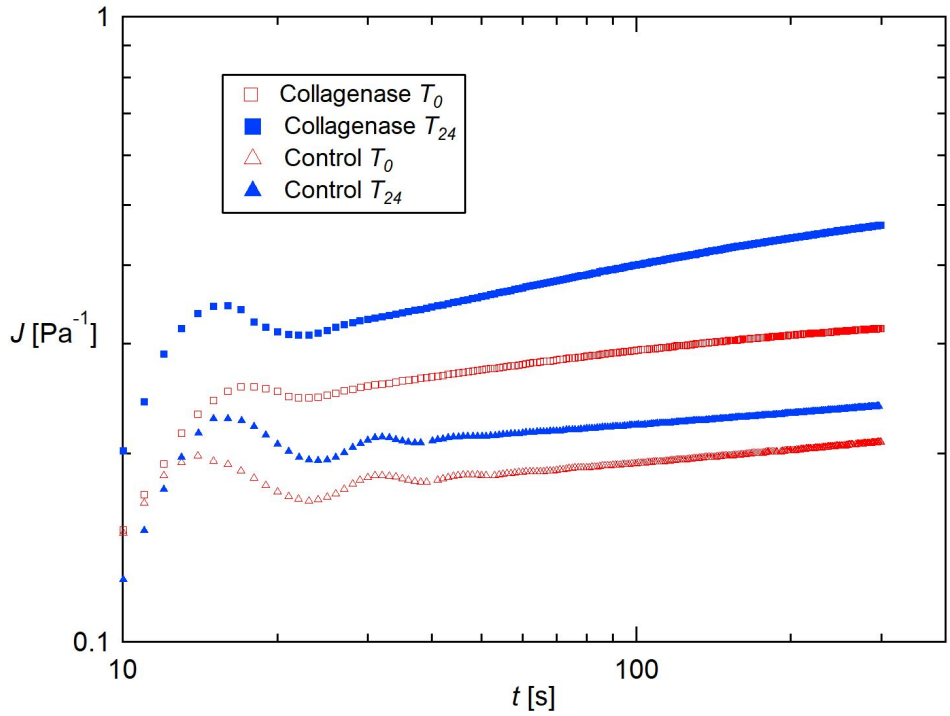


Figure 3.4: Examples of the creep compliance curve for one eye from each group (indicated with Collagenase or Control) before the injection (T_0), and 24 hours after the injection (T_{24}).

Table 3.1: The mean and standard deviation values of viscosity and creep compliance intercepts for both groups

Labels		**Creep compliance intercept**		**Viscosity**	
Injection	$t(h)$	**Mean**	**Standard deviation**	**Mean**$\times 10^3$	**Sd**$\times 10^3$
Collagenase	0	0.196	0.068	14.5	4.95
Collagenase	1	0.326	0.107	10.1	5.07
Collagenase	24	0.715	0.361	6.96	4.46
PBS	0	0.160	0.081	16.8	6.02
PBS	1	0.150	0.071	21.6	10.5
PBS	24	0.207	0.091	14.3	4.07

significant difference between the values of creep compliance from group 1 versus group 2 before the injection (with mean of 0.196 [Pa^{-1}] vs 0.160 [Pa^{-1}] respectively). One hour after the injections, creep compliance values are significantly different between group 1 and 2 ($P< 2.4 \times 10^{-6}$). The same trend is observed 24 hours after the injection and the result of the unpaired t-test is $P< 1.0 \times 10^{-7}$ with the average creep compliance intercept values of 0.715 [Pa^{-1}] and 0.207 [Pa^{-1}] for group 1 and group 2 respectively.

Data comparison within each group from T_0 to T_1 indicates that the change in the J_s values of group 1 is statistically significant (Table 3.3), however the changes in the group 2 values are not considerable as shown in Table 3.4. The average J_s in group 1 increased from 0.196 [Pa^{-1}] to 0.326 [Pa^{-1}] with a p-value less than 1.3×10^{-5}, compared to group 2 where the average decreased from 0.160 [Pa^{-1}] to 0.150 [Pa^{-1}] over the same period of time (p-value is around 0.6). Twenty four hours after the injection the J_s increases in both groups, however the change is only significant in group 1.

3.3.2 Viscosity

At T_0 viscosity values are not significantly different between the two groups (with mean values of 14.5×10^3 [Pa.s] vs 16.8×10^3 [Pa.s]). However, the statistical results reported in Table 3.2 shows that the viscosity values of the two groups are significantly different from one another 1 hour and 24 hours after the injection. Table 3.3 indicates the statistically significant decreases in group 1 between each two time points (e.g., T_0 vs T_{24}) individually. The same comparison on group 2 data showed no statistically significant changes for the data over time, except for T_0 vs T_1 which had a P-value just under 0.05 (Table 3.4). Figure 3.6 indicates the changes in the viscosity values for each group at every time point, where the overall trend within each group is shown as well as in comparison with the other group.

3.3.3 Vitreous humor statistical analysis

Exact P-values for the comparisons between the groups are reported in Table 3.2. For each eye the steady state values of the viscosity and creep compliance intercept are used.

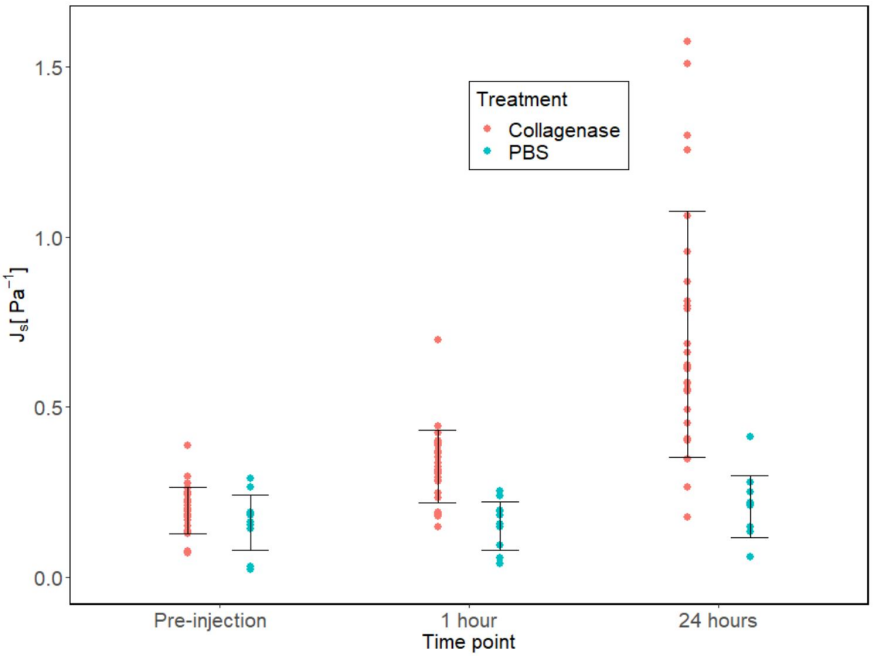


Figure 3.5: Comparison of compliance intercept values for the porcine eyes injected with PBS, Collagenase and no injection over time, Standard deviation of the values are shown with the bars. The differences between the collagenase injected eyes at 1 hour compared to the values of the same time from the PBS injected eyes is statistically significant (with the mean values of 0.326 [Pa^{-1}] compared to 0.160 [Pa^{-1}]). The same holds for the difference between the groups at 24 hours (with the mean values of 0.715 [Pa^{-1}] for group 1 and 0.207 [Pa^{-1}] for group 2). The eyes in group 1 show significant increases from T_0 to T_1, and T_1 to T_{24}. In group 2 the value decreases from T_0 to T_1 and increases from T_1 to T_{24} and none of the changes are statistically significant.

