AN ABSTRACT OF THE THESIS OF

Danielle Lurisa Leiske for the degree of Master of Science in Chemical Engineering presented on December 15, 2004.
Title: Molecular and Rheological Characterization of Sodium Hyaluronate (HA) and Equine Synovial Fluid

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Willie E. Rochefort

Sodium hyaluronate (HA) is a polysaccharide found in all parts of the body. Although it performs important functions in the eye, the coagulation process and other parts of the body, its contribution to synovial fluid is particularly important. As the major component of synovial fluid, HA is responsible for the viscoelastic properties important in joint lubrication and cartilage protection. In this thesis, molecular and rheological characterization techniques were used to study; i) commercial HA materials and HA synovial fluid supplements; ii) equine synovial fluid from different joints of both live and deceased horses; iii) equine synovial fluid from a clinical study of intra-articular HA supplementation in the hock joints of a group of six horses.

Commercial HA materials and HA intended for intra-articular, intravenous and oral supplementation were studied using size exclusion chromatography combined with inline multi-angle laser light scattering (SEC-MALLS), dilute solution capillary viscometry to obtain intrinsic viscosity ([η]), and steady

shear and dynamic oscillatory shear rheology. The molecular weight range of the HA samples was 2.88×10^5 to 1.96×10^6 Da. The molecular weight and intrinsic viscosity were correlated and a Mark-Houwink-Sakurada equation for HA in phosphate buffer solution (PBS) was found to be $[\eta] = 0.17\ Mw^{0.68}$. The "a" value of 0.68 indicates HA behaves as a random coil in PBS which is consistent with values reported in literature. Zero shear viscosities of the samples at a concentration of 2.5 mg/ml ranged from approximately 0.06 to 0.5 Pa-s and were found to have a nearly linear relationship with the product of molecular weight and concentration ($\eta_o \propto cMw^{1.08}$). All samples exhibited both viscous and elastic properties in the dynamic oscillatory shear tests. The correlation between molecular weight and rheological properties of pure HA indicates that these techniques may be used in the future to characterize HA materials and possibly to discern the connection between molecular properties of HA and their lubrication properties in synovial fluid.

The techniques used to characterize pure sodium hyaluronate samples were then applied to equine synovial fluid from a number of live and deceased horses (euthanized for reasons unrelated to this study) in an attempt to elucidate the role of HA in joint lubrication. The concentration and molecular weight of the HA-protein complex in synovial fluid was measured using SEC-MALLS. Steady shear and dynamic oscillatory shear tests were also performed on synovial fluid samples. The investigation of the properties of synovial fluid in normal equine joints reported in this thesis is the beginning of

an attempt to establish baseline values for comparison with diseased joints. The molecular weight of HA in synovial fluid ranged from $1.5x10^6$ to $6.5x10^6$ Dalton (Da) and concentration ranged from 0.11 to 0.84 mg/ml, both are in the range of values reported in literature. The steady shear viscosity of synovial fluid samples ranged from about 0.001 to 1 Pa-s, with slight upturns at low shear rates in some samples, indicative of aggregation. The molecular characterization is difficult due to the complexity of the fluid. Although each sample exhibits unique rheological properties, at this time changes in viscosity or elasticity cannot be correlated to changes in either molecular weight or concentration of HA.

Finally, a preliminary study of the intra-articular injection of HA was completed using six healthy horses and rheological characterization of synovial fluid from tarsocrural hock joints. The horses were divided into three groups: the experimental group (2 ml of Hyvisc, an HA supplement, in each hock); the positive control group (2 ml of Lactated Ringers Solution (LRS) in each hock); and the negative control group (no treatment). The horses received the above treatments after aspiration of synovial fluid for the samples that were treated as the baseline value for each hock joint. Synovial fluid samples were also taken one and two weeks after treatment. Cytology, including total protein and nucleated cell counts, was performed to monitor the health of the joints throughout the study.

Rheological properties of synovial fluid in the experimental group increased one week after treatment compared to the control groups. This could be due to exogenous HA remaining in the joint after treatment, indicating that it takes longer than one week for exogenous HA to be cleared from the joint. Two weeks after treatment all test groups returned to the pre-treatment state. This was most likely due to the fact that the joints were aspirated one week after treatment, negating both treatment and non-treatment effects by the second week of the study.

Molecular and Rheological Characterization of Sodium Hyaluronate (HA) and Equine Synovial Fluid

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CHAPTER 1

INTRODUCTION

Sodium hyaluronate (also referred to as hyaluronic acid, or HA) is the largest molecular component of synovial fluid and contributes both viscous (lubricating) and elastic (shock-absorbing) properties that are important in the lubrication and protection of cartilage. Hyaluronate (HA) is a polymer found in all parts of the body but is of particular importance in articular joints. The key to the viscoelastic behavior of synovial fluid is molecular weight and concentration of HA. Hyaluronate's important role in joint lubrication has made it of particular interest in the biomedical field.

Certain types of joint diseases have been attributed to the breakdown of HA in the joint. Although the exact nature of the degradation of HA is not yet fully understood, it has been credited to the presence of deleterious enzymes and radicals. Any decrease in the size of HA molecules will result in a decrease of viscoelastic properties of synovial fluid, which could lead to cartilage damage. Viscosupplementation is a treatment developed to care for joint disease. It involves either intra-articular or intravenous injections of HA supplements to the diseased joint or patient. Initially this idea was developed to boost the viscoelastic properties of synovial fluid, however it was later realized that HA performs a

biochemical role in joints in addition to its well described mechanical role. Nevertheless, the mechanical role of HA in joint fluid is important and worthy of study.

Many clinical studies analyzing the efficacy of HA supplementation in the treatment for arthritis have been published and will be discussed in Chapter 4. Most appear to agree that supplements decrease lameness, pain and further cartilage damage. However, few researchers have actually analyzed the mechanical properties of synovial fluid itself before and after HA treatment to determine the mechanism of viscosupplementation.

The objective of this research was to: i) study both the molecular characteristics and rheological (flow) properties of pure HA samples (derived from bacterial fermentation, chicken combs, and human umbilical cords); ii) study the molecular and rheological properties of equine synovial fluid as a function of joint and disease state; and iii) conduct a preliminary clinical HA supplementation study on a group of healthy equine joints to establish whether rheological characterization techniques could be used to measure changes in synovial fluid from healthy and diseased joints.

Chapter 2 begins with the characterization of pure HA samples, some frozen and some intended as joint supplements. The steady shear and dynamic oscillatory

shear properties were measured and correlated to molecular weight as determined by light scattering. The intrinsic viscosity values for each sample were also measured and compared to molecular weight to obtain a Mark-Houwink-Sakurada equation. The study of pure sodium hyaluronate samples will be used to develop characterization techniques for application to HA in synovial fluid.

Chapter 3 continues with a similar analysis of equine synovial fluid. It includes rheological characterization of synovial fluid in addition to light scattering analysis which determines molecular weight and concentration of HA. Fifteen samples were measured and divided into three categories for analysis; healthy hock joints, healthy stifle joints, and diseased joints. This chapter describes preliminary work of this research group to establish a normal range of values for each joint. The ultimate goal was to determine the differences between synovial fluid from diseased joints and synovial fluid from healthy joints based on mechanical and molecular properties.

Chapter 4 is a summary of a preliminary study on live horses completed in conjunction with the OSU College of Veterinary Medicine. Commercial HA supplement was injected in the tarsocrural hock joints of three live horses. Synovial fluid samples were taken before injection, one week after injection and two weeks after injection. The rheological and viscous properties of each sample were analyzed and compared to samples taken from three live control horses.

This was treated as a preliminary study to determine whether intra-artciular injection of HA changed the properties of equine synovial fluid, and whether rheological techniques could be used to measure any differences.

CHAPTER 2

MECHANICAL AND MOLECULAR CHARACTERIZATION OF SODIUM HYALURONATE

2.1 INTRODUCTION

Sodium hyaluronate (HA) is a biologically ubiquitous polymer. It consists of repeating glucuronic acid and N-acetylglucosamine units and is typically in the range of 0.2-10 million g/mol (or Dalton, Da). Figure 2-1 shows a diagram of the repeat units of HA. Commercial sources of high molecular weight HA include chicken combs, umbilical cords, and bacterial fermentation. Supplemental HA is very expensive, as much as $6,000 per gram. This polymer is highly studied because it is found in many parts of the body including the skin, the vitreous humor of the eye, and synovial fluid.

Since this polymer is so prevalent in the body, HA is a natural target for scientific attention. This attention can be divided into three main categories: the biochemical role of HA in body processes; mechanical function; and molecular characterization of HA. This thesis will be concerned with characterizing the mechanical properties of HA with a comparison to molecular size for application to synovial fluid.

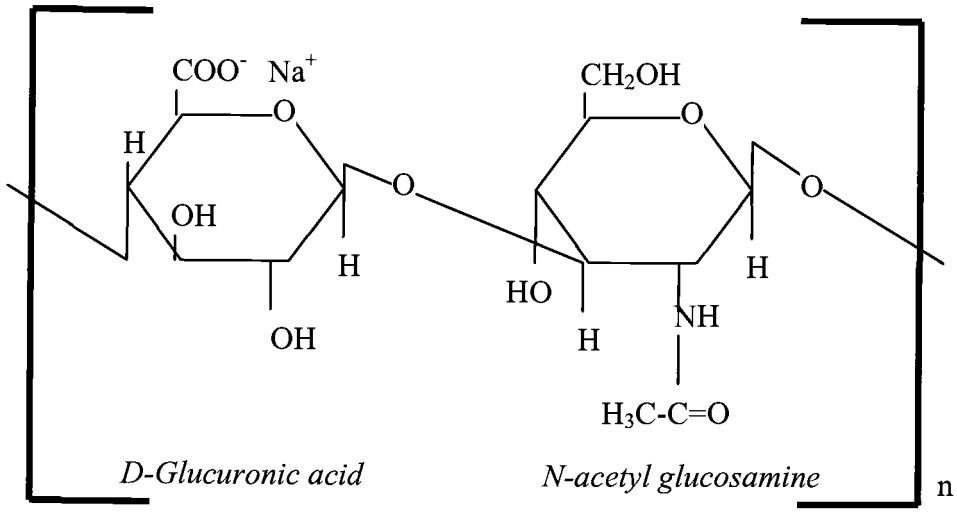


Figure 2- 1 Repeat unit of sodium hyaluronate

All high molecular weight molecules are capable of both viscous and elastic behavior depending on molecular configuration, weight, and concentration in solution. The molecular weight of HA in the body is typically around 10^6 Da which is sufficient for it to exhibit both elastic and viscous behavior depending on the stresses applied to it. The degree to which it behaves elastically is quite dependent on both molecular weight and concentration, which combined determine how entangled the molecules are in solution. The purpose of this chapter is to develop molecular and rheological characterization techniques and use them to explore the relationship between the rheological properties of HA and the size of molecules.

2.2 Literature Review

Sodium hyaluronate is an important biological polymer, so it is natural that it has received much attention in the literature. Many topics relating to sodium hyaluronate have been investigated; however this review will delve into the rheological and molecular characterization only. While these topics have been explored extensively by past researchers, few have coupled a rheological study to molecular characteristics. It should be noted that most rheological measurements of HA have been done in the concentrated regime, at concentrations much greater than those found in the body.

In 1993, both the steady and dynamic oscillatory shear rheology of high molecular weight rooster comb HA was measured by De Smedt et. al. (1993). Based on the zero shear viscosity they determined that HA has an overlap concentration, c*, value of about 1 mg/ml. This concentration is important in determining the rheological behavior of polymers in solution. Concentrations below c* are considered dilute with little or no intra-molecular interactions. If the concentration is greater than c* molecular entanglements will dominate solution behavior. De Smedt et. al. also explored the distance between entanglements in a polymer solution, elastic modulus, G', dependence on concentration ($G' \propto c^{2.82}$) and Newtonian or zero shear viscosity dependence on concentration ($\eta_o \propto c^{3.76}$). Relationships between flow properties and concentration can be used to predict concentration based on behavior or vice versa.

Kobayashi, Okamoto and Nishinari (1994) explored the dynamic properties of one weight percent HA solutions of molecular weight range $5x10^5$ to $2x10^6$ Da. They described HA-HA interactions using competitive inhibition and showed a decrease in viscous and elastic modulus with the addition of salt (due to electrostatic shielding). Hyaluronate is a polyelectrolyte, making ionic strength a factor in how densely individual molecules are coiled.

Bothner and Wik (1987) examined the effects of molecular weight and concentration on viscosity. They measured the molecular weight using low angle light scattering and found a linear relationship between log of zero shear viscosity and the product of molecular weight and concentration. Molecular weight and concentration both contribute to flow properties making this relationship very descriptive of HA rheology.

Molecular characterization of HA has been performed by many researchers using a wide variety of techniques. Light scattering was used to determine molecular weight in the research done by Hoksputsa et. al. (2003). They compared HA molecular weight measured by size exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) to sedimentation equilibrium. It was determined that SEC-MALLS was the more consistent method (using a dn/dc value of 0.167). They measured HA samples in the molecular weight range of

$4.8x10^4$ to 1.45 million Da, the upper end is in the same range as the HA samples measured for this thesis.

Soltes et. al. (2002) also used SEC-MALLS to measure the molecular weight and distribution of some commercially available HA products. Light scattering techniques can be difficult to perform, especially for high molecular weight molecules and aqueous systems. Information gained by these researchers regarding experimental conditions is invaluable.