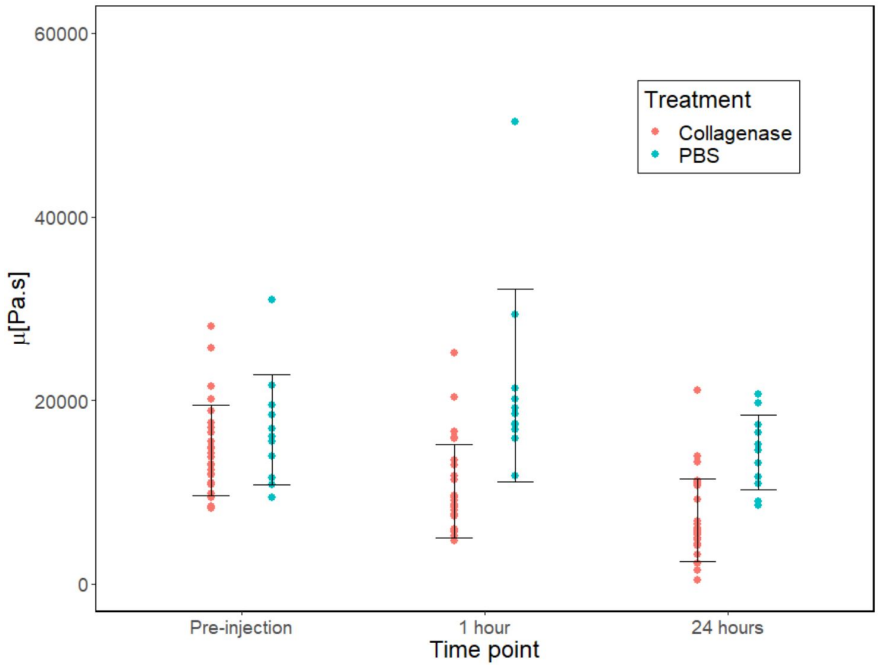


Figure 3.6: Comparison of viscosity values for the porcine eyes injected with PBS versus Collagenase over time. Standard deviation of the values are shown with the bars. The differences between the collagenase injected eyes at 1 hour compared to the values of the same time from the PBS injected eyes is statistically significant (with the mean values of 10.1×10^3 compared to 21.6×10^3). The same holds for the difference between the groups at 24 hours (with the mean values of 6.96×10^3 for group 1 and 14.3×10^3 for group 2). The eyes in group 1 show significant decrease from T_0 to T_1, and T_1 to T_{24}. The values in group 2 do not present a constant trend.

Table 3.2: Unpaired t-test results for creep compliance and viscosity of the vitreous in both groups over time

Properties	**Pre-injection**	**1 hour**	**24 hour**
Creep compliance intercept (J_s)	0.21	2.4×10^{-6}	1.0×10^{-7}
Viscosity (μ)	0.29	4.5×10^{-3}	8.3×10^{-5}

Table 3.3: Paired t-test results for creep compliance and viscosity values of the vitreous in group 1 over time

Properties	T_0 **vs.** T_1	T_0 **vs.** T_{24}	T_1 **vs.** T_{24}
Creep compliance intercept (J_s)	1.3×10^{-5}	2.0×10^{-7}	8.2×10^{-6}
Viscosity (μ)	1.4×10^{-3}	1.3×10^{-6}	6.4×10^{-3}

Therefore, each eye has three viscosity and three creep compliance intercept values. The unpaired t-test compares the values from all the eyes from group 1 versus all the eyes in group 2 at each time point, Table 3.2. The paired t-test is used to compare the changes of the values over time within each group. As discussed earlier, values from different time points within each group are compared over time and reported in Table 3.3 and 3.4.

Table 3.4: Paired t-test results for creep compliance and viscosity values of the vitreous in group 2 over time

Properties	T_0 **vs.** T_1	T_0 **vs.** T_{24}	T_1 **vs.** T_{24}
Creep compliance intercept (J_s)	0.6	0.10	0.08
Viscosity (μ)	0.07	0.3	0.06

3.4 Vitreous changes due to the enzyme activity

Previous studies have investigated the vitreous gel from many aspects including but not limited to its appearance [13], molecular structure [23], and biochemistry [89, 31]. In addition, recent studies have focused on the potential effects of changes in the vitreous characteristics on the vitreoretinal diseases [10, 82, 14, 67, 90]. However, the limited number of studies on the viscoelastic behavior [37, 75, 76, 73, 24, 7, 91] of the vitreous minimizes our ability to understand its relationship to the pathology and pathobiology of many vitreoretinal diseases.

As previously mentioned, rheology is a suitable method to quantify the fluid properties of the vitreous gel. To be more precise, rheological studies can measure bulk or molecular level of flow behaviors. Generally, rheology refers to measurements of the bulk fluid behavior instead of the molecular level which is known as microrheology. Previous rheological experiments studied the mechanical features [39, 24, 78, 74] of the vitreous and the effects of enzymes on vitreous properties by parallel plate setup [25] on a low number of samples. In a parallel setup, the vitreous is extracted from the vitreous cavity and the vitreoretinal connection is broken. After this connection is broken, the vitreous starts to dehydrate, and major alteration to the structure happens quickly that might alter the captured rheological data. In addition, the volume of each sample varies due to the dissection which may impact the accuracy and reliability of the results [24, 25, 7].

Microrheology is a method that provides data on the microscopic properties of material by tracing the thermal motion of the tracer particles in the substance [57, 68]. Microrheology measures the local properties and has many advantages [57, 92] due to its setup but lacks the certainty provided by bulk rheological methods [58]. There is ongoing research in this field to improve this method and to increase its accuracy [57, 93, 94]. Microrheology is used to measure the mechanical properties of the dissected vitreous humor [59] but this method provides less precise information. Similar to the parallel plate setup, microrheology requires the vitreous to be extracted from the eye or a full perpendicular cut.

The results reported in this *in-situ* study, provide a better understanding of the variations of the mechanical properties of the vitreous structure due to the injection of collagenase type

II in comparison with PBS injection over time. Alteration to the network of the vitreous structure is minimized as there is no need for a dissection. It is noteworthy that, the vitreous structure is primarily comprised of collagen type II [39, 24, 25, 56]. Evidence suggests that the presence of collagen type II bundles may be a sign of the vitreous liquefaction [95, 96]. Therefore, it is crucial to quantify the effects of the collagen type II degradation in the vitreous mechanical characteristics, which can be achieved by collagenase type II injection.

The data evaluations show that the slope of a typical creep curve of group 1 is steeper and that the intercept of the line with the J-axis is greater 24 hours after the injection compared to the curve prior to the injection (Figure 3.4). This result suggests that the viscosity and elasticity of the vitreous in this group has decreased. The common creep curve of group 2 is reported before the injection and 24 hours afterwards (Figure 3.4). There are no significant changes in the slope of the steady state region of the creep curve or the J-axis intercept. Hence, the viscosity and elasticity of the vitreous are not significantly changing after the PBS intravitreal injection. The reported changes in group 2 might be due to the degradation of the vitreous over time. In addition, PBS diffuses over time which might lead to a decrease in the viscosity. These changes are not significant over time and in comparison with group 1.

It is hypothesized that vitreous mainly owes its elastic behavior to the collagen in its network [15]. The increase in the creep compliance intercept for group 1 indicates the decrease in the elasticity of the vitreous (Figure 3.5). Collagenase breaks the peptide bonds that exist in the collagen content of the vitreous. The reduced number of bonds in the chemical structure provides a network that is easier to deform which leads to a decrease in the elasticity of the flow. The results in this study are aligned with the expectations of the biochemistry.

The viscosity values were not significantly different between the two groups at T_0. The values decreased significantly in group 1 compared to group 2 over time (Figure 3.6). This result suggests that collagen fibrils have an important role in establishing the viscous behavior of the vitreous.

In addition to the comparison between the groups, the values for each group are also evaluated individually over time. P-values of the statistical analysis indicate no significant variations in the viscosity and creep compliance intercept values of group 2 over time (Table 3.4). By contrast, in group 1 the changes are statistically significant after the injection in comparison with the pre-injection results. The difference in values between one hour and 24 hours after the injection is also statistically significant. The exact trend of variations is not the focus of this study.

In this study, it is assumed that the evaporation of the vitreous content was kept to a minimum by keeping the eyes in a closed insulated box and in diluted PBS. Therefore the effects of dehydration did not significantly change the results, as confirmed by the values from group 2. The overall results from both groups verify the hypothesis of the expected alterations on the vitreous content due to the injection of collagenase type II compared to PBS.

Further studies can lead to new information on the extracellular matrix and mechanical properties of the vitreous humor after different enzymatic degradation can lead to improved diagnostic and treatment tools. In addition, it could expand the new era of intravitreal drug delivery [96] and might potentially prevent the need for surgical treatments in some cases.

3.5 Conclusion

We studied the changes caused by collagenase type II on the vitreous network structure and its effects on the mechanical behavior of the vitreous network over time in comparison with a control group (PBS injected). The analysis showed a significant decrease in the elasticity of the vitreous which could be the result of the active enzyme degradation on the collagen component within the vitreous network.

Future studies can use the same method to understand the effectiveness of other active enzymes on the vitreous network that can be helpful in further understanding the macromolecular structure of the vitreous and its potential role in some vitreoretinal conditions. In addition, modeling the trend of the effectiveness of active enzymes could help to predict

the results and develop new potential therapeutic options. We believe this method can be useful in the investigation of the effectiveness of current pharmacological injections used to treat vitreoretinal conditions such as liquefaction or aging.

CHAPTER 4

Modeling of the vitreous structure

In this chapter we investigate the changes in the viscoelastic behavior of separate components of the vitreous network. As mentioned earlier, the viscoelastic behavior of vitreous gel is due to the presence of biopolymers in its structure. Fluid properties of the vitreous is mainly the result of interactions between the characteristics of collagen type II and hyaluronic acid (HA) networks. Having a better understanding of the structure of each component and their changes during aging and various diseases such as diabetes can lead to better monitoring and treatment options.

We study the effects of collagenase type II on 44 samples of porcine vitreous using an *in situ* rheological experiment in comparison with 18 eyes in a control group injected with Phosphate Buffered Saline Solution. We analyze the behavior of each component over time in both groups. We focus on the changes of viscosity and elasticity of the collagen network within the vitreous gel. The results of the analysis in this study show that the changes in the fluid properties of the vitreous after collagenase injection is primarily lead by the structural alterations of the collagen network. Creep compliance and retardation time values of the collagen network are significantly higher in the first group compared to the control group one hour and twenty-four hours after the injection. In contrast, creep compliance of the HA network shows no statistically significant change one hour after the injection in both groups. The results of the reported analysis of individual components in this study support the previous findings on the alterations within the vitreous structure in its entirety.

4.1 Introduction

Vitreous humor is a clear gel between the retina to the lens in the eye globe. As we discussed earlier, studying the structure of the vitreous gel [89], its roles in the functions of the eye [44] and in the pathology of vitreoretinal diseases [65], were brought to attention in the late 1900s. Prior to which it was thought vitreous does not play an important role within the eye globe [16]. It is now clear that the vitreous has many roles in protecting the structure and providing the means for the functions of the eye [10]. In addition, vitreous humor supports the lens [97] and regulates the intraocular Oxygen [11], the functions of the vitreous were discussed in details in Chapter1.

The vitreous gel is attached to the retina which results in a direct connection between the changes in its network and the pathology of many vitreoretinal diseases [67, 24]. As described previously, network of the vitreous gel has two main biopolymers, collagen and hyaluronic acid (HA) [89, 98]. Collagen has high tensile strength and HA provides a network to support the collagen fibrils within the vitreous [99, 100, 98]. It is hypothesized that alterations in the structure of the vitreous gel are due to the variations of the characteristics of its components. Therefore, a more detailed study of each one of the two networks is helpful to further understand the behavior of the vitreous gel in its entirety. It also allows us to find the cause of changes in the vitreous humor properties in natural processes such as aging. A deeper understanding of the vitreous structure and its properties can help us to improve our methods of intraocular drug delivery [56, 97] and surgical treatment options, to create a better engineered substitute [101], and to develop new methods of treatments. Several studies use the rheological properties of the vitreous humor to develop a better substitute with similar viscoelastic behavior [102, 103].

Degeneration of the vitreous network leads to many vitreoretinal conditions. This process can be due to many reasons such as aging and diseases [104]. The structure of the vitreous gel changes over time which causes a separation of the liquid part in the vitreous (i.e., water and HA) from the collagen fibers in the form of bundles [67]. The collagen fibers are the connected monomers (Figure 4.1) and can form tangled networks. This process is one form

of the liquefaction of the vitreous. Liquefaction of the vitreous gel can alter vitreoretinal interface and lead to posterior vitreous detachment (PVD), vitreomacular traction (VMT) syndromes [105], and eventually cause more serious conditions such as retinal tear (RT) or retinal detachment (RD) [66]. Diseases such as diabetes can also change the vitreous structure in different ways and lead to retinopathy [106, 107]. These conditions can be treated by Pars Plana Vitrectomy (PPV), a surgical procedure to remove the vitreous humor and replace it with a fluid as a substitute [66]. Recently an enzymatic intravitreal Jetrea injection (Ocriplasmin; Thrombogenics, Inc) has been found to induce pharmacologic vitreolysis by means of proteolysis of the vitreoretinal connections [108]. In addition, studies introduced intravitreal injection of certain gases to exert mechanical forces and remove the adhesion of the vitreous gel to the retina [109, 110]. Further investigations are essential to fully characterize and improve the effects of the mentioned treatments on the vitreous gel.

Understanding the fluid properties of a polymeric network can shed light on the characteristics of its structure. Rheology is an indirect method to measure the properties of fluid. Prior investigations show that the vitreous humor is a viscoelastic gel [39, 24, 78]. Similar to the molecular structure of the vitreous, its viscoelastic properties are mainly due to two main components; collagen and HA network. The viscoelastic properties of each component within the vitreous gel were studied previously using shear rheological methods [24, 74]. In addition, the enzymatic degradation of the vitreous gel was studied using the same method where the vitreous gel was dissected [25, 56].

As mentioned before, microrheological experiments do not require full dissection of the gel [37, 75, 59]. However, because only the localized characteristics are reported, the accuracy of the techniques needs improvement [58]. Furthermore, in Chapter 2 we extensively described that using *in situ* rheological experiments, we can measure the effects of enzymes on the fluid properties of the vitreous gel over time without any major alterations to the structure of the eye globe [111]. Using this method, it is possible to further investigate the changes in the structure of the vitreous humor and relate it to each one of the separate components. We analyzed the data from the eyes in two groups of injections over time to quantify the alterations in the properties of each network individually (i.e., collagen and HA). This is the

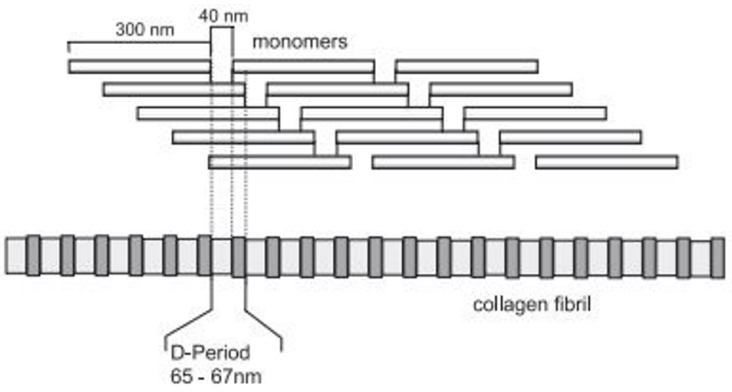


Figure 4.1: Collagen fibers and the connection of the monomers. [4]

first *in situ* study to analyze the characteristics of the two main components of the vitreous gel over time.

We analyze the changes in the creep compliance and retardation time of each component as well as the steady state viscosity of the vitreous gel. We would like to emphasize that the reported values in this study are an indication of the fluid properties of the individual components of the vitreous gel and the main purpose is to show the variations of values over time and due to the enzymatic degradation as opposed to the exact values of the properties.

4.2 Enzymatic degradation methodology

Fresh porcine eyes were harvested and shipped on dry ice within the same day by Sierra Medical Supplies (Whittier, CA, USA). In total we tested 44 eyes in the first group and 18 eyes in the control group. The protocol of the experiments are exactly as described earlier in Chapter 2. A small triangular incision was made on the pars plana of the eye to access the vitreous gel. This incision provided an access to the vitreous for testing and injections.

A stress-controlled rheometer (TA instruments, AR 2000) was used with a 0.87mm diameter cylindrical probe. The rheological procedure used in this study is creep flow, one of the oldest and commonly used methods to understand flow characteristics of fluids. In this test a constant torque/shear stress (τ) is applied and the caused deformation (γ) is recorded. Creep compliance (J), which is an indicator of the elasticity of the fluid, is derived from the

deformation using $J(t)=\gamma(t)/\tau$. After the incision was made, we secured the eye to avoid extra movements during the experiment. The probe was fully inserted into the eye to run the test close to the center of the vitreous cavity. We applied a torque of 1 μNm with zero normal force over 6 minutes on each eye. This first test was performed before any injections and we refer to it as the pre-injection result (T_0). Immediately after the pre-injection test, the eye was injected with 50μL of collagenase type II (group 1) or the same volume of PBS in group 2. We recorded the time of the injection and repeated the test one hour (T_1) after the injection. The scleral opening was protected during the first hour by inserting a small capped plastic vial on top of the opening to reduce the alteration of the vitreous gel. Subsequent to this test, eyes were individually stored at 4 °C in a container filled with PBS to avoid evaporation of the water content of the vitreous gel. Eyes were brought back to the room temperature (25°C) 15 minutes before the last experiment to prevent alterations of the results due to temperature changes. The last test was 24 hours after the injection (T_{24}) to observe changes over a longer period of time. We were limited to repeat the test beyond 24 hours as vitreous would naturally degrade. A schematic of the experimental procedure is shown in Figure 4.2.

The creep curves in this study are highly nonlinear with many local minima, hence we used Python non-linear regression model to improve the accuracy of the fittings. Non-linear regression model is an optimization technique to solve highly nonlinear problems using the least square minimization approach. This method was used to provide the best fit for the creep curve of each eye at all three time points. We used viscoelastic discrete spectra model with two Voigt-Kelvin elementsin series to analyze the fluid characteristics of the elements present in the vitreous humor network [24].