Some have used molecular characterization to explore the configuration of HA in solution. This is done primarily by constructing a Mark-Houwink-Sakurada (MHS) equation using intrinsic viscosity and molecular weight measurements. Mendichi et. al. (2003) found an "a" value of 0.783, indicating HA behaves as a random coil polymer in solution. They also divided the HA data into three groups based on molecular weight (Mw): $Mw < 100,000$, $100,000 < Mw < 1,000,000$, and $Mw > 1,000,000$. They found smaller molecules tend to be stiffer than large molecules, with "a" values of 1.056, 0.778, and 0.604 respectively. It is more difficult for small molecules coil, the extreme example is a two-monomer molecule. A molecule with two monomers must be linear, but as monomers are added to the chain the backbone becomes more flexible with a greater ability to coil. This is important to note because the molecular behavior of different

molecular weight polymer solutions can vary not just because of degree of entanglement, but molecular flexibility.

Another group led by Vercruysse (1995) found values for the MHS and other polymer theory equations for HA in the 10,000 to $1x10^6$ Da molecular weight range. Their "a" value for an MHS relationship was 0.72 which is similar to Mendichi et. al.

Another point of interest of HA research has been conformation changes with pH. Both Reed et. al. (1989) and Park et. al. (1978) found that the measurement of molecular weight measured by light scattering is constant between pH 2-10, although the radius of gyration appears to decrease below pH 5. It is good to know that large pH differences between solvents should not affect light scattering results, making the light scattering of HA in any salt solution comparable to other studies.

2.3 Theoretical Background

2.3.1 Polymer Theory

Polymers are very large molecules and are subject to a distribution of molecular weight. This distribution can very greatly depending on method and conditions of synthesis thus the molecular weight can be described in different ways. The first moment of molecular weight is the number average molecular weight, Mn, defined by equation 2-1. The number average molecular weight measures as if each

polymer molecule has an equal probability of being measured. Where M_i is the molecular weight of the i^{th} molecule and n_i is the number of molecules, i.

$$Mn = \frac{\sum n_i M_i}{\sum n_i} \qquad \text{Eqn. 2- 1}$$

The second moment of molecular weight is the weight average molecular weight, Mw. The weight average molecular weight counts as if each monomer has an equal chance of being picked, so a larger molecule has a greater chance of being counted. The weight average molecular weight will always be larger than the number average molecular weight.

$$Mw = \frac{\sum n_i M_i^2}{\sum n_i M_i} \qquad \text{Eqn. 2- 2}$$

The z average molecular weight, Mz is the largest molecular weight value. It is gives the high molecular weight molecules even more importance than Mw does and can be useful in the identification of high molecular weight tails.

$$Mz = \frac{\sum n_i M_i^3}{\sum n_i M_i^2} \qquad \text{Eqn. 2- 3}$$

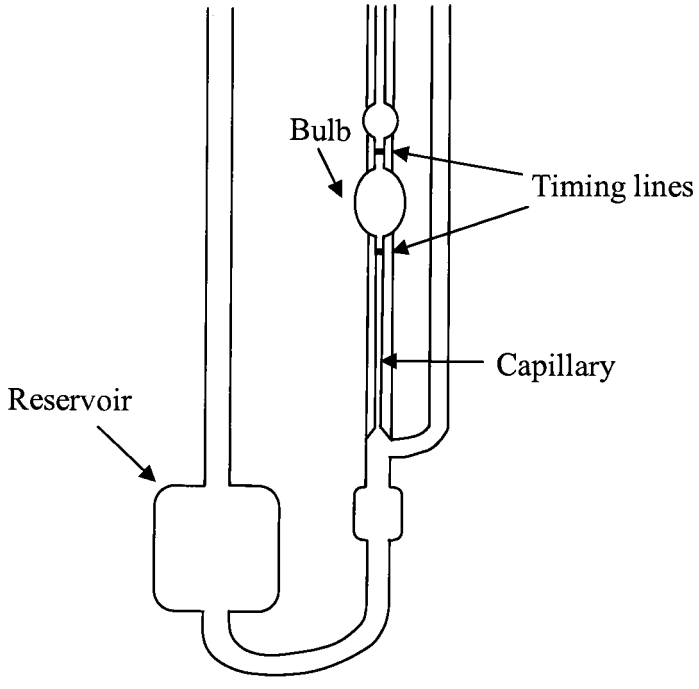
These different ways of measuring molecular weight lead to molecular weight distribution which is an important value in characterizing any polymer system.

2.3.2 Intrinsic Viscosity

The intrinsic viscosity, [η], of a polymer solution is measured in the dilute regime. If a polymer solution is diluted enough, the effects of *single* molecules on flow rate can be observed which allows determination of the molecular weight.

Intrinsic viscosity measurements are taken in viscometers (Figure 2-2). Although there are many types of viscometers, they all have some basic features. These include a bulb with timing lines above and below and a thin capillary which limits the flow of the solution. Many viscometers have a large reservoir at the bottom so the concentrations can be diluted in the viscometer itself. To perform a measurement, the liquid is pulled up through the capillary and above the first timing line. The time, t, it takes for the meniscus of polymer solution to pass between the two lines is proportional to solution viscosity, η.

Solution viscosity is a result of many factors, including solvent, solute, solvent-solute interactions, concentration, and temperature. The flow times of a polymer solution are compared to the flow time of the solvent to remove temperature and solvent effects.



(Geisler, 2004)

Figure 2- 2 Diagram of a Cannon-Ubbelohde dilution viscometer

The specific viscosity (equation 2-4) is the viscosity of the polymer solution minus solvent viscosity relative to the solvent (where η_s and t_s are the viscosity and flow time of the solvent respectively). Flow time and viscosity are proportional, therefore they can be substituted for each other in any of the following equations, equations 2-4 through 2-6, however only the viscosity will be shown.

$$\eta_{sp} = \frac{\eta - \eta_s}{\eta_s} = \frac{t - t_s}{t_s} \qquad \text{Eqn. 2- 4}$$

Reduced viscosity is the specific viscosity, η_{sp}, with the effects of concentration removed. Specific viscosity is divided by the solute concentration, c, to yield the reduced viscosity, η_{red}.

$$\eta_{red} = \frac{\eta_{sp}}{c} \qquad \text{Eqn. 2- 5}$$

An extrapolation to zero concentration removes all entanglement effects (polymer-polymer interactions) and yields the intrinsic viscosity, $[\eta]$.

$$[\eta] = \lim_{c \to 0} \frac{\eta - \eta_s}{\eta_s c} \qquad \text{Eqn. 2- 6}$$

The intrinsic viscosity is essentially a model of the contribution of a single molecule to overall solution viscosity. It is especially useful in the study of polymers for many reasons. First, it can be related to the entanglement concentration, c*. This is the concentration where polymer molecules begin to overlap and entangle resulting in a change of solution characteristics.

$$[\eta] = \frac{1}{c^*} \qquad \text{Eqn. 2- 7}$$

Intrinsic viscosity can also be related to molecular weight by the Mark-Houwink-Sakurada Equation (Sperling, 2001). This equation is specific to a polymer and solvent system. If the constants "K" and "a" are known, the intrinsic viscosity is a very powerful number. An MHS equation will be derived for HA in PBS in this chapter.

$$[\eta] = KM_w^{\ a} \qquad \text{Eqn. 2- 8}$$

The "a" value is a very telling parameter of polymer configuration and polymer-solvent interactions. If the "a" value is 0.5, the solvent is poor for that polymer (theta solvent). If "a" is between 0.6-0.8 the polymer is in random coil configuration in solution, and if the "a" value greater than 0.8 the polymer behaves as a rigid rod. DNA is an example of a rigid rod polymer; the helical shape of the back bone makes the molecule very stiff (Sperling, 2001).

2.3.3 Light Scattering

Light scattering is a dilute solution characterization method that can be used to determine absolute weight average molecular weight, molecular size through the radius of gyration, and the second virial coefficient. (The second virial coefficient (A_2) is an indication of the solvent quality, an A_2 value of zero is a poor solvent.) This measurement can be performed statically in batch mode and in combination with size exclusion chromatography (SEC) using a flow through cell modes.

2.3.3.1 Batch Light Scattering

Light of a uniform wavelength is emitted from a source, typically from a helium-neon laser, and passes through a glass cell containing the solution of interest (Figure 2- 3). Eighteen detectors are positioned at varying angles (ranging from 15°-163°) to simultaneously measure the intensity of scattered light, $I(\Theta)$. This technique is often referred to as multi-angle laser light scattering (MALLS) The intensity of light scattered is proportional to the product of polymer mass and

concentration, whereas the angular variation of scattered light is related to size (radius of gyration).

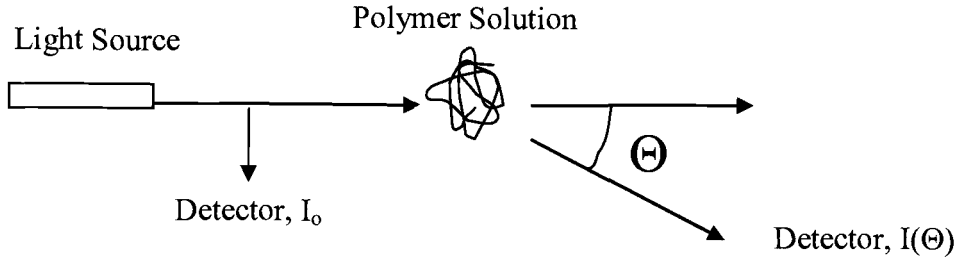


Figure 2- 3 Path of light scattered through a polymer solution

In batch light scattering, multiple concentrations of the same polymer material are sent through the flow cell and the intensity of light is measured at all 18 angles. This data is interpreted using the method developed by Zimm. Some light is split from the original beam to measure the incidental intensity, I_o.

The incident light and scattered light in light scattering experiments are related by the Rayleigh Ratio (equation 2-9).

$$R(\Theta) = \frac{I(\Theta)}{I_o} \qquad \text{Eqn. 2- 9}$$

$P(\Theta)$ is the scattering function which relates angular variation of scattering intensity to the radius of gyration, $\langle r_g \rangle$ where n_o is the solvent refractive index and λ is the wavelength of light.

$$\frac{1}{P(\Theta)} = 1 + \frac{16\pi^2 n_o^2}{3\lambda^2} \sin^2 \frac{\Theta}{2} \langle r_g^2 \rangle \qquad \text{Eqn. 2- 10}$$

The basic light scattering equation developed by Zimm (1948) is as follows:

$$\frac{K^* c}{R(\Theta)} = \frac{1}{M_w P(\Theta)} + 2A_2 c \qquad \text{Eqn. 2- 11}$$

A_2 is the second virial coefficient which denotes solvent/solute interactions (A_2 is greater than zero for a good solvent). Mw is the weight average molecular weight. The variable c is the concentration of solute, and K* is the optical constant defined below (equation 2-12).

$$K^* = \frac{4\pi^2 n_o^2 \left(\frac{dn}{dc}\right)^2}{N_A \lambda^4} \qquad \text{Eqn. 2- 12}$$

where:

dn/dc = differential refractive index of a solution

n_o = refractive index of solvent

λ = wavelength of light

N_A = Avogadro's number

In the basic light scattering equation, all variables on the left hand side of the equation are known or measured quantities. Thus the molecular weight and size can be obtained using the right hand side of the equation and a double

interpolation of data using a Zimm plot. The basic light scattering equation is plotted on the y-axis of a Zimm plot vs. equation 2-13.

$$\text{x axis: } \sin^2\frac{\Theta}{2} + kc \qquad \text{Eqn. 2- 13}$$

where:

k = "stretch factor," arbitrarily chosen to make the plot readable

First, the zero angle interpolation of the basic scattering equation removes all angular (size) effects, thus leaving only concentration effects. An examination of equation 2-10 will reveal, as $\Theta \rightarrow 0$, $P(\Theta) \rightarrow 1$, leaving an intercept of 1/Mw and the second virial coefficient embedded in the slope.

$$\frac{K^* c}{R(0)} = \frac{1}{M_w P(0)} + 2A_2 c \qquad \text{Eqn. 2- 14}$$

The interpolation of zero concentration eliminates the concentration contribution to the solution behavior, leaving only angular effects. The inverse of molecular weight is the intercept of this line as well, and the radius of gyration can be obtained from the slope.

$$\frac{K^* c}{R(\Theta)} = \frac{16\pi^2 n_o^2}{3\lambda^2}\left\langle r_g^2 \right\rangle \sin^2\frac{\Theta}{2} + \frac{1}{M_w} \qquad \text{Eqn. 2- 15}$$

Below is an example of a Zimm plot for polystyrene in toluene. Note the zero concentration and zero angle interpolation lines are marked on the graph. The y-intercept is the inverse of the weight average molecular weight, Mw.

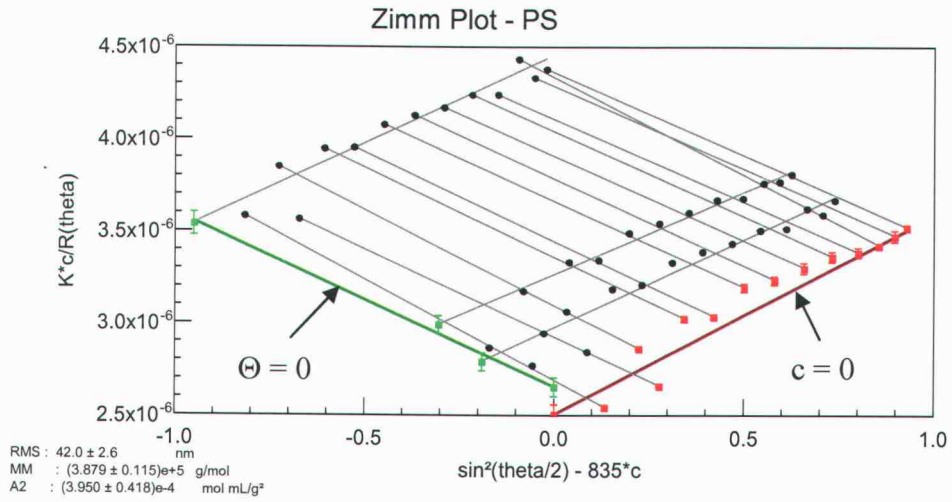


Figure 2- 4 An example of a Zimm plot of polystyrene in toluene constructed at OSU

Although static light scattering is a very useful method to gather information about molecular size and weight, it reveals nothing about the molecular weight distribution. Moreover, the concentration of polymer in solution must be known. If the desire is to measure the polymer concentration in a biological fluid, for example, the concentration is not known. The concentration would have to be determined in another method prior to light scattering. Size exclusion chromatography (SEC) light scattering allows one to measure both molecular weight and concentration in one test which can be both convenient and useful.

2.3.3.2 Size Exclusion Chromatography with Multi-angle Laser Light Scattering

Gel permeation chromatography separates molecules by size. A typical set-up for SEC-MALLS is shown below.

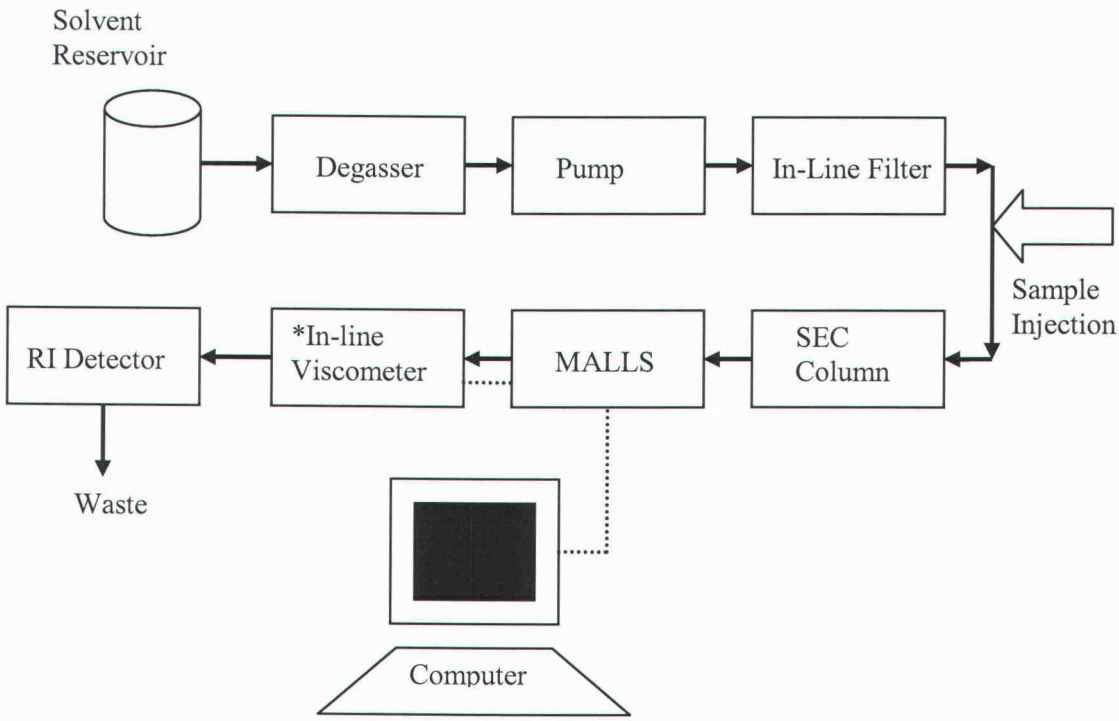


Figure 2- 5 Typical SEC-MALLS flow diagram; solvent enters the degasser and is pumped through filters before the sample is injected and passes through columns and detectors

*Note: In-line viscometer not a part of the OSU system, but was used at Wyatt Technology Corporation

Solvent is held in a reservoir where gas is removed in a degasser to ensure no gas comes out of solution during the pressure changes in the system. The solvent is thoroughly filtered to ensure the cleanliness necessary for light scattering before

the sample is injected. The solvent carries the sample through one or more columns where size separation occurs. Columns are typically packed with porous plastic or ceramic. Smaller molecules will enter the pores of the packing material, while large molecules will be excluded from the pores. Larger molecules elute from the column more rapidly due to a shorter path length.

Following elution from the columns the solution enters the light scattering detector where peaks are recorded at each of the eighteen angles. The refractive index detector (RID) also records a peak, but of the change in refractive index. If the dn/dc value of the sample is known, the RID peak can be integrated to yield concentration. Additionally, an-line viscometer can be used to measure sample viscosity as it elutes from the system.

The light scattering data can be used to determine molecular weight; however a Zimm plot cannot be constructed because only one concentration is measured. Thus a Debye plot is used instead. Figure 2-6 is an example of a Debye plot for on of the HA samples in phosphate buffer solution.

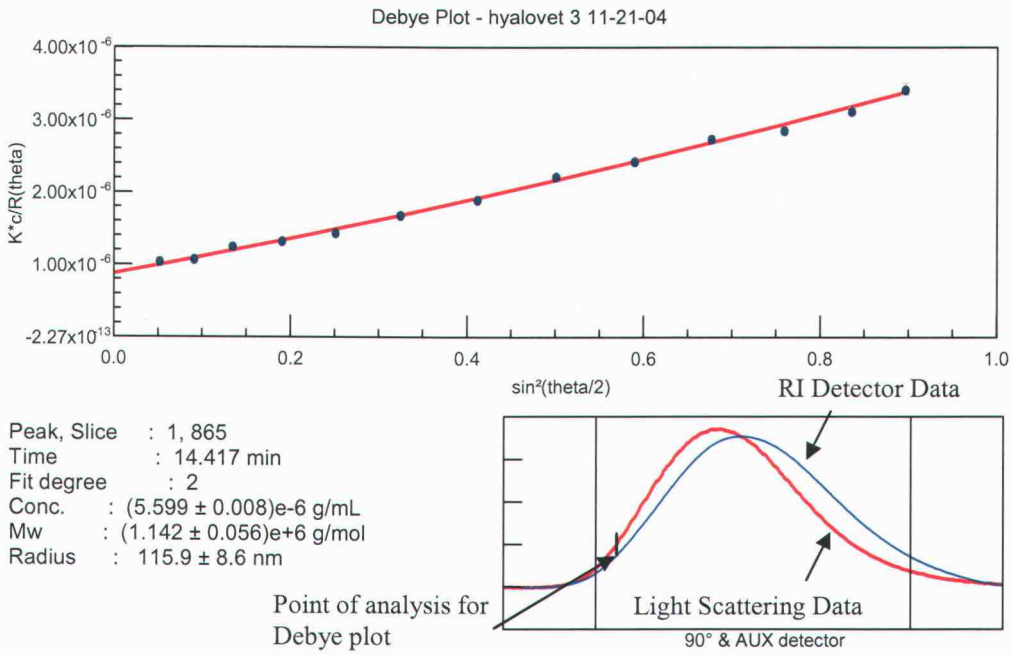


Figure 2- 6 Debye plot of Hyalovet, an HA supplement, tested at OSU

The Debye plot is similar to a single concentration line from a Zimm Plot, and gives molecular weight and radius information. Note that it has the same axes as a Zimm plot. Because there is no interpolation to zero concentration, the second virial coefficient is unobtainable using this method. One Debye plot with a unique molecular weight can be constructed from every point of the elution peak in the light scattering data. When this information is matched with the RI detector data, one can calculate molecular weight distribution of the sample.

If an in-line viscometer is used, the intrinsic viscosity and even the "K" and "a" values of the MHS equation may be found. Due to size separation in the columns, the polymer is extremely dilute as it elutes into the detectors. In fact, the concentration is so dilute that it can be theoretically considered "zero" concentration; therefore the viscosity measured by the viscometer is actually the intrinsic viscosity. To measure viscosity, the viscometer splits the flow into two sides. On one half the sample flows through a capillary. On the other side the sample enters a column to delay its elution, followed by a capillary. As this half of the sample is delayed, solvent is running through the capillary. The pressure difference between the two capillaries is measured, which is the difference between solvent and sample flow. The pressure reading is used to calculate viscosity. Since molecular weight is being measured simultaneously with intrinsic viscosity, a Mark-Houwink-Sakurada plot can be constructed from a single if the molecular weight distribution of the sample is broad (usually $Mw/Mn > 2$) (Sperling, 2001; Wyatt 1992).

2.3.4 RHEOLOGY

Rheology is the study of how materials deform in response to outside forces. Rheological studies are most commonly applied to non-Newtonian fluids and gels to characterize viscosity as a function of shear rate, yield stress, elasticity, and other fluid properties. A Newtonian fluid, such as water, has a constant viscosity for any shear rate and no elasticity. The measurement of elasticity relies on

polymer entanglements within the solution. However entanglements can occur because of large molecules or high concentrations and it can be difficult to discern which makes a greater contribution to rheological properties.

2.3.4.1 Steady Shear Rheology

Figure 2-7 is an example for the experimental set-up for a steady shear experiment. The fluid is placed on a flat plate and a truncated cone is lowered in contact with the fluid. (Here the cone angle ζ is greatly exaggerated, for rheological measurements the angle of the cone is between 1-5°.) The distance between the cone and plate is such that if the cone were not truncated the tip of the cone would just be in contact with the plate. This distance is called the gap. The flow geometry will be approximated by a non-truncated cone in contact with the plate.

Once the fluid is appropriately loaded onto the plate, the cone rotates at a specified shear rate, $\dot{\gamma}$. All polymeric fluids have some resistance to flow, which is measured by the torque, T, acting on the plate. The measured torque is used to calculate the shear stress, τ, throughout the fluid. The shear stress divided by the shear rate is the viscosity, η.

$$\eta = \frac{\tau}{\dot{\gamma}} \qquad \text{Eqn. 2- 16}$$

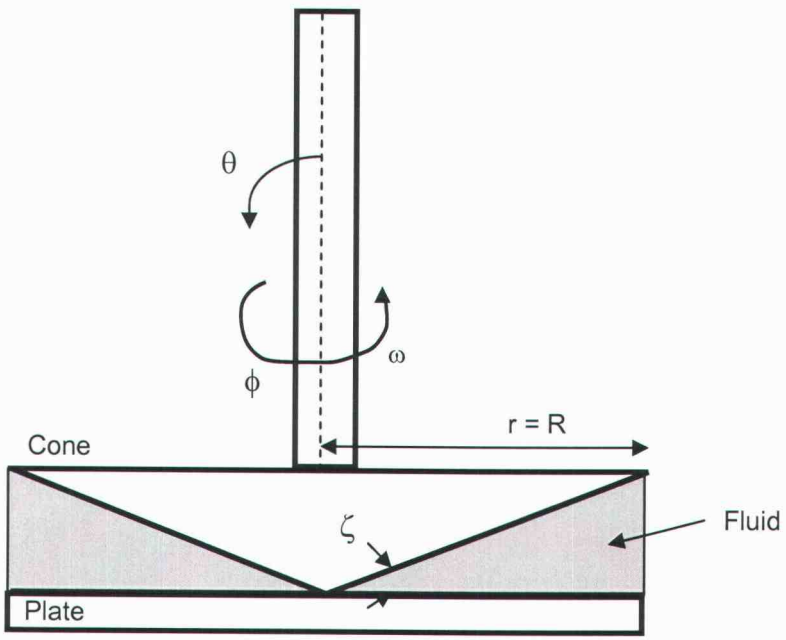


Figure 2- 7 Diagram of cone and plate geometry in a rheometer

Velocity in the θ and r directions is assumed to be zero so only velocity in the ϕ direction, v_ϕ, will be addressed. The system is also symmetric about r = 0 so the partial derivative in the ϕ direction, $\delta/\delta\phi$, will be taken as zero. While there is a velocity gradient in the r direction, stress in the r and ϕ plane, $\tau_{r\phi}$, is ignored since it is not measured by the cone and does not contribute to viscosity calculations. Pressure gradients and gravity effects are also ignored. It is also assumed that the system is isothermal and all physical properties (excluding viscosity) are constant. This is a very reasonable assumption since the fluids are incompressible and all tests are completed in thirty minutes making temperature change negligible.

Before modeling the slightly complex flow between a cone and plate, consider simple shear flow between two parallel plates where the upper plate is moving at velocity, v (Figure 2-8).

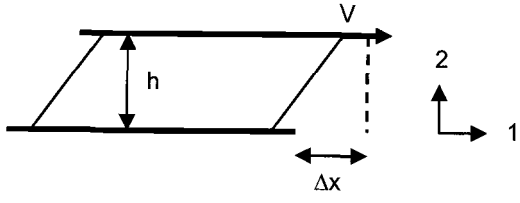


Figure 2- 8 An example of flow between two parallel plates where the top plate is moving at velocity, v

Here the shear rate, $\dot{\gamma}$, imparted on the fluid is defined as the velocity, V, of the plate divided by the plate spacing, h.

$$\dot{\gamma} = \frac{V}{h} \qquad \text{Eqn. 2- 17}$$

If there is no pressure drop in the direction of flow, the velocity in the x direction, v_x, be linearly related to the distance from the top plate according to equation 2-18 (here the axis is taken as x-y instead of 1-2).

$$v_x = V\frac{y}{h} \qquad \text{Eqn. 2- 18}$$

The stress tensor is defined as:

$$\tau = \begin{bmatrix} \tau_{11} & \tau_{12} & 0 \\ \tau_{21} & \tau_{22} & 0 \\ 0 & 0 & \tau_{33} \end{bmatrix} \qquad \text{Eqn. 2- 19}$$

where τ_{11}, τ_{22}, and τ_{33} combine to make the normal stresses. The stresses τ_{12} and τ_{21}, are equal and necessary to calculate the viscosity.

Now consider the cone-and-plate shear flow in question. Directions 1 and 2 in Figure 2-8 will be ϕ and θ of Figure 2-7 respectively. As discussed previously, velocity is imparted on the fluid in the ϕ direction only and the only component of the stress tensor included in the calculation of torque is $\tau_{\phi\theta}$.