To perform statistical analysis on these test results, R-studio was used. R-studio is an integrated development environment used for statistical computing. Mixed ANOVA analysis are used in all of the statistical calculations. In these analysis the injection types are the between subjects factor (collagenase type II or PBS) and the three time points that the experiments are repeated for all the samples are the within subjects factor [112]. The significant differences reported by the ANOVA analysis are further investigated using paired or

unpaired t-tests (depending on the repeated measure comparison or between subject analysis) adjusted by Bonferroni correction. These results are also compared to the Welch's t-test. The range of p-values are reported as the p-values are not exactly the same. However, both tests were in agreement on the existence of a significant difference for each test. The details of the statistical results are discussed later in this chapter.

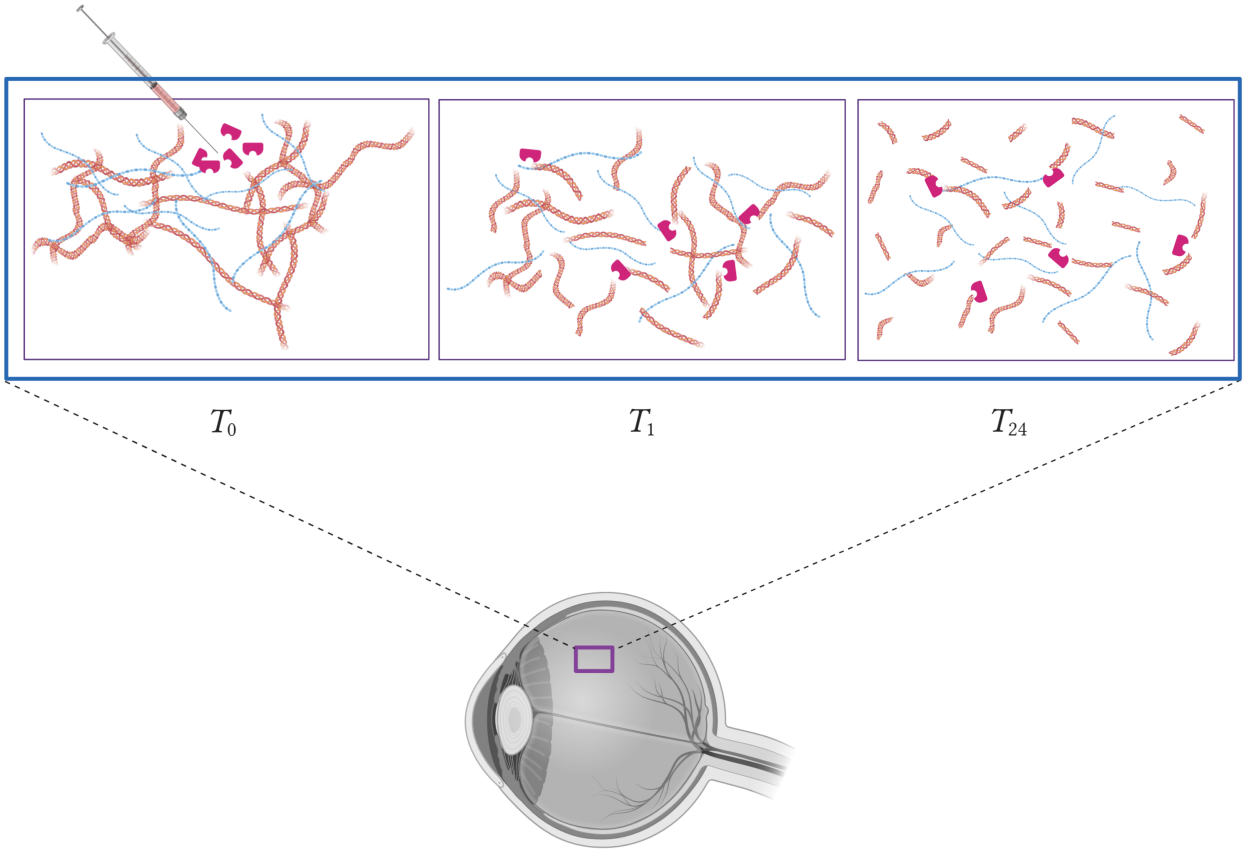


Figure 4.2: Schematic of the experimental procedure shown for group1. After the first experiment, T_0, the porcine eye is injected with collagenase. One hour and 24 hours after the injection, T_1 and T_{24}, the test is repeated. Collagen network is affected by the enzyme which results in chopped collagen network. This schematic is created with Biorender.com.

The creep curves in this study are highly nonlinear with many local minimum, hence we used Python non-linear regression model to improve the accuracy of the fittings. Non-linear regression model is an optimization technique to solve highly nonlinear problems using the least square minimization approach. This method was used to provide the best fit for the creep curve of each eye at all three time points. Fitting parameters and the fitting criteria are explained extensively in Section 4.3.1.

4.3 Viscoelastic behavior of components

In this section the details of the of the fitting analysis are reported. The statistical analysis and its results are also shown for both groups of the eyes. We also elaborate on the model used for the fitting and analyze parameters of individual components.

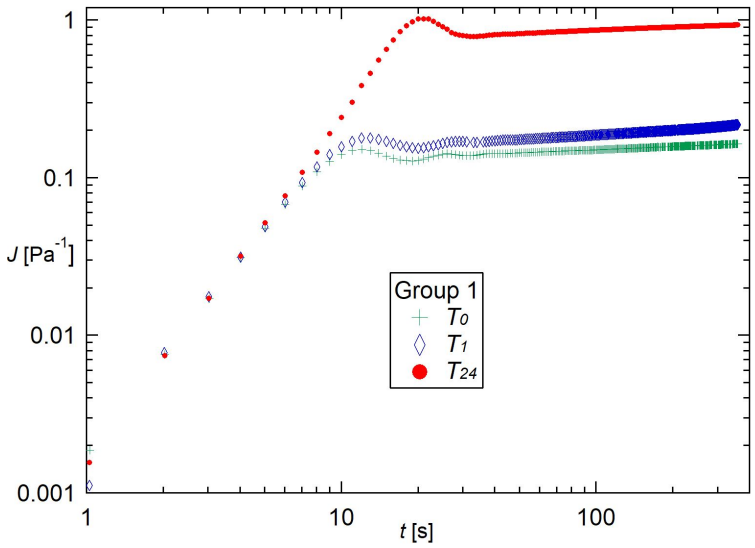
4.3.1 Fitting parameters

Typical creep curves for all three different time points (T_0, T_1, T_{24}) are shown in Figure 4.3 a & b for one eye from each group. The properties of each component of the vitreous gel can be characterized by its creep compliance (J_k) and retardation time (t_k). These properties and any subsequent changes in their values affect the creep compliance of the vitreous gel. The viscoelastic discrete spectra model [8, 24] is used (Equation 4.1) to analyze the behavior of the vitreous gel as a result of the interaction of its two components.

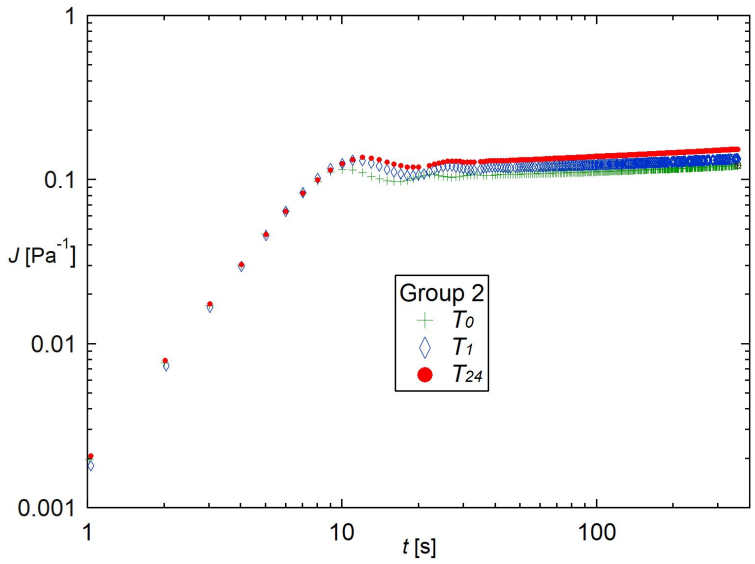
$$J(t) = \sum_k J_k(1 - r^{(-t/t_k)}) + t/\eta_m \tag{4.1}$$

There are 5 parameters in this equation. Viscosity of the vitreous gel (η_m) in the steady state region (i.e., the linear segment of the creep curve when the change in the slope is not significant). Each one of the components within the network of the vitreous gel (i.e., collagen network and HA) has two parameters related to their fluid properties, creep compliance (J_k) and retardation time (t_k). Creep curves of the eyes are collected and analyzed individually. Variations in creep compliance (J_k) and retardation time (t_k) values of each component lead

to changes in the fluid properties of the vitreous gel.



(a)



(b)

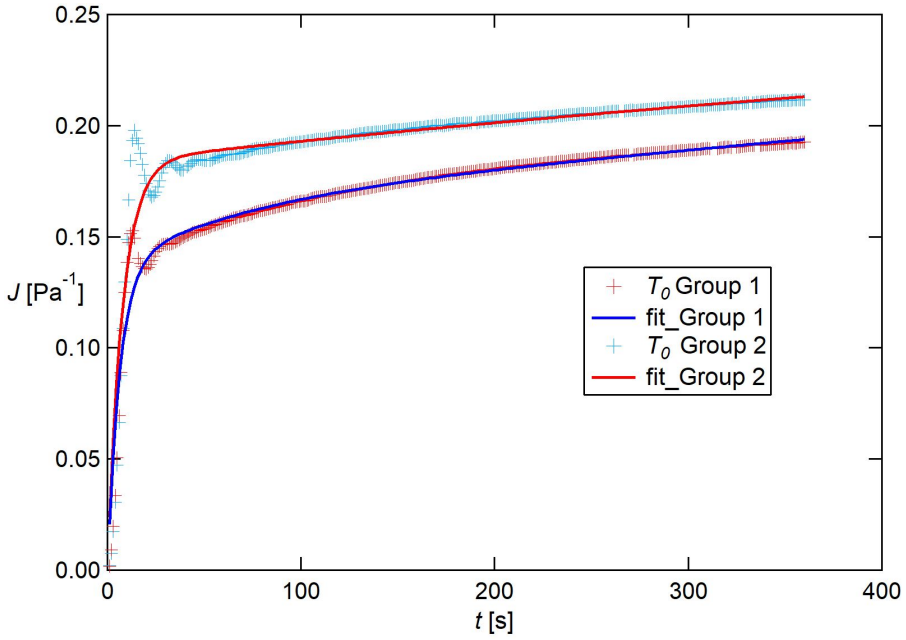
Figure 4.3: a) Creep curves for group 1 at all three time points in comparison with, b) creep curves for group 2 over time.

4.3.2 Creep curve fitting

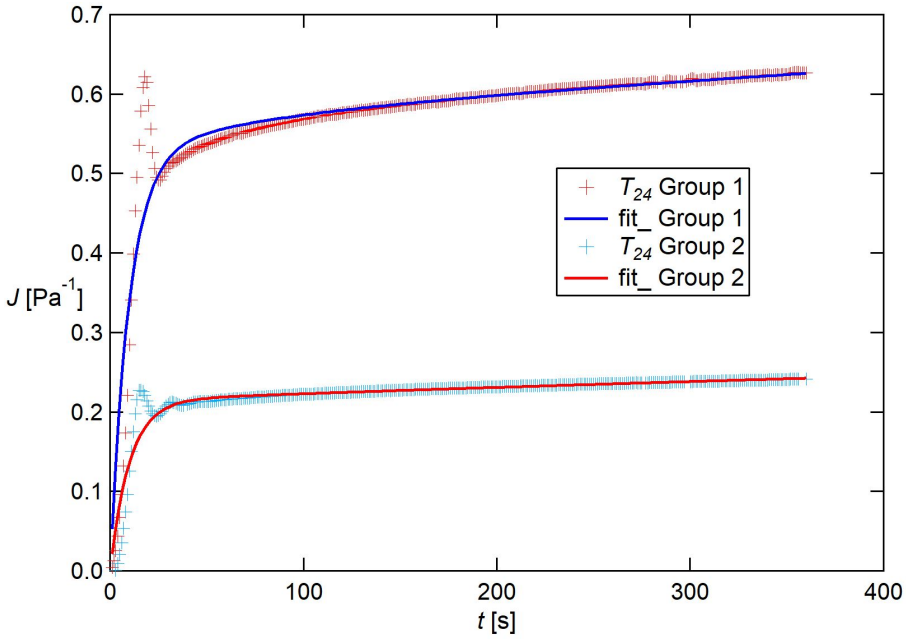
As shown before, there are three creep curves for each eye (T_0, T_1, T_{24}), in this section we cover the fitting procedure used to evaluate the behavior of each component. Viscosity of the gel at the steady state was calculated using the method explained in Chapter 2, for each one of the eyes using the slope of the linear segment of the creep curve between t=240 s and t=300 s. Using the calculated viscosity, we modeled the entire creep curve using the Python non-linear regression model to find the rest of the parameters for each experiment. The fit of the pre-injection curve provided the values for J_1, t_1, J_2, and t_2. Figure 4.4.a shows common creep curves with the fittings for both groups at T_0, where the behavior of the curves are similar although not exactly the same. Values are further analyzed statistically.

The calculated value of t_2 (i.e., retardation time of HA network) from the first fit (T_0) was kept constant for T_1 and T_{24} modeling of that eye in both groups. The hypothesis is that the effect of time is the same in both groups on the retardation time of the HA network, hence the value of t_2 should not change significantly over time. This hypothesis is validated by calculating the value of t_2 the curve using the found values of J_1, t_1, and J_2. The fitted results are minimally different from the used value of t_2. Figure 4.5 shows both fitted curves on the same creep curve at T_{24} for an eye from group 1. Fitted curve 1 is plotted using the calculated parameters with a fixed t_2 from T_0 while fitted curve 2 is fitting the curve with all parameters fixed but t_2.

As it can be seen from Figure 4.4.b, creep curves for the eyes show different behavior in the slope. It is worth mentioning that for this particular example the creep curve for the eye from group 1 at T_0 is actually below the curve for eye from group 2, whereas at T_{24} the curve for from group 1 shifts significantly higher than the curve from group 2.



(a)



(b)

Figure 4.4: a) Creep curves for one eye from each group before the injection with the fitted curve, b) creep curves for the same eye in each group 24 hours after the injection with the fitted curve.

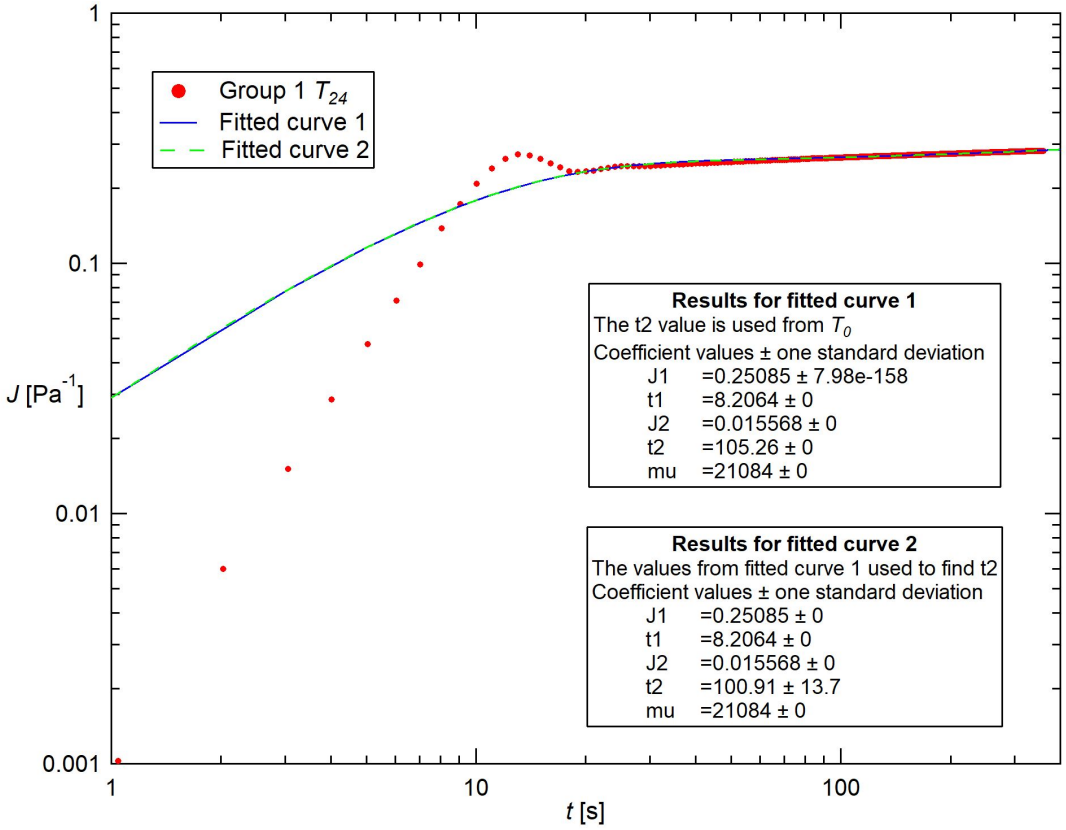


Figure 4.5: This curve shows a sample of the verification of the t_2 values. The value is originally found using the T_0 curve and kept constant for the two other time points. This curve is an example from group 1 at T_{24}. The found value (100 s) is minimally different from the constant value used to find the other three parameters (105 s). Values of all 5 parameters are provided for each fitted curve.