The above example can be used to determine the velocity profile of the fluid of the cone-and-plate model. For the fluid at the top of the gap in contact with the cone, the velocity in the ϕ direction is defined by ωr, where ω is the rotation rate of the cone and r is the radial position. At the center of the cone $r = 0$, $v_\phi = 0$, and at the edge of the cone $r = R$ and $v_\phi = \omega R$. The vertical distance from the plate to any point in the fluid is described by equation 2-20 and the total height of the gap is described as equation 2-21 where ζ is the cone angle. For very small x, $\sin(x) \approx x$. Since the quantities $(\pi/2-\theta)$ and ζ are very small, the distance between the plate and the fluid and the total height of the gap can be approximated by $r(\pi/2-\theta)$ and $r\zeta$ respectively.

$$y = r\sin(\frac{\pi}{2} - \Theta) \approx r(\frac{\pi}{2} - \Theta) \qquad \text{Eqn. 2- 20}$$

$$h = r\sin(\zeta) \approx r\zeta \qquad \text{Eqn. 2- 21}$$

This yields the following equation for velocity distribution for rotation rate, ω:

$$v_\phi = \omega r \frac{\pi/2 - \theta}{\zeta} \qquad \text{Eqn. 2- 22}$$

Next the shear rate as a function of position can be determined. The shear rate in the ϕ, θ direction, $\dot{\gamma}_{\phi\Theta}$ is given as equation 2-23 where v_ϕ is the velocity in the ϕ direction (the direction of interest) and ρ, ϕ and θ are axial positions.

$$\dot{\gamma}_{\phi\theta} = \frac{\sin\theta}{r}\frac{\delta}{\delta\theta}\left(\frac{v_\phi}{\sin\theta}\right) + \frac{1}{r\sin\theta}\frac{\delta v_\theta}{\delta\phi} \qquad \text{Eqn. 2- 23}$$

As previously determined, the velocity in the θ direction, v_θ, is equal to zero. The gap between the cone and plate is very small, so θ can be approximated as π/2, and sinθ = 1. This simplifies equation 2-23. Recall that ω is the rotation rate and ζ is the cone angle.

$$\gamma_{\phi\theta} = \frac{1}{r}\frac{\delta v_\phi}{\delta\theta} = -\frac{\omega}{\zeta} \qquad \text{Eqn. 2- 24}$$

Thus the shear rate is constant throughout the fluid and the shear stress is constant. Now a relationship between torque and shear stress can be developed (recall that torque is the measured by a transducer during experiments). Torque (T) is the integral of shear stress in the ϕ,θ direction, multiplied by the moment arm (r) over the total area:

$$T = \int_0^{2\pi R}\int_0^{} \tau_{\phi\theta} r^2 dr d\phi \qquad \text{Eqn. 2- 25}$$

$$\tau_{\phi\theta} = \frac{3T}{2\pi R^3} \qquad \text{Eqn. 2- 26}$$

The viscosity (η) can now easily be calculated from equation 2-24 and 2-26 ($\dot{\gamma} = -\dot{\gamma}_{\phi\Theta}$):

$$\eta(\gamma) = \frac{\tau_{\phi\theta}}{\dot{\gamma}} = \frac{3T\zeta}{2\pi R^3 \omega} \qquad \text{Eqn. 2- 27}$$

where:

T = torque

$\tau_{\phi\theta}$ = shear stress is ϕ,θ direction

r = radial position

R = outer radius of the cone

ω = rotation rate

At low shear rates many non-Newtonian solutions exhibit a viscosity that is independent of shear rate. This area is often called the Newtonian plateau, viscosity values in this range are referred to as zero shear viscosity (η_o). For an ideal, monodisperse polymer, zero shear viscosity is proportional to the weight average molecular weight to the first power for dilute regimes and it is proportional to the weight average molecular weight to the 3.4 power in entangled solutions

Another measure of Newtonian behavior is through the power law index, n. In a power law fluid, shear stress is proportional to shear rate to the n power, where n is known as the power law index.

$$\tau = k\dot{\gamma}^{n} \qquad \text{Eqn. 2- 28}$$

For a Newtonian fluid, n is equal to one and k becomes the viscosity. The lower the n value the more shear thinning the fluid and the less Newtonian it is (Sperling, 2001; Bird et. al. 1987; Walters 1975).

2.3.4.2 Dynamic Oscillatory shear Rheology

Samples are loaded in the same manner as steady shear tests (Figure 2-7) using the cone and plate geometry. Instead of rotating at a steady speed the cone oscillates, applying a sinusoidal stress (σ) to the sample. Equation 2-29 describes the sinusoidal stress, where ω is the angular frequency.

$$\sigma = \sigma_o \sin \omega t \qquad \text{Eqn. 2- 29}$$

The strain is the displacement from the "zero point" of the cone at a given frequency. In a Hookian solid all energy is preserved and the strain (ε) is given as:

$$\varepsilon = \varepsilon_o \sin \omega t \qquad \text{Eqn. 2- 30}$$

In an entanglement the stress and strain will not be in phase; some energy will be lost via viscous dissipation. The difference between stress and strain is δ, the

phase angle. The in-phase (σ') and out-of-phase (σ") components of the stress are described by equation 2-31 and 2-32.

$$\sigma' = \sigma_o \cos\delta \qquad \text{Eqn. 2- 31}$$

$$\sigma'' = \sigma_o \sin\delta \qquad \text{Eqn. 2- 32}$$

Where:

σ = stress

σ' = in-phase stress component

σ" = out-of-phase stress component

σ_o = amplitude of stress

δ = phase angle

The elastic modulus, G', is the in-phase modulus and describes any elastic behavior in a fluid. An example of a purely elastic system is a rubber band. If a stress is placed on a rubber, the energy can be returned retained and the rubber will return to its original shape. Conversely, a Newtonian fluid such as water will have a G' value of zero because there is no energy retention. A physical model for a pure elastic system is a spring, where all energy put into the system can be retrieved. Mathematically, the elastic modulus, G', is described by equation 2-33. Recall that ε_o is the amplitude of the strain and σ' is the in-phase component of the stress.

$$G' = \frac{\sigma'}{\varepsilon_o} = G^* \cos\delta \qquad \text{Eqn. 2- 33}$$

The viscous, G" modulus is the out of phase modulus, calculated using the out-of-phase component of stress and strain. The viscous modulus (equation 2-34) describes to what extent the substance being measured behaves like a fluid. It can is also referred to as the "loss" modulus, since any stress or deformation imparted on a Newtonian fluid is lost. A physical model of this system is a dashpot.

$$G'' = \frac{\sigma''}{\varepsilon_o} = G^* \sin\delta \qquad \text{Eqn. 2- 34}$$

The complex modulus, G* is related to G' and G" by equation 2-35.

$$G^* = G' + iG'' \qquad \text{Eqn. 2- 35}$$

A polymer system will have both elastic and viscous properties. Because the molecules are so large, their degree of entanglement can contribute elastic properties to the system. Examples of results from the dynamic oscillatory shear test are in Figure 2-9, dynamic data of National Bureau of Standards (NBS) 1490, a polyisobutylene sample. If G" is greater than G', the fluid is in the viscous region meaning it behaves like a lubricating fluid. If the converse is true the fluid is behaving like a rubber. It is important to note that regardless of the region describing the polymer fluid behavior, it will always have both *viscous* and *elastic* properties. This is why polymer solutions are called *viscoelastic* fluids.

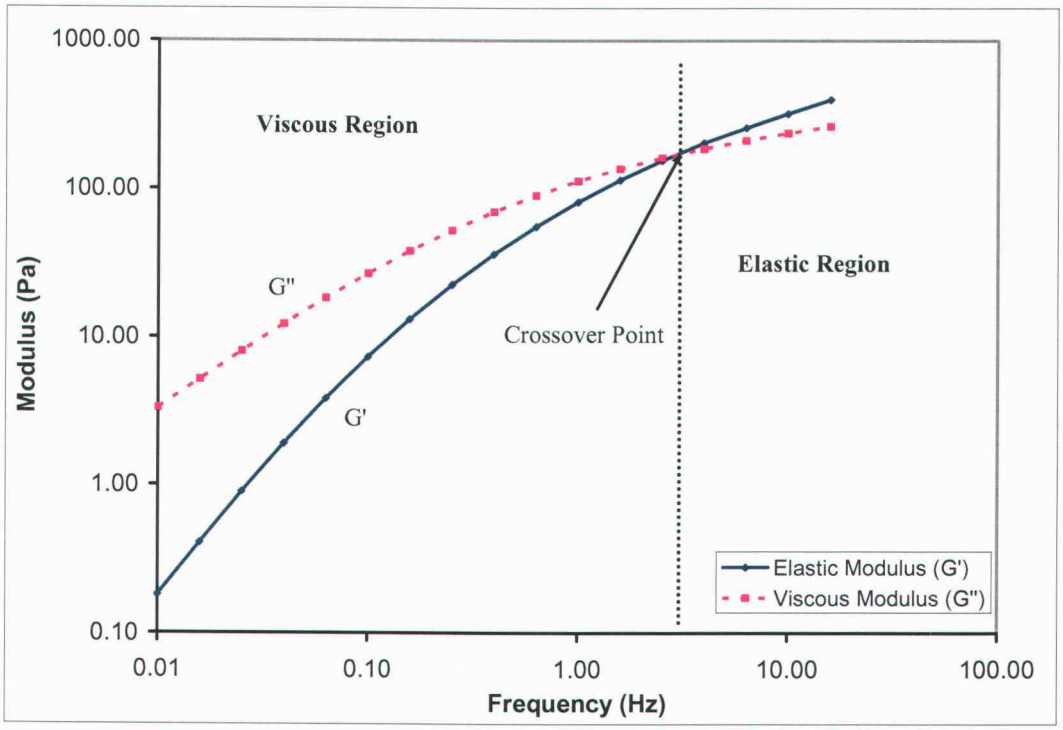


Figure 2- 9 Example results from a dynamic oscillatory shear test of NBS 1490, a polyisobutylene sample

The point where G' becomes higher than G" is called the crossover point. This point occurs at a crossover frequency, ω_c, which is equal to the inverse of relaxation time, τ_R. The relaxation time is the time required for a polymer system to relax after a stress is imposed. If the length of test time is longer than the relaxation time the polymer will be able to fully relax. Then it will act like a viscous fluid. If the test time is too fast and does not allow the polymer to relax, the system will act like a gel. If the polymer solution is a true gel, G' and G" will

be parallel to each other, with G' dominating. Relaxation time, τ_R and crossover frequency, ω_c, can be related to molecular weight, Mw, by equation 2-36.

$$\tau_R \propto (\omega_c)^{-1} \propto M^{3.4} \qquad \text{Eqn. 2- 36}$$

A larger polymer will have a greater relaxation time (due to a higher degree of entanglement), and will consequently exhibit a crossover point at a lower frequency.

Additionally, the complex viscosity, η^* can be defined as follows.

$$\eta^* = \frac{\left[(G')^2 + (G'')^2\right]^{1/2}}{\omega} \qquad \text{Eqn. 2- 37}$$

Notice the complex viscosity contains both viscous and elastic components which can provide additional information to the viscosity behavior of viscoelastic fluids. The steady shear viscosity measures viscous properties only. As the frequency becomes low, the elastic modulus becomes small and the viscous modulus dominates the complex viscosity term. For this reason, at low frequencies the complex viscosity approaches steady shear viscosity, known as the Cox-Merz Rule (Sperling, 2001; Bird et. al. 1987; Walters 1975).

2.4 MATERIALS AND METHODS

2.4.1 SAMPLES

Ten different HA samples were examined using the described characterization methods. Information regarding the sources, estimated molecular weights and intended uses is summarized below in Table 2-1.

Table 2- 1 Summary of sodium hyaluronate samples used in study

Sample Name	HA Source	Intended Use	*Mw (Da)	HA Conc. (mg/ml)
[1]HAC3Na	Chicken Combs	Laboratory	1.0×10^6	Frozen
[1]HAC2Na	Chicken Combs	Laboratory	0.9×10^6	Frozen
[1]HAC1Na	Chicken Combs	Laboratory	0.6×10^6	Frozen
[1]HA1NaL	Umbilical Cords	Laboratory	0.5×10^6	Frozen
[1]HA2NaF	Umbilical Cords	Laboratory	0.8×10^6	Frozen
[2]Synthovial 7	Fermentation	Human Oral	2.0×10^6	3
[2]Hyalun	Fermentation	Equine Oral	2.0×10^6	5
[3]Legend	Fermentation	Equine IV	N/A	10
[4]Hyalovet	Chicken Combs	Equine IA	N/A	10
[5]Hyvisc	"natural sources"	Equine IA	N/A	11

Manufacturer
[1]Biozyme Laboratories, San Diego, CA
[2]Hyalogic™, Edwardsville, KS
[3]Bayer Pharmaceuticals Corp, West Haven, CT
[4]Wyeth, Madison, NJ
[5]Anika Therapeutics, Woburn, MA
*Manufacturer's estimation of Mw

The five samples from Biozyme Laboratories were donated by Biozyme. These samples were lyophilized and frozen; they were intended for laboratory use. The other HA samples are marketed by their respective companies as oral, intra-articular, or intravenous supplements for treatment of arthritis in horses and humans. The samples from Hyalogic were also donated to the lab.

Many of the experiments required dilution of HA. In all cases, these dilutions were performed in a phosphate buffer saline (PBS). PBS is often used to mimic plasma (the base component of synovial fluid) because of ionic concentration and pH. The recipe used was 7.813 milli-molar (mM) sodium phosphate dibasic, 21.875 mM sodium phosphate monobasic and 150 mM sodium chloride.

2.4.2 Intrinsic Viscosity

All intrinsic viscosity measurements were performed in a #75 Cannon-Ubbelohde Micro-Viscometer from Cannon Instrument Company (http://www.cannon-ins.com/). Hyaluronate solutions were prepared at initial concentrations of about 0.5 mg/ml. A minimum of five dilutions were performed until a final concentration of about 25% of the original was obtained.

2.4.3 SEC-Light Scattering

Only combined size exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) was performed on HA samples with PBS as the mobile

phase. Stock solutions were prepared by dissolving or diluting HA in 0.02 μm filtered PBS. Seven of the ten sodium hyaluronate samples listed in Table 2-1 were tested at WTC, and all samples (except Hyvsic) were tested at OSU.

The facilities at OSU include the apparatus shown in Figure 2-10. All HA samples (except Hyvisc) were tested at concentrations around 0.5 mg/ml at flow rates of 0.2 ml/min. At this concentration the samples were subject to a phenomenon known as viscous fingering, which will be discussed in detail in Section 2.5.2. Additionally, light scattering was performed a second time at OSU (light scattering and RI detector only) for the samples Sythnovial 7, HAC1Na, and Hyalovet at a concentration of about 0.1 mg/ml and a flow rate of 0.5 ml/min.

At OSU, the pump used was a Hitachi La Chrom with built-in degasser. A Rheodyne manual injector was attached with a sample loop of 500 μl at OSU. A Polymer Labs (PL) Aquagel OH 8 μm pre-column and column were used for separation. The light scattering detector was a Dawn EOS by Wyatt Technologies Corporation, followed by a La Chrom RI Detector by Hitachi.

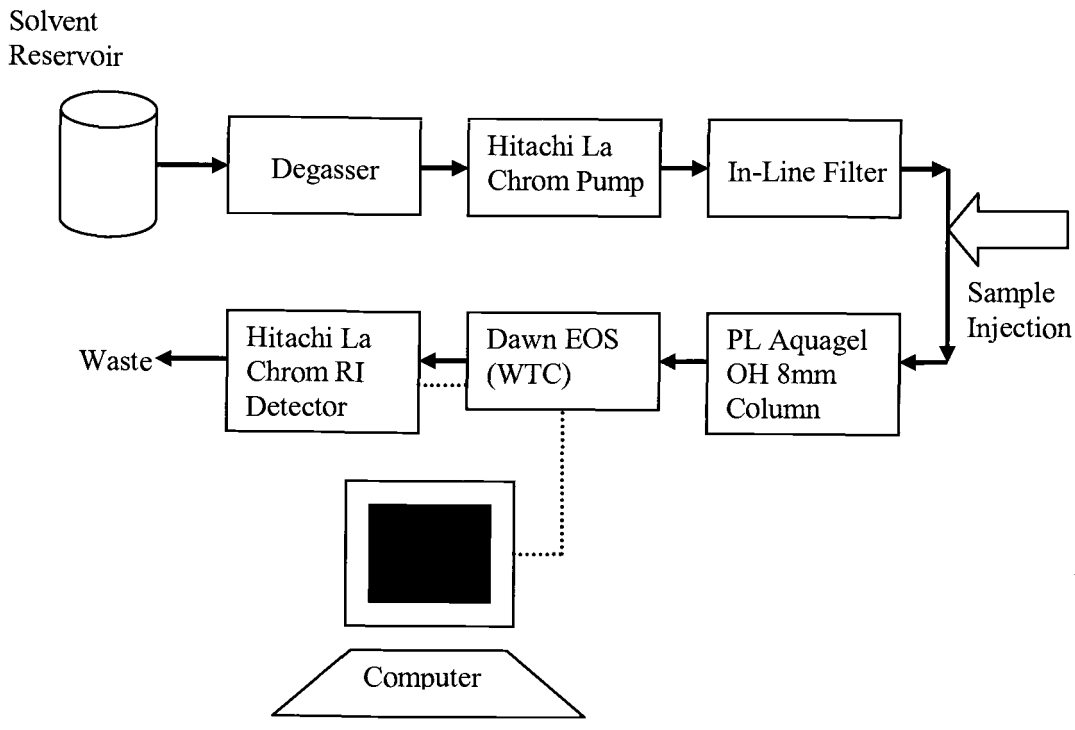


Figure 2- 10 SEC-MALLS system at Oregon State University

Seven of the ten HA samples listed in Table 2-1 were tested at Wyatt Technology Corporation (WTC, www.wyatt.com) in order do SEC-MALLS followed by an in-line viscometetry using their ViscoStar. This allows for the determination of intrinsic viscosity and the Mark-Houwink-Sakurada equation for a single sample (Figure 2-11). Hyaluronate was dissolved in filtered PBS to concentrations of about 0.1 mg/ml. Samples tested at WTC were separated using the same polymer lab columns as at OSU, but a WTC Optilab® Rex refractive index detector and Viscostar viscometer were used. In addition, a 900 µl injection loop was used to

inject samples. Astra 5 was used to analyze data collected at WTC and Astra 4 was used at OSU. Second order Zimm formalism was used to analyze all HA data.

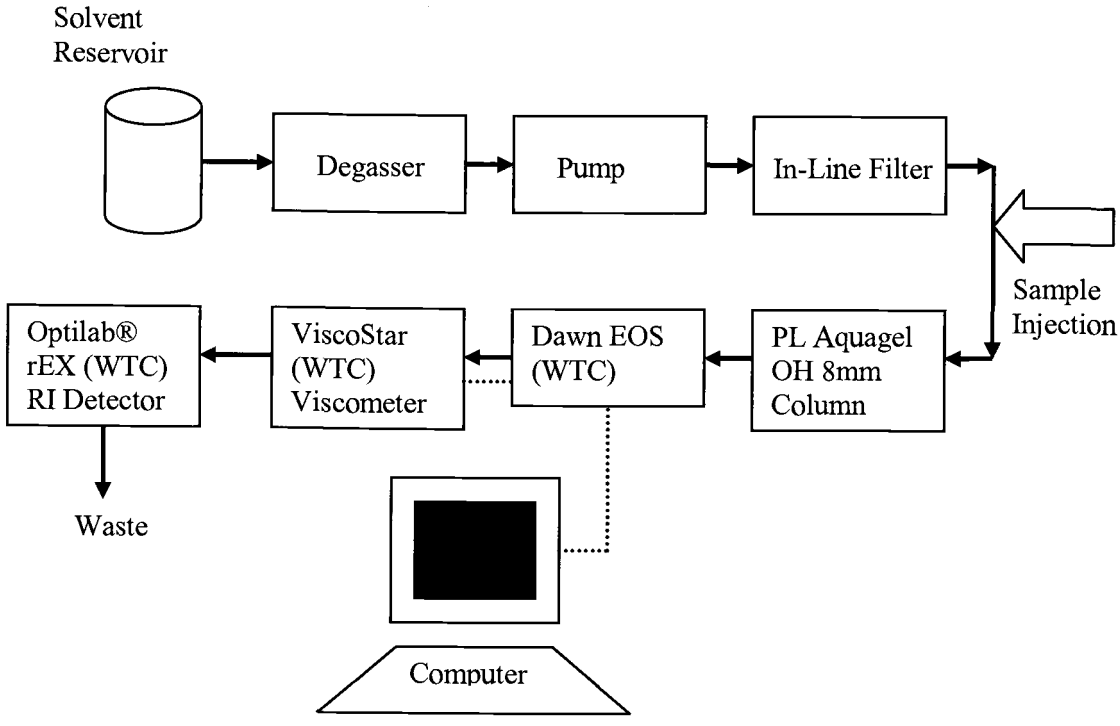


Figure 2- 11 SEC-MALLS equipment at Wyatt Technology Corporation

2.4.4 RHEOLOGY

Since each supplemental HA sample was in solution at different concentrations, it was deemed most appropriate to perform rheology at a single uniform concentration. Later comparisons between pure HA and synovial fluid will be made, so a concentration within the estimated range of synovial fluid was chosen.

All rheological experiments were performed at 2.5 mg/ml (dilutions made in PBS). All experiments were performed with the Rheometrics Fluids Spectrometer (model RFS II) using a cone-and-plate geometry with a 50 mm diameter, 1° cone.

2.4.4.1 Steady Shear

One goal of the steady shear tests was to measure the zero shear viscosity of HA solutions to compare to sample molecular weight. Thus viscosity at the lowest possible shear rate was measured. Viscosity was measured at shear rates between 0.1-100 or 1000 s^{-1} however reliable data was not always collected at these low shear rates.

2.4.4.2 Dynamic Oscillatory Shear

All dynamic tests were performed in the oscillatory frequency range of 0.01-10 Hz. Strain values ranged from 50%-100% (0.5-1.0 strain units) depending on the sample. In all cases, the lowest possible strain to obtain reliable data was used.

2.5 Results and Discussion

2.5.1 Molecular Characterization

2.5.1.1 Intrinsic Viscosity

Understanding intrinsic viscosity results requires one to think of single molecules in dilute solution. Each solution is diluted multiple times to estimate the effects on viscosity of a single molecule which is assumed to be a "random coil" in solution.

Examples of intrinsic viscosity graphs lie below. The Mark-Houwink-Sakurada equation discussed in Section 2.3.2 shows that the greater the intrinsic viscosity the greater the molecular weight.

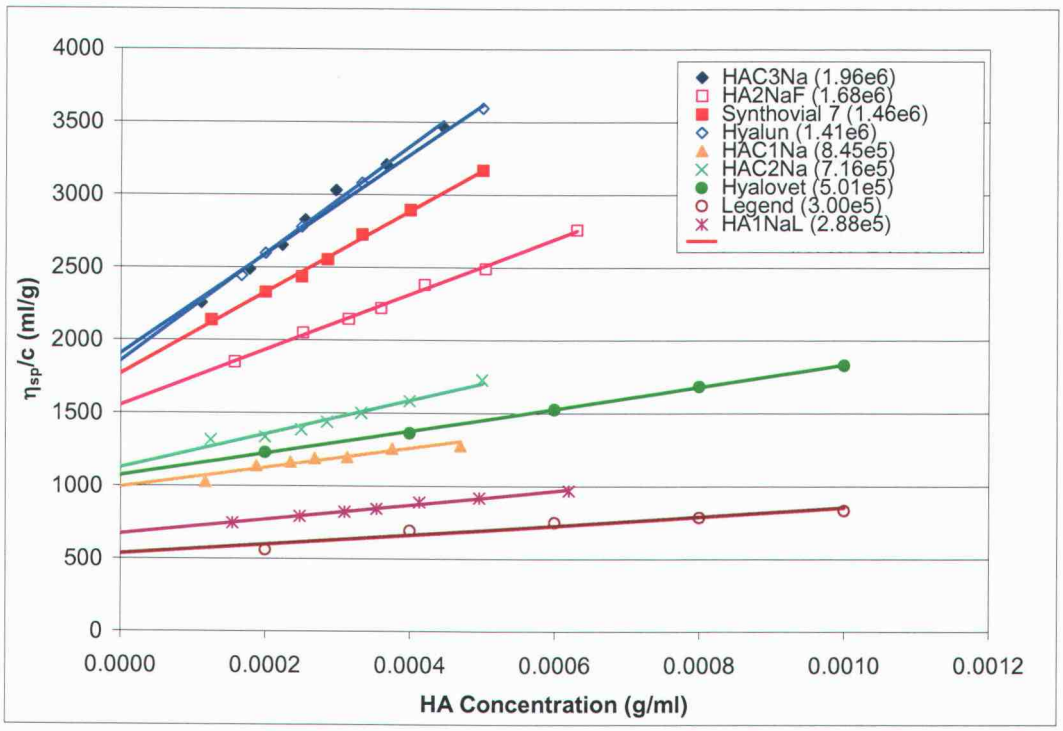


Figure 2- 12 Intrinsic viscosity of all HA samples in PBS (molecular weight values in parentheses from Table 2-3)

The linearity of each sample confirms that these measurements were done in the dilute regime. Any polymer entanglements would result in a slight upturn at of η_{sp}/c at higher concentrations. The intrinsic viscosity values are summarized in Table 2-2. They will be compared to intrinsic viscosity values measured by the ViscoStar in Table 2-3 and 2-4.

Table 2- 2 Intrinsic viscosity of each HA sample measured at OSU

Sample	[η] (ml/g)
HAC3Na	1856.50
HAC2Na	1067.80
HAC1Na	995.00
HA1NaL	674.15
HA2NaF	1555.50
Synthovial 7	1770.70
Hyalun	1909.00
Legend	533.40
Hyalovet	990.00
Hyvisc	N/A

2.5.1.2 SEC-MALLS

Recall that only the SEC-MALLS technique was used to measure the molecular weight of HA samples instead of the batch technique. This was done in order to determine the molecular weight distribution and to establish the technique for later use with synovial fluid samples. Each HA sample was run at a slightly different concentration, so a direct comparison of the light scattering and RI traces for each sample would yield little information. However, an example of the raw data collected at WTC (c~0.1 mg/ml) using the Viscostar and Astra 5 to analyze the data will be compared data collected at OSU (c~0.5 mg/ml) using Astra 4 for the sample Legend.