4.3.3 Collagen parameters

The statistical results are provided in this section comparing collagen parameters over time between the mean of the two groups. The characteristics of the collagen component of the vitreous network is analyzed over time. At each time point the average values for creep compliance and retardation time of the collagen network are compared between the groups over time (Figure 4.6 and Figure 4.7). In addition, statistical comparison of J_1 values for each group over time (paired t-test) is shown in Table 4.2 and Table 4.3. The results of unpaired statistical analysis for all three parameters using both described methods are reported in Table 4.1.

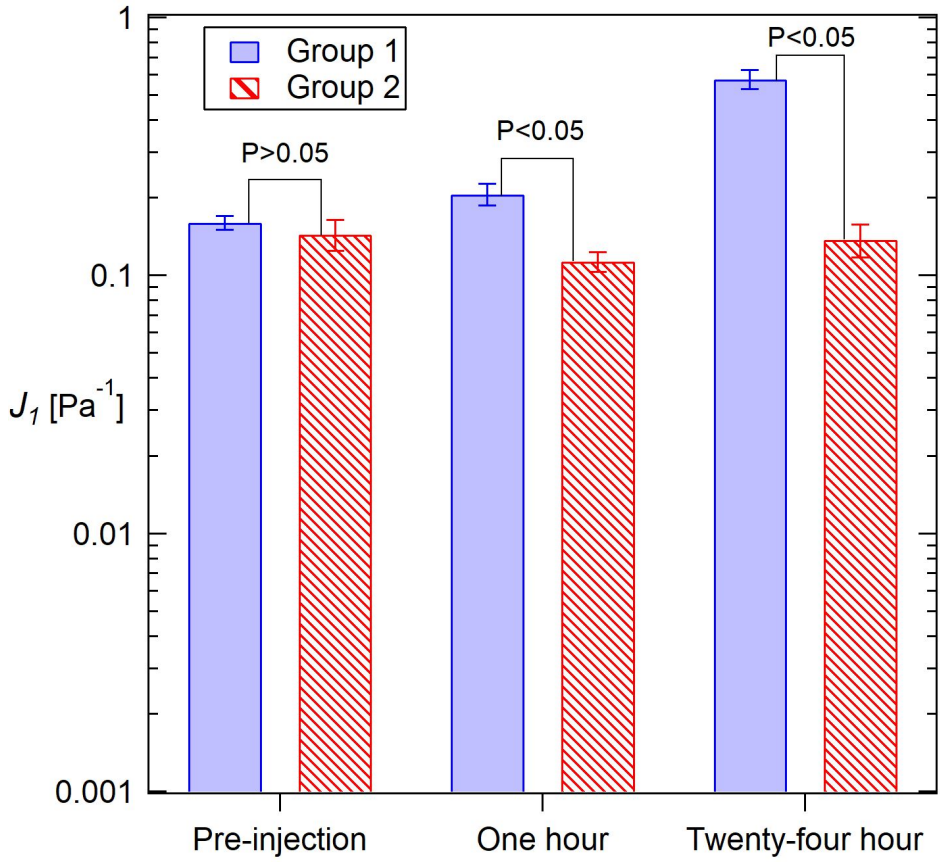


Figure 4.6: Average values for the creep compliance of the collagen network within the vitreous structure for both groups. P-values are provided to compare the two groups at each experiment. At time zero there is no statistically significant difference between the two groups while at the other two time points the two groups are statistically different.

The average value of creep compliance of collagen network (J_1) does not differ significantly

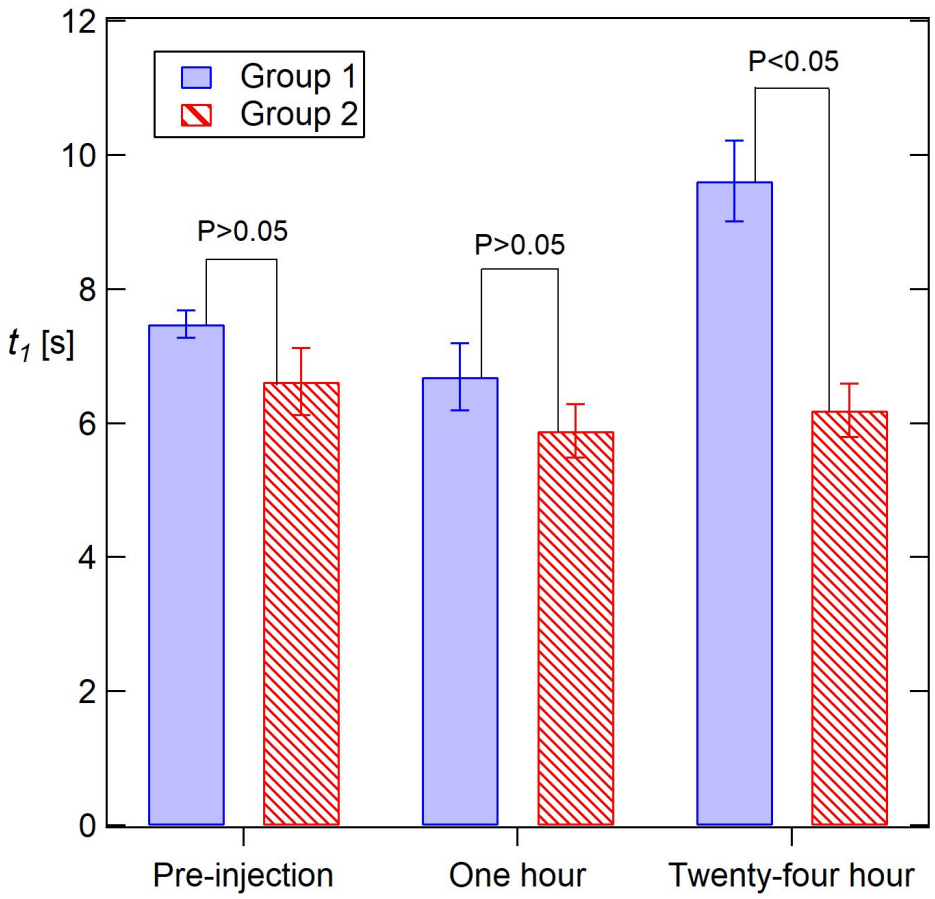


Figure 4.7: Average values for the retardation time of the collagen network within the vitreous structure for both groups. P-values are provided to compare the two groups at each experiment. There are no statistical significant differences between the two groups at the first two time points. Twenty-four hours after the injection the average value of t_1 is significantly higher in group 1 compared to group 2.

between the two groups before the injection with an average of 0.16 Pa^{-1} for group 1 and 0.14 Pa^{-1} for group 2. However, results from the experiments at T_1 and T_{24} in group 1 are significantly higher than the ones in group 2, the ranges of p-values are reported in Figure 4.2. The average values for J_1 in group 1 are 0.21 Pa^{-1} at T_1 and 0.58 Pa^{-1} at T_{24} compared to 0.11 Pa^{-1} and 0.14 Pa^{-1} for group 2. Moreover, J_1 increases noticeably over time in group 1, whereas in group 2 the increase is not significant (Table 4.2)

It is noteworthy to mention that there are no statistically significant differences between the average values of the retardation time of the collagen network, t_1, between the two groups at T_0,with values of 7.48 s and 6.62 s for group 1 and group 2 respectively, and at T_1 with an

Table 4.1: Unpaired t-test results for both groups over time using Welch's t-test and the Bonferroni adjusted t-test to decrease the possibility of type I error. All three parameters are shown here in separate parts of the table

Welch's P-values	**0**	**1 hour**	**24 hour**
J_1	0.49	1.3×10^{-4}	1.4×10^{-11}
t_1	0.12	0.21	2.6×10^{-5}
J_2	0.12	0.06	1.2×10^{-4}
Bonferroni P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
J_1	0.42	3.2×10^{-3}	2.6×10^{-7}
t_2	0.06	0.33	1.2×10^{-3}
J_2	0.09	0.21	0.03

Table 4.2: Paired t-test results for creep compliance of the collagen network in both groups over time

Mean	**0**	**1 hour**	**24 hour**
Group 1	0.16±0.01	0.21±0.02	0.58±0.05
Group 2	0.14±0.02	0.11±0.01	0.14±0.02
Welch's P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	0.03	3.2×10^{-11}	1.1×10^{-9}
Group 2	0.12	0.80	0.34
Bonferroni P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	0.049	7.8×10^{-11}	1.1×10^{-8}
Group 2	0.37	1.00	1.00

Table 4.3: Paired t-test results for retardation time of the collagen network in both groups over time

Mean	**0**	**1 hour**	**24 hour**
Group 1	7.48±0.2	6.69±0.5	9.61±0.6
Group 2	6.62±0.5	5.89±0.4	6.19±0.4
Welch's P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	0.15	1.8×10^{-3}	4.2×10^{-4}
Group 2	0.25	0.51	0.59
Bonferroni P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	0.36	7.8×10^{-3}	1.9×10^{-3}
Group 2	0.26	1.00	1.00

average value of 6.69 s for group 1 and 5.88 s for group 2. Whereas t_1 =9.61 s is significantly higher in group 1 compared to t_1 =6.19 s for group 2 at T_{24} (Figure 4.7).