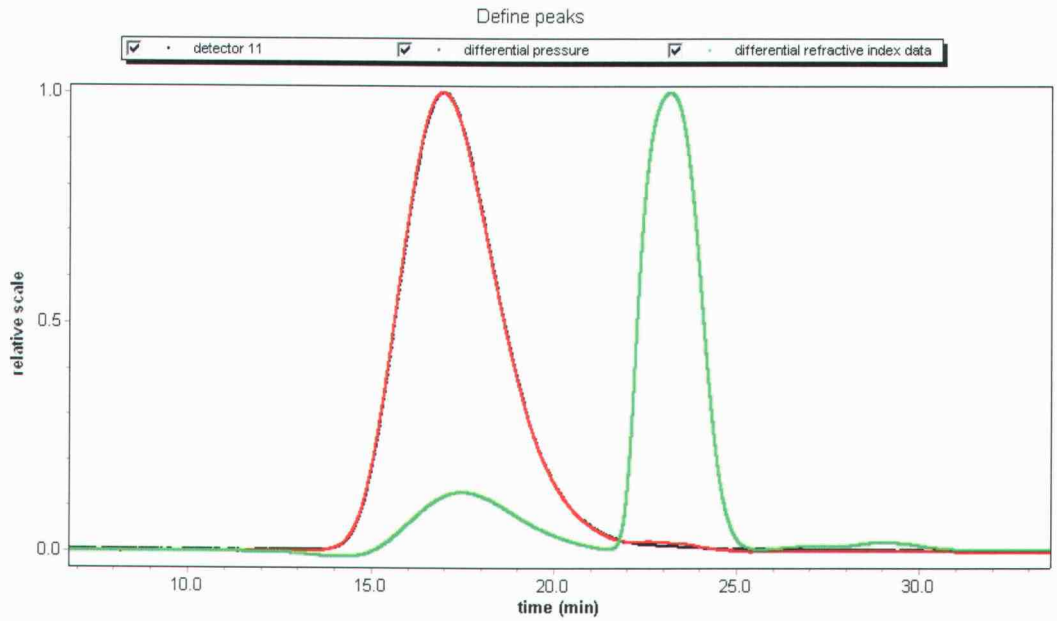


Figure 2- 13 Light scattering trace and RI trace of Legend, c~0.1 mg/ml

Figure 2-13 is an example of the raw light scattering and refractive index (RI) detector data taken at WTC. The lighter line is the RI detector trace and the darker line is both the 90° detector of the light scatterer and the differential pressure from the viscometer. It should be noted that the difference in elution time for each detector has been eliminated by aligning the peaks. The HA comes out in a single, smooth peak, indicating a fairly monodisperse sample. The second peak from the RI detector is due to a difference in salt concentration; the salt concentration in the sample was slightly higher than the salt concentration of the mobile phase.

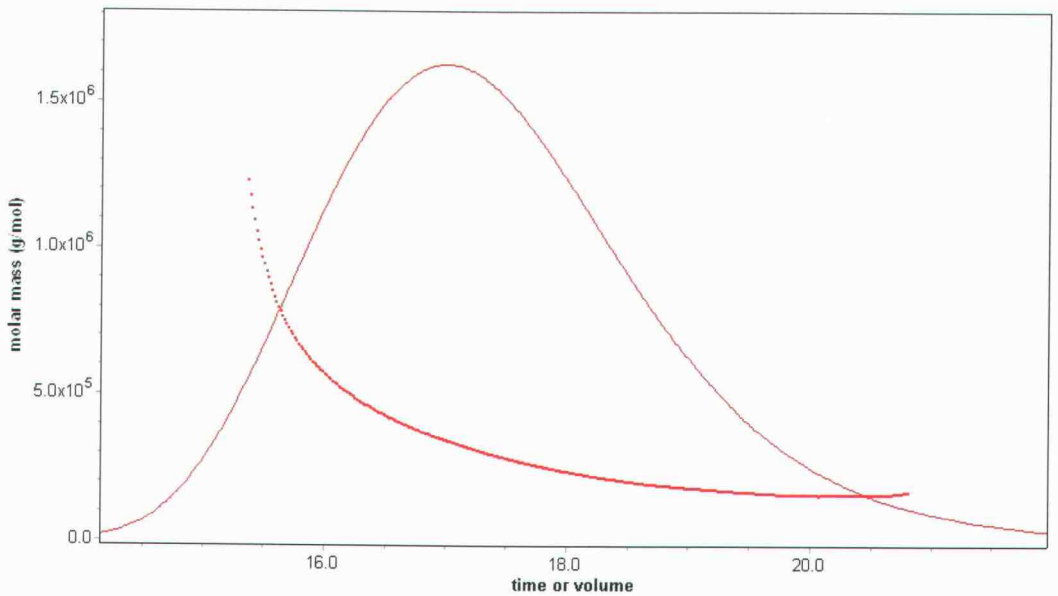


Figure 2- 14 Molecular weight by time of Legend (WTC)

Figure 2-14 shows the RI trace (peak) and the calculated molecular weight (line) at each point during the trace for Legend. Notice the first molecules to elute from the system are the highest molecular weight (around $1.2x10^6$ Da) and the lower molecular weight molecules follow. Similar plots were constructed for each HA sample, and the values of Mw and Mw/Mn were recorded for each.

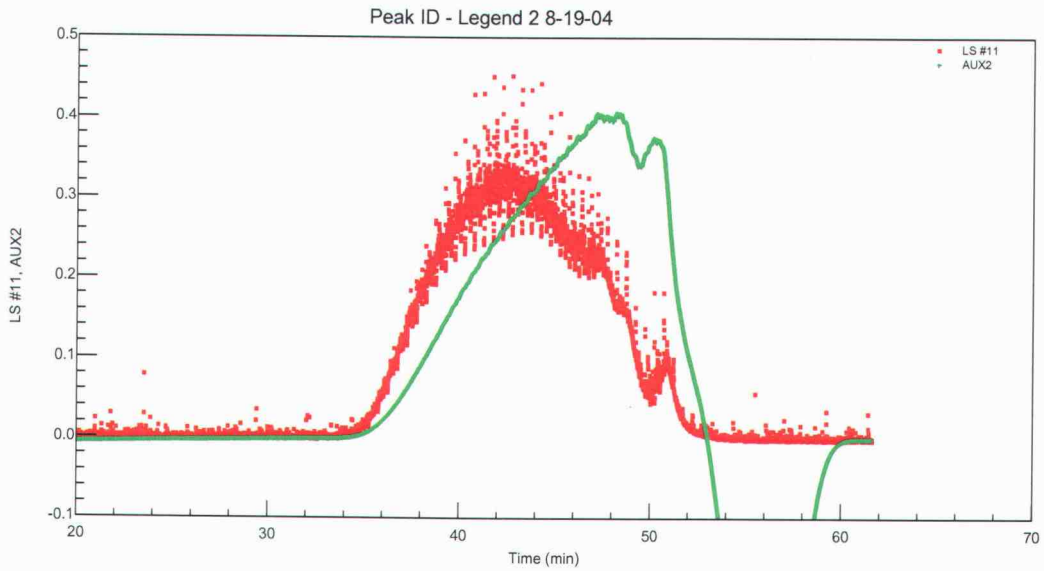
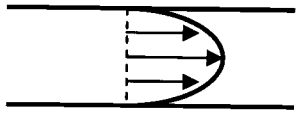
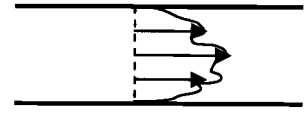


Figure 2- 15 Light scattering and RI trace for Legend at OSU (c~0.5 mg/ml) which exhibits viscous fingering effects

Figure 2-15 is a plot of the same sample, Legend, taken at OSU with a concentration around 0.5 mg/ml. (The negative RI peak is due to a difference in salt concentration between sample and mobile phase.) Notice the difference in the shape of the peak between Figure 2-13 and Figure 2-15 (ignore elution time as samples were measured at different flow rates). Both the light scattering and RI traces in Figure 2-13 are very smooth giving a nicely shaped peak. Conversely, the peak in Figure 2-15 is bumpy and rough. This is due to viscous fingering. Viscous fingering occurs in chromatography when the viscosity of the sample is approximately two or more times the viscosity of the mobile phase.



No Viscous Fingering:
Sample in uniformly distributed in the tube



Viscous Fingering:
Velocity profile has abnormal shape leading to non-uniform distribution

Figure 2- 16 Diagram of sample velocity profiles with and without viscous fingering

When the sample passes through the light scattering and RI detectors, measurements are taken at one point only in the flow-through cell. Under normal circumstances, the velocity profile of the sample will be uniform in the tube and the sample will be well mixed, allowing the detector to get consistent readings with time. When viscous fingering occurs the velocity profile of the sample is not smooth and fractions of high or low molecular weight molecules can exist in the "fingers." In this case, the amount of light scattered or refractive index at the same point along the sample cell will be inconsistent, which leads to messy curves.

Although this phenomenon will not necessarily cause large amounts of error in the data, it will contribute some. Originally, samples were measured at OSU at concentrations of about 0.5 mg/ml, a concentration at high enough to cause viscous fingering. This error was corrected for the samples taken at WTC,

therefore molecular weights measured at WTC will be considered correct. The three samples not measured at WTC, Synthovial 7, HAC1Na and Hyalovet, were re-measured at OSU at a lower concentration to avoid viscous fingering.

In addition to molecular weight, the intrinsic viscosity of each sample was measured at WTC using the ViscoStar. Unfortunately, Mark-Houwink plots were not available for every HA sample tested at WTC. This is mostly due to the narrow molecular weight distribution of the HA samples. However, a beautiful Mark-Houwink plot was constructed from the data collected of Legend and is shown in Figure 2-17.

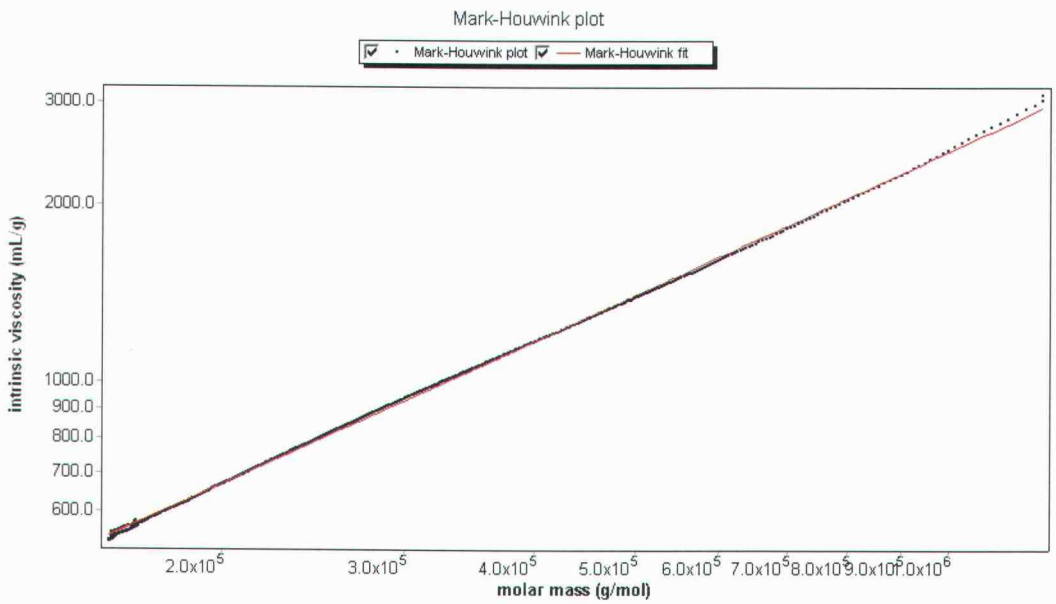


Figure 2- 17 Mark-Houwink Plot of Legend, constructed using Astra V by WTC

This Mark-Houwink plot of Legend yielded an MHS equation as follows.

$$[\eta] = 3.227 \times 10^{-2} (Mw)^{0.81} \qquad \text{Eqn. 2- 38}$$

This is an MHS equation based on a single HA sample, with the various molecular weight fractions within the sample providing the data for the intrinsic viscosity versus molecular weight correlation. This is significantly different then a MHS plot constructed from a variety of samples with a calculated average intrinsic viscosity. The latter is more common and will be described in the following section. An MHS plot will be constructed using intrinsic viscosity and molecular weight values for all HA samples.

2.5.1.3 Summary of Molecular Results

The following tables summarize the molecular weight and intrinsic viscosity collected both at OSU and Wyatt Technology Corporation.

Table 2-3 and Table 2-4 summarize the molecular properties of HA samples as measured at OSU and WTC, respectively. With the exception of two of the low molecular weight samples, Legend and Hyalovet, and HAC1Na, the molecular weights measured at OSU and WTC are within 10% of each other. These differences can likely be attributed to the viscous fingering that occurred with the OSU samples.

Table 2- 3 Summary molecular properties measured at OSU

Sample	†From OSU (with Viscous Fingering)				
	Mw (Da)	Mw/Mn	[η] (ml/g)	[1]c_o (mg/ml)	‡c_o/c^*
HAC3Na	1.77E+06	1.06	1856.50	0.45	1.43
HAC2Na	6.95E+05	1.22	1067.80	0.50	0.81
HAC1Na	6.55E+05	1.40	995.00	0.47	N/A
HA1NaL	2.68E+05	1.62	674.15	0.62	0.48
HA2NaF	1.77E+06	1.19	1555.50	0.63	1.67
Synthovial 7	1.55E+06	1.01	1770.70	0.50	N/A
Hyalun	1.50E+06	1.00	1909.00	0.50	1.30
Legend	1.66E+05	1.61	533.40	1.00	0.92
Hyalovet	3.84E+05	1.30	990.00	1.00	N/A
Hyvisc	N/A	N/A	N/A	N/A	N/A

[1]Highest concentration used for intrinsic viscosity measurements
†Viscous fingering effects Mw and Mw/Mn values only
‡c* is based on intrinsic viscosity measured at WTC, $c^*=1/[\eta]$

Table 2- 4 Summary of molecular properties measured at WTC

Sample	From WTC (no Viscous Fingering)			
	Mw (Da)	Mw/Mn	[η] (ml/g)	c* (mg/ml)
HAC3Na	1.96E+06	1.06	3210.30	0.31
HAC2Na	7.16E+05	1.14	1622.00	0.62
†**HAC1Na**	8.45E+05	1.18	N/A	N/A
HA1NaL	2.88E+05	1.23	776.10	1.29
HA2NaF	1.68E+06	1.05	2646.00	0.38
†**Synthovial 7**	1.46E+06	1.13	N/A	N/A
Hyalun	1.41E+06	1.45	2590.80	0.39
Legend	3.00E+05	1.19	916.00	1.09
†**Hyalovet**	5.01E+05	1.13	N/A	N/A
Hyvisc	1.75E+06	1.03	2690.00	0.37

†Mw and Mw/Mn measured at OSU

The intrinsic viscosity measurements however are very different, with OSU measurements 10-40% lower than WTC values. The ViscoStar is a calibrated piece of equipment, so the intrinsic viscosity values collected at WTC were considered correct. One possible explanation for the error is that the samples were in the shear thinning region during intrinsic viscosity measurements at OSU. Shear rates in the capillary, calculated by equation 2-39, ranged from about 70 s^{-1} to 200 s^{-1}.

$$\dot{\gamma} = \frac{4Q}{\pi d^3} \qquad \text{Eqn. 2- 39}$$

where:

Q is the volumetric flow rate

d is the capillary diameter

Although the HA concentrations measured in the intrinsic viscosity tests were much lower than those measured in rheology tests, the steady shear results in Section 2.5.2 will show that this 70s^{-1} to 200 s^{-1} is in the shear thinning range for HA solutions. If this is the case in the dilute solutions, the intrinsic viscosity values would be underestimated in the Cannon-Ubbelohde capillary viscometers that were used. To correct this, a viscometer with a smaller capillary should be used to decrease shear rate. These measurements will be done at a later date.

Because manual measurements of intrinsic viscosity were found to be inaccurate, the Mark-Houwink-Sakurada plot (Figure 2-18) for HA in PBS used the intrinsic viscosity values and molecular weight values measured at WTC (data from Table 2-4). Therefore only seven samples are represented on the graph instead of all ten.

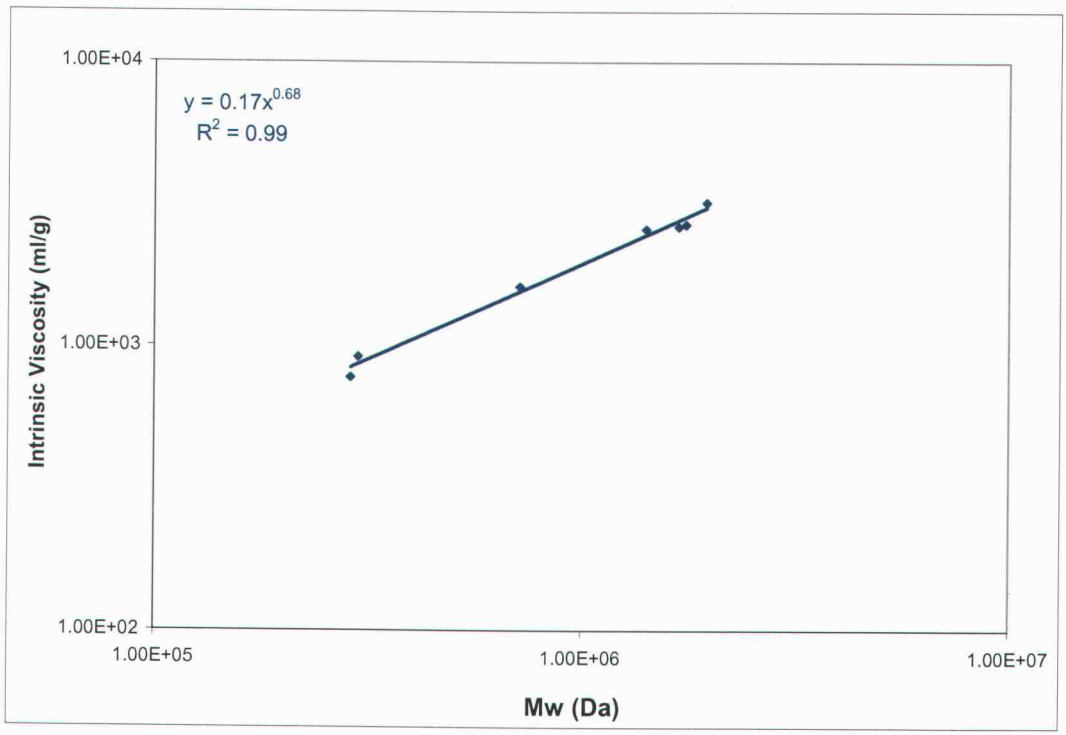


Figure 2- 18 Mark-Houwink Sakurada plot for HA in PBS (data from Table 2-4)

The resulting MHS equation for HA in PBS is:

$$[\eta] = 0.168(Mw)^{0.68} \qquad \text{Eqn. 2- 40}$$

The "a" value of 0.68 is a reasonable number for a random coil polymer in a good solvent like PBS. The "a" value for the Legend sample alone run at WTC was

0.81 which is significantly higher than the value for all HA samples combined, but Legend is a low molecular weight HA sample (approximately 300,000 Da from Table 2-3).

This value for "a" is similar to those collected by Mendichi et. al. (2003) and Vercruysse (1995) who found "a" values of 0.783 and 0.72 respectively. Mendichi was measuring HA samples from below 100,000 Da to over 1,000,000 Da, and Vercruysse measured a molecular weight range of 10,000 Da to 1,000,000 Da. The range measured for this thesis was 500,000 Da to 2,000,000 Da. Since Mendichi's and Vercruysse's molecular weight ranges included lower molecular weight samples, it makes sense that their "a" values would be higher than the one calculated for this thesis (since lower molecular weight molecules appear to be stiffer).

2.5.2 RHEOLOGY

The discussion of the rheological characterization results will be separated into steady shear and dynamic oscillatory shear results. The samples will be divided into frozen HA and HA supplements.

2.5.2.1 Steady Shear

In a polymer solution, several factors can contribute to the measured rheological properties. These include temperature, solvent, molecular weight, concentration,

molecule stiffness, and hydrodynamic volume. To eliminate the concentration and solvent effects, all rheological measurements were taken at a 2.5 mg/ml in PBS. This value was chosen because it is approximately the concentration for HA in synovial fluid and is greater than the overlap concentration, c* (c/c* ~ 2-8), which ensures some degree of molecular interactions for all samples. Since all other variables are the same, a higher viscosity or modulus will be due to higher molecular weight.

2.5.2.1.1 Frozen HA Samples

Below (Figure 2-19) are the steady shear results for the frozen HA samples. The sample names in the legend are explained in Table 2-1. The numbers in the parenthesis are the molecular weights in Da (without viscous fingering). The complex viscosity from a dynamic oscillatory shear experiment ($\eta^*(\omega)$) are presented in the section so that trends in the viscosity data can be compared. The Cox-Merz rule states that in the limit of low shear and low frequency the viscosity and complex viscosity are approximately equal, and that the complex viscosity should fall off faster than steady shear viscosity.

$$\lim_{\dot{\gamma}\to 0}\eta(\dot{\gamma}) = \lim_{\omega\to 0}\eta^*(\omega) \qquad \text{Eqn. 2- 41}$$

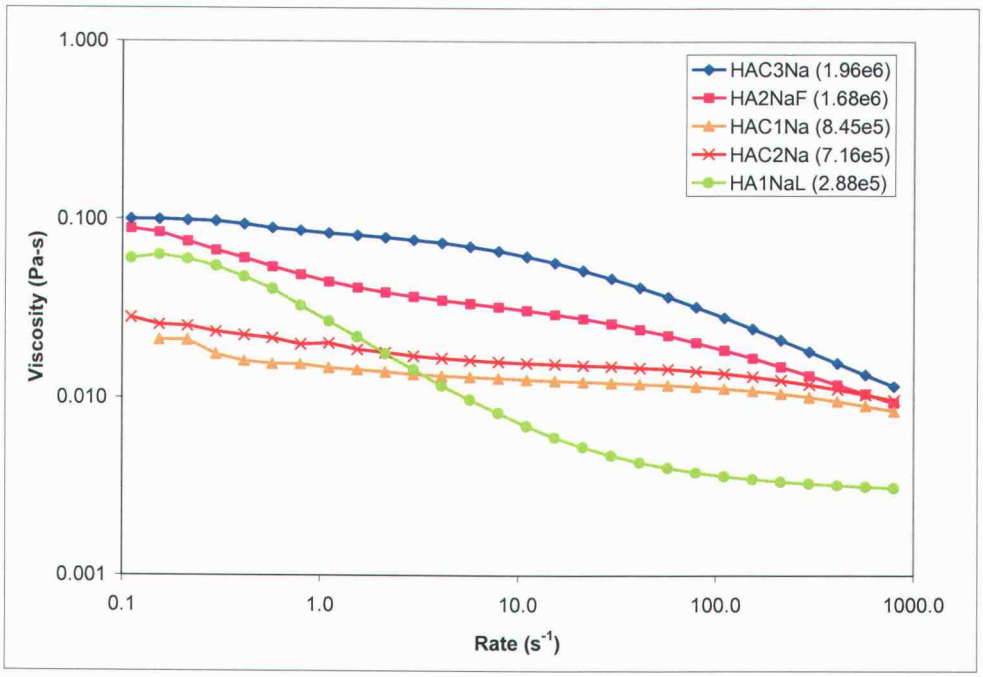


Figure 2- 19 Steady shear of all frozen hyaluronate acid samples at 2.5 mg/ml in PBS (molecular weight in Da)

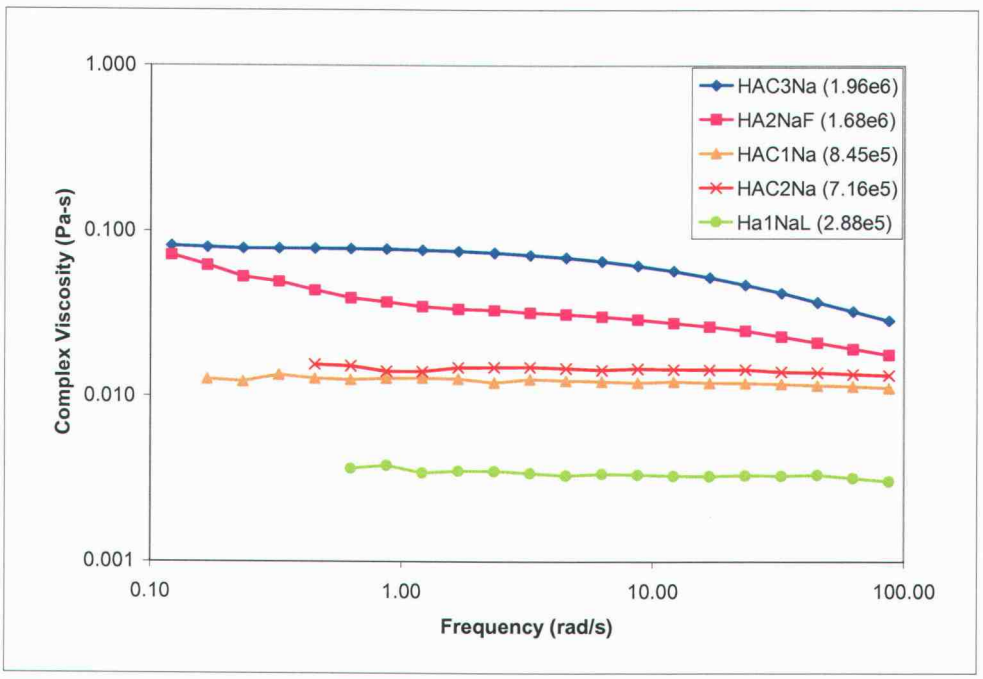


Figure 2- 20 Complex viscosity of frozen hyaluronate samples at 2.5 mg/ml

At low shear rates, each sample approaches a Newtonian plateau where viscosity is independent of shear rate. The viscosity at this point is called the zero shear viscosity, which is proportional to molecular weight. These zero shear viscosity values all occur at a viscosity range of 0.02 through 0.1 Pa-s (the viscosity of water is 0.001 Pa-s). At these low shear rates the polymer molecules remain entangled and the viscosity does not respond to a slight increase of shear rate. Once a critical shear rate is reached the polymers begin to disentangle; at these higher shear rates the viscosity begins to decrease in the shear thinning region. The critical shear rate is molecular weight dependent. The complex viscosity closely mirrors the steady shear viscosity both in magnitude and shape for most samples.

The anomalous viscosity versus shear rate curve is HA1NaL, which is the lowest molecular weight sample in this group. It exhibits a high zero-shear viscosity followed by a much faster drop-off with shear rate than the other frozen samples. Then it enters what appears to be a second Newtonian plateau. This shape is characteristic of a sample with associations, which may be present in this sample because of different processing conditions. The complex viscosity for this sample does not follow this behavior pattern; it is nearly constant with frequency; but data was not obtained in the lowest shear region. The HA2NaF sample also exhibits a slight upturn in viscosity at low shear rates and this behavior is magnified in the lower frequency complex viscosity data. This type of behavior in general may be

indicative of sample aggregation. Both HA1NaL and HA2NaF are from human umbilical cords (the only two samples from this source), so this may be an indication that something in the processing of these materials leads to aggregation behavior in solution.

Because temperature and polymer concentration were kept constant, viscosity is dependent only on molecular weight. Thus the molecular weight of the frozen samples in decreasing order, not including HA1NaL, should be HAC3Na, HA2NaF, HAC2Na and HAC1Na which roughly agrees with the light scattering results.

2.5.2.1.2 Sodium Hyaluronate Supplements

The steady shear results of the supplements are summarized in Figure 2-21. The HA supplements have shapes similar to the frozen samples, as would be expected. All the supplement samples, except for Hyvisc, have a slight upturn at low viscosities, which again may be an indication of associations. This behavior is more pronounced in the complex viscosity graph for Hyalun and Synthovial 7. Hyalovet and Legend however appear very flat in the complex viscosity graph, but once again the data at low shear rates was not obtained.

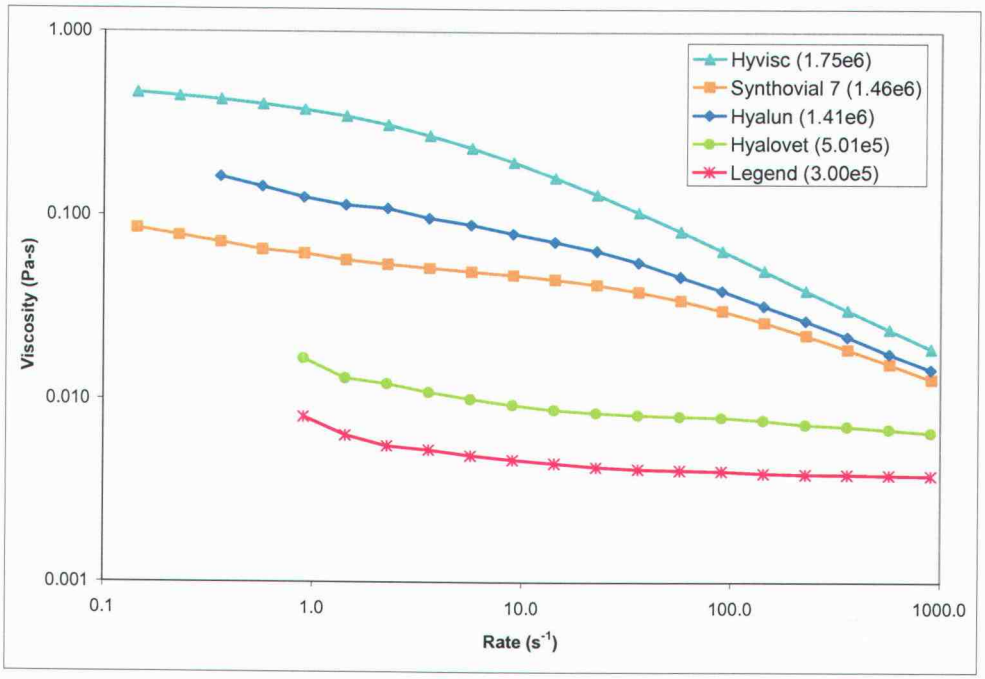


Figure 2- 21 Steady shear results for sodium hyaluronate supplements at 2.5 mg/ml

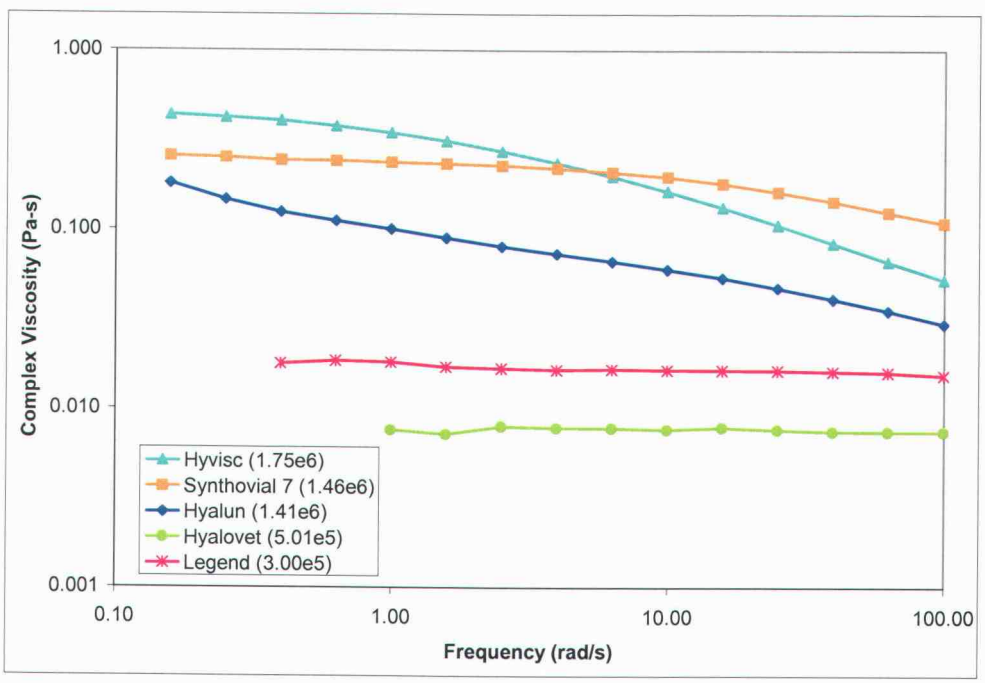


Figure 2- 22 Complex viscosity of HA supplements, 2.5 mg/ml

The rheological data indicates that Hyvisc is a very clean product (well-defined curves) of high molecular weight. This will be confirmed by the dynamic data in the following section. To summarize, Hyvisc exhibits the highest viscosity, followed by Hyalun, Synthovial 7, Hyalovet and Legend. The order of sample viscosity agrees with molecular weight.

2.5.2.2 Dynamic Oscillatory Shear

The dynamic oscillatory shear data for HA samples will be compared in plots of the viscous and elastic moduli versus frequency. The complex viscosity, obtained from the dynamic measurements, was discussed in the previous section.

2.5.2.2.1 Frozen HA Samples

The elastic and viscous moduli, G' and G" respectively (see Figure 2-9), for these samples lie between 0.01-10 Pa (Figure 2-23). Just as in the steady shear results, if all other parameters are equal, a higher modulus can be equated with a higher molecular weight (higher degree of entanglement). Based solely on this fact, the molecular weight again should be (in decreasing order) HAC3Na, HA2NaF, HAC2Na, HAC1Na, and HA1NaL, which agrees with molecular weight measurements except the juxtaposition of HAC2Na and HAC1Na. Within the frequency measured by the instrument, all HA solutions were in the viscous region. However, the two higher molecular weight samples (HAC3Na and HA2NaF) do approach a crossover point (G' = G") at high frequencies. It should be noted that

the flattening of the elastic modulus data for HA2NaF at low frequency is indicative of aggregation.

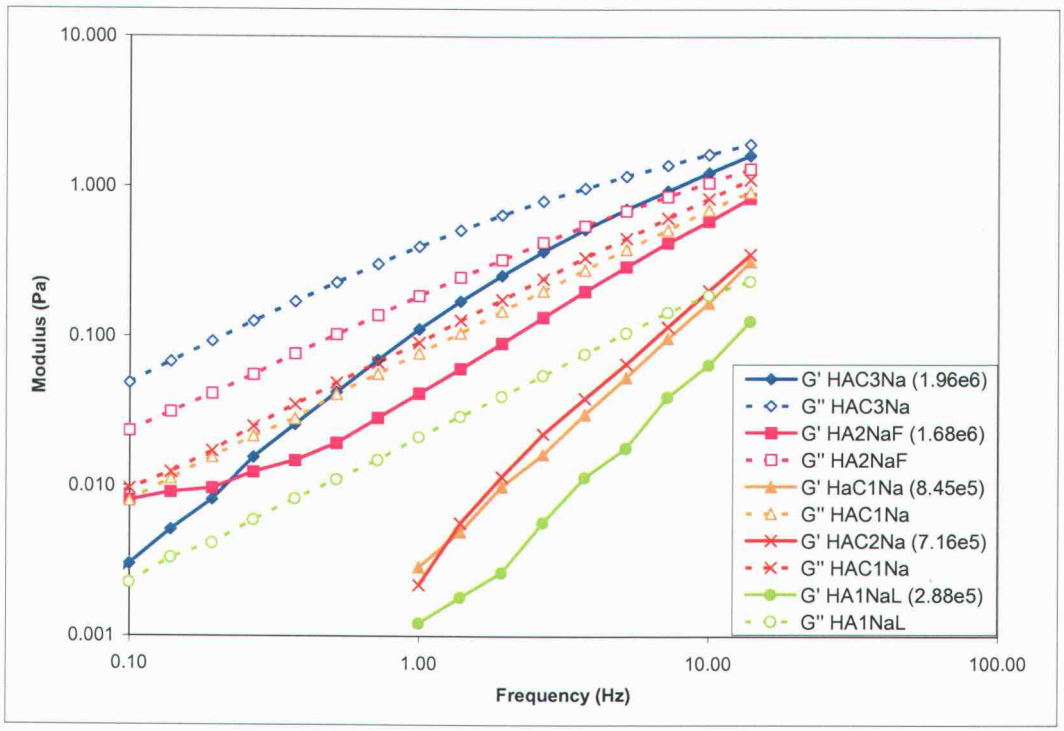


Figure 2- 23 Dynamic oscillatory shear results for frozen HA samples

Also of note is the fact that the three low molecular weight samples have very low G' values. If all HA samples could be measured at higher frequencies, the crossover points would occur at much greater frequencies than HAC3Na and HA2NaF. It is also worth noting that the G' values for HAC2Na, HAC1Na and HA1NaL were very poor between 0.1-1.0 Hz which is why they were omitted

from the graph. This low amount of elasticity is another indication of lower molecular weight.

2.5.2.2.2 HA Supplements

The graph from the HA supplements (Figure 2-24) looks very similar to the frozen HA samples.

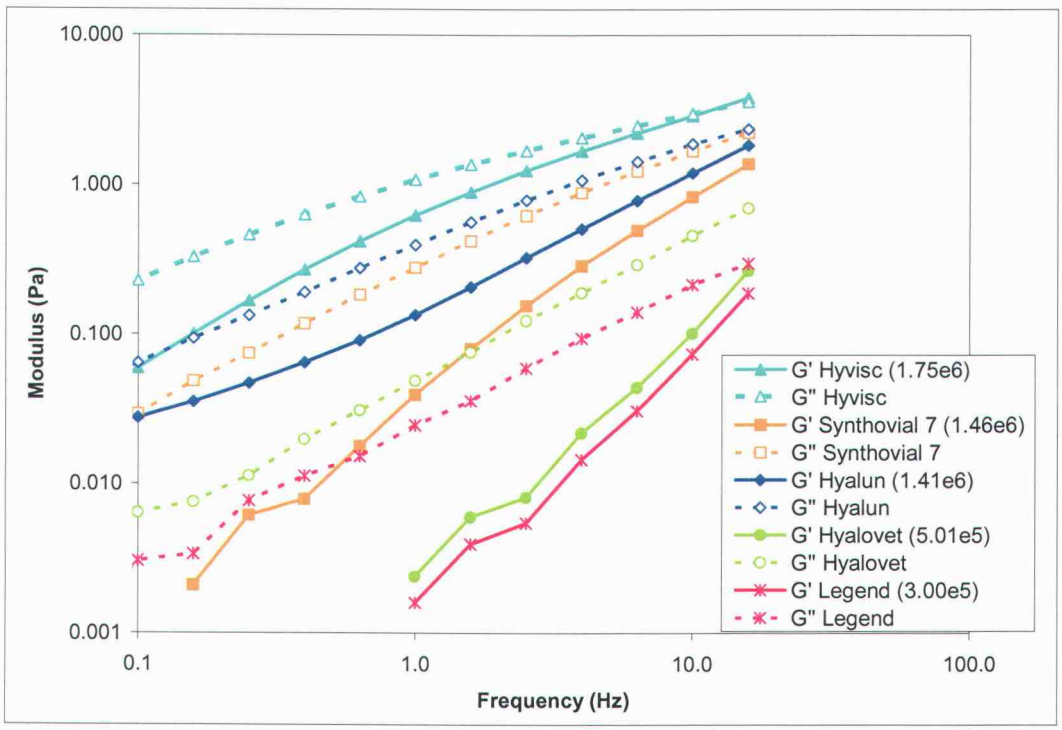


Figure 2- 24 Dynamic oscillatory shear HA supplements

The moduli range is 0.01-10 Pa and only Hyvisc exhibits a crossover point at about 10 Hz, and once again appears to be the cleanest and most viscoelastic of all

the samples tested. Synthovial 7 and Hyalun, the next highest molecular weights, do not exhibit a crossover point but they do approach it. Legend and Hyalovet, the lower molecular weight samples, have very weak G' values below a frequency of 1 Hz.

2.5.2.3 Summary of Rheology Results

Table 2-5 is a summary of zero shear viscosity and power law index, n, compared to molecular weight.

Table 2- 5 Summary of molecular weight and steady shear results of HA samples

	Mw (Da)	η_o (Pa-s)	n
HAC3Na	1.96E+06	0.100	0.59
HAC2Na	7.16E+05	0.029	0.81
HAC1Na	8.45E+05	0.018	0.84
HA1NaL	2.88E+05	0.060	0.44
HA2NaF	1.68E+06	0.089	0.65
Synthovial 7	1.46E+06	0.097	0.61
Hyalun	1.41E+06	0.114	0.57
Legend	3.00E+05	0.009	0.98
Hyalovet	5.01E+05	0.025	0.92
Hyvisc	1.75E+06	0.476	0.48

With the exception of HA1NaL and the juxtaposition of HAC1Na and HAC2Na, higher molecular weight samples have higher zero shear viscosities and lower power law indices. All samples were tested at the same concentration so higher

molecular weight samples must have a larger degree of entanglement. Legend and Hyalovet exhibit nearly Newtonian behavior with indices of 0.98 and 0.92 respectively and the lowest power law indices are 0.44 and 0.48 of HA1NaL and Hyvsic, respectively. Hyvisc exhibits a low power law index (indicative of high shear thinning) because as mentioned it appears to be the most viscoelastic of all supplements. The low "n" value for the low molecular weight sample HA1NaL is indicative of shear thinning behavior due to the break-up of aggregate associations. These two behaviors are quite different and supply important information on the expected behavior of the sample in applications. If one is looking for a highly lubricating product (high viscoelasticity) then Hyvsic would be the sample of choice.

The molecular weight