4.3.4 HA parameters

The same properties, creep compliance J_2 and retardation time t_2, can be calculated for the HA network. As mentioned before in Section 4.3.2, at T_0 the first set of data and the viscosity value are used to calculate all four parameters (J_1, t_1, J_2, and t_2). For the other two sets of data (T_1 and T_{24}), the calculated t_2 is used in order to run the same Python non-linear regression model with the same population number. Due to the high non-linearity of the data and fittings, we hypothesized the changes are not significant. This hypothesis was validated by calculating the t_2 values at T_1 and T_{24} using the previously fitted parameters.

Average values of the creep compliance of the HA network are shown in Figure 4.8 as well as the range of p-values from unpaired t-test analysis between the two groups at each time point. The actual p-values using the mentioned unpaired t-tests are also reported in Table 3.2. The mean values and standard errors for the creep compliance of HA network are provided along with p-values from the paired t-test for each group over time in Table 4.4.

The average value (J_2) does not differ significantly between the two groups at T_0 with 0.024 Pa^{-1} for group 1 compared to 0.018 Pa^{-1} for group 2 and T_1, with average values of 0.030 Pa^{-1} and 0.012 Pa^{-1} for group 1 and group 2 respectively. However, result from the experiment at T_{24} in group 1, 0.37 Pa^{-1}, is significantly higher than the one in group 2 (0.014 Pa^{-1}), results of the unpaired t-test p-values are reported in Figure 4.8. Moreover, J_2 at T_{24} is higher compared to the other two time points in group 1, whereas in group 2 the increase is not significant (Table 4.4). All the results are further discussed in Section 4.4.

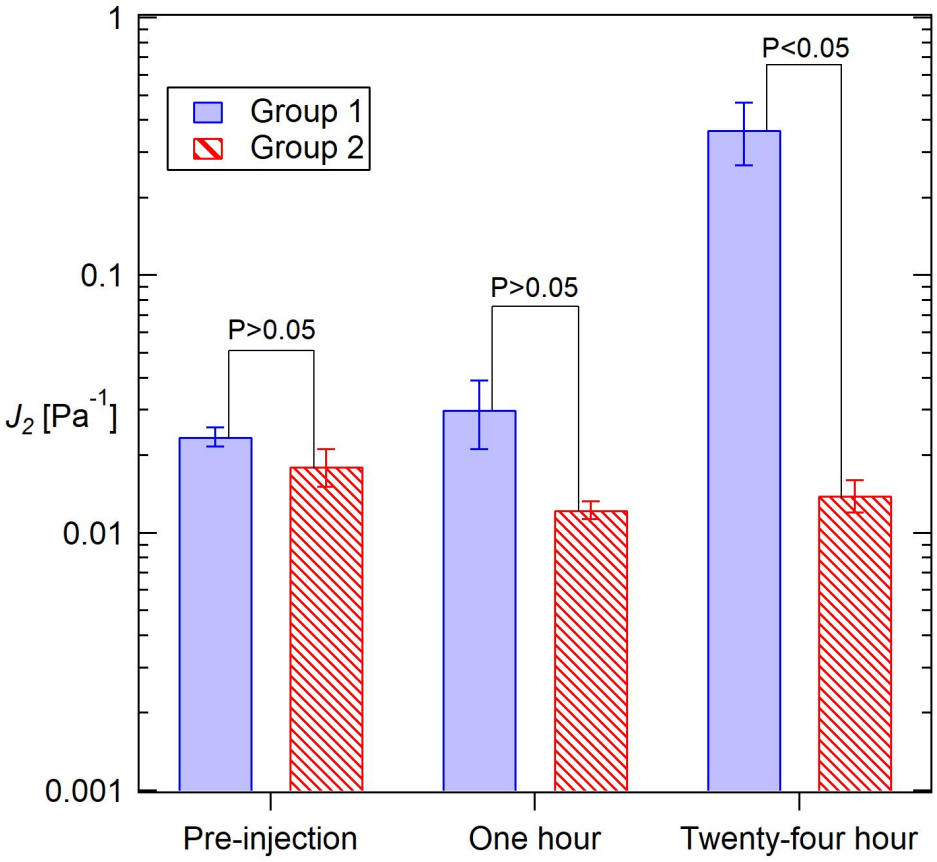


Figure 4.8: Average values for the creep compliance of the HA network within the vitreous structure for both groups. P-values are provided to compare the two groups at each experiment. Average values of J_2 are not statistically different at the first two time points. However, there is a significant difference between the average values 24 hours after the injection between the two groups.

Table 4.4: Paired t-test results for creep compliance of the HA network of the vitreous gel in both groups over time

Mean	**0**	**1 hour**	**24 hour**
Group 1	0.024±0.001	0.030±0.01	0.366±0.1
Group 2	0.018±0.003	0.012±0.001	0.014±0.002
Welch's P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	0.48	1.5×10^{-3}	1.9×10^{-3}
Group 2	0.10	0.25	0.40
Bonferroni P-values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	1.00	4.9×10^{-3}	4.3×10^{-3}
Group 2	0.22	0.81	0.91

4.4 Characterization of individual components

Vitreous gel has a complicated fluid structure which is due to its polymeric network and molecular structure [31, 24]. HA network and collagen fibrils are the main components of this structure with distinct fluid characteristics. Aging can alter the structure of the vitreous gel where it becomes more liquid-like. This happens mainly as a result of the cross linking of collagen fibrils [113] that may lead to a pulling force on the retina at the vitreoretinal interface. This pulling can cause a full PVD but in some cases the vitreous gel remains partially attached to the retina and causes point forces at certain locations which could result in RT or RD. If a patient becomes symptomatic, PPV surgery can help removing the force [110, 114, 109, 115]. As PPV is an invasive course of treatment, there are many studies that focus on alternative options such as pharmacologic vitrectomy, and pharmacologic or gas induced vitreolysis [110, 116, 117].

There have been many rheological studies to characterize the effects of the aging on the structure of the vitreous. Comparison of the creep results on ovine eyes from three different ages, showed a decrease in both loss and storage modulus with age which is due to the breakdown of the collagen network [118]. A rheological study on the dissected human vitreous samples shows the decrease in the viscoelasticity of the vitreous humor which is related to the liquefaction of the eye [119]. Another study on the vitreous humor of human at different ages showed higher stiffness and viscosity for the solid phase of the older vitreous and lower viscosity for the liquid phase of the older vitreous gel [74]; however, the discrete retardation analysis did not show significant correlation, which could be due to the dissection of the vitreous as well as the low number of samples tested. These findings are in contradiction with the studies that measure the vitreous properties in its entirety. Our findings in group 1 are in agreement with the aging analysis of the vitreous as one gel; we assume that the similarity is due to the injection of the collagenase which breaks the bonds in the collagen network.

The molecular structure of the vitreous gel and the interaction of its networks of biopolymers are very important in understanding of the pathology of many vitreoretinal diseases.

It can also provide information about the possible approaches to slow down the degenerative aging process or to treat the resulting conditions. To be able to invent better quality of a substitute for the vitreous gel, one should be fully aware of its properties and roles [53, 116, 102, 120, 103]. Previous studies could identify the composition of the vitreous network [113, 31, 13] but due to the fragile structure of the vitreous gel, reported direct measurements require dissection and are limited [73, 39, 25, 74].

Previous studies successfully showed that the behavior of the vitreous network is due to the presence of two main networks of biopolymers [24, 74]. Each one of these networks has its own properties and changes in one of the components can lead to alterations in the vitreous properties. Microrheological experiments show that the probe can be trapped in one of the components and lead to localized information. For instance if the probe is located in the fibrils of collagen network the results would drastically differ compared to when the probe is in the liquid pockets within the vitreous [59]. Therefore, it is important to better understand properties of the main components on the macro scale and study the structure of both biopolymers to be able to investigate the changes in the characteristics of vitreous. In addition, exploring the behaviour of mentioned components after enzymatic degradation can provide better constraints for the design of new treatments for vitreolysis. There have been no *in situ* rheological studies of the vitreous network over time as with the normal shear rheological setup of the experiments the structure of the eye globe is disturbed. Hence, after the first run of the test, the eye must be discarded. There are reported less invasive microrheological methods both *in vivo* and *ex vivo* however, the spatial size limitation of the experiment and localized characteristics constraint still exist [121].

It is important to quantify the time variation on vitreous humor. The ability to repeat the experiment allows us to characterize the degradation due to time and/or different enzymes on the rheological properties of the vitreous. There are reported studies on rheological measurement of the dissected vitreous properties over time. Silva and co-workers reported the rheological properties of the dissected gel and liquid parts of the vitreous individually over time [78].

In our *in situ* experimental setup, the vitreous stays intact inside the eye globe therefore,

we can repeat the test at different time intervals (T_0, T_1, and T_{24}) [111]. We measured the viscosity and creep compliance of the vitreous and their changes due to the injection of collagenase over time. In the present study we modeled the individual components of the vitreous and their changes as a result of collagenase injection in comparison with a control group. The results reported here are in agreement with the previous studies that used the viscoelastic discrete spectra model to analyze porcine and human vitreous to show two distinguished components [24, 74]

The slope of the creep curve in the steady state for group 1 is the smallest for the pre-injection curve with an average value of 6×10^{-5} [1/Pa.s]. One and 24 hours after the injection the slope increases, average values are 9×10^{-5} [1/Pa.s] and 2×10^{-4} [1/Pa.s] respectively. Creep curves at all three points for one eye from group 1 are shown in Figure 4.3.a. On the other hand, group 2 has approximately the same slope for all three time intervals, where the average value for all the eyes in group 2 varies from 4×10^{-5} [1/Pa.s] to 5×10^{-5} [1/Pa.s]. All three curves for one of the eyes in group 2 are shown in 4.3.b to show the similarity between the curves at different points for the same specimen.

Characteristics of the collagen network are shown by J_1 and t_1. Creep compliance is directly related to the inverse of elasticity. As shown before J_1 values only increase significantly in group 1 and are statistically different from group 2 at T_1 and T_{24}. These results suggest that elasticity of the collagen network has significantly decreased in group 1. This could be explained by having less bonds present in the network of collagen due to the enzymatic effect of the collagenase. Less bonds in the biopolymer structure makes it easier to elastically deform the network. This result supports the hypothesized changes on the collagen network and confirms that the change in the elasticity of the vitreous is due to the alterations of the collagen component of the gel.

The results from group 1 show a different behavior for collagen retardation time compared to its creep compliance. There are no statistically significant differences between the average values of the retardation time, t_1, between the two groups at T_0 and T_1. At T_{24} the value increases significantly in group 1 as shown in Figure 4.7 which makes it statistically different from group 2. The retardation time of collagen network increases over time in group 1

however, its average value is only noticeably higher at T_{24} compared to the other two time points. The increase in the retardation time suggests that there is a change from solid-like behaviour to more liquid-like behaviour in the collagen network. This change can be due to the presence of less bonds in the collagen networks. This data validates the previous findings on the changes of the vitreous network as one gel [111].

There are no statistically significant differences observed for t_1 values in group 2 and it decreases over the first hour followed by an increase at T_{24}. This could have been the result of the diffusion of the injected PBS or changes due to the effect of time. These results show that we can map the effects of various active and placebo injections on the specific parameters of the vitreous gel.

The average value of HA creep compliance does not differ significantly between the two groups at T_0 and T_1. The analysis show an increase in J_2 at T_{24} in group 1 which is also significantly higher than the one in group 2. Therefore, the results suggest that elasticity of the HA network has decreased 24 hours after the injection of collagenase. This could be due to unavoidable alterations on the HA network after the degradation of the enzyme in addition to the effect of time on the bonds in its network.

As it was mentioned earlier, PPV is an invasive surgery with longer time for the rehabilitation of the vision [122]. Enzymatic vitreolysis can induce PVD to remove the traction force of the vitreous on the retina. However, discovering the right enzymatic injection can be difficult. Jetrea (Ocriplasmin, Thrombogenics) is the first intravitreal drug injection that was approved by FDA in 2012. There have been many studies on the efficacy of Jetrea [90, 123]. While this method has many advantages compared to PPV, it is limited to some patients with specific conditions. In addition, there are many side effects associated with the Jetrea injection such as lens instability and macular hole enlargement [122].

There are case studies reporting temporary disturbances or loss of vision in patients after receiving the injection which can be due to the enzymatic digestion of the retina and/or vitreoretinal connections [124]. Other studies found that the induced acute visual loss is due to the ellipsoid zone changes [125, 122]. It has been recommended to use a quantification

method on the optical coherence tomography (OCT) imaging of patients, which has provided a better outcome to identify the ellipsoid layer loss [125].

Effects of Jetrea should be more extensively studied as a treatment option, as its roles as a protease may not be limited to the vitreoretinal interface. The protease might attack the retina as well as the vitreous gel itself [126] and cause the aforementioned side effects. Many other enzymes are used during the vitrectomy procedure to induce liquefaction of the vitreous and/or dehiscence the vitreoretinal interface [127]. Enzymatic treatments can be a great substitute for the surgical methods with lower risks and costs as well as shorter rehabilitation time. Further studies are required to understand the actual effects of an enzyme on separate components of the vitreous. This can potentially help patients with diabetic retinopathy or abnormal vitreoretinal adherence to have an induced PVD which removes the need for surgical intervention.

We investigated the changes on the components of the vitreous structure due to an active enzyme injection on the specific components of the network over time. Effect of time can be more precisely modeled by adding more time intervals to the experimental procedure (i.e., add time points between 1 hour and 24 hours) and to predict the changes at other time points without the experimental procedure. In future, our method can help finding and evaluating possible enzymatic treatments and to predict the effects of other potential injections over time and their possible roles in the treatment of some vitreoretinal conditions.

4.5 Conclusion

In this chapter, we investigated the changes on the components of the vitreous structure due to the enzyme injection on the specific component of the network over time compared to a control group injected with PBS. Effect of time can be more precisely modeled by adding more time intervals to the experimental procedure (i.e. add time points between 1 hour and 24 hours) to predict the changes. In future, our method can help finding and evaluating possible enzymatic treatments and to predict the effects of other potential injections over time and their possible roles in the treatment of some vitreoretinal conditions.

Our method provides the opportunity to measure the potential changes on the characteristics of the vitreous humor due to enzyme injections. This method can further evaluate the changes in the properties of each component over time. Collagenase has been used as a sample of active enzymes to validate the proposed method.

CHAPTER 5

Conclusion

In this dissertation, we present the first *in situ* time study of the porcine vitreous gel viscoelastic properties over time. We investigated the changes in the characteristics of the vitreous gel after enzymatic injections compared to a control group. The changes were fitted using the appropriate mechanical analogy and statistically analyzed.

We further analyzed the two main networks of biopolymers in the vitreous gel. We modeled the fluid behavior of each component within the vitreous gel after enzymatic injection using a two element viscoelastic discrete retardation spectra model. We used rheological concepts to characterize the parameters of each network over time. We hypothesized that the first part of the creep curve is due to the presence of the collagen type II network, which shows more of a viscoelastic solid response, followed by a viscoelastic liquid behavior that is related to the role of the hyaluronic acid in the vitreous gel. Each component has two parameters, the retardation time and elasticity, that can be affected by the enzyme, time, and unavoidable evaporation.

We have chosen three certain time points to model the behavior of the vitreous gel over time. Due to the delicate structure of the vitreous network and unavoidable evaporation of its water content we did not repeat the experiment more than three times and after 24 hours. However, the effect of time can be more precisely modeled by repeating the experiment at more time points or by picking time points closer to one another (i.e. add time points between 1 hour and 24 hours) to predict the behavior. This could help us to evaluate the efficacy of pharmaceutical intravitreal injections (e.g., Jetrea) on the characteristics of the vitreous gel and further study its possible adverse effects. This method can be used to find the suitable enzyme that would either prevent the liquefaction or slow down its rate.

To conclude, in this dissertation we further proved the accuracy of our novel *in situ* rheological technique to measure the changes in the vitreous humor after injections over time. We could further relate the changes in the vitreous viscoelastic properties by investigating the macromolecular structure of the vitreous humor. The results of this study can be used to improve the current surgical and pharmaceutical treatments for vitreoretinal conditions. Further studies can lead to development of less invasive therapeutic options. These finding could also be beneficial to find better substitutes for the vitreous gel after its surgical removal in a vitrectomy surgery.

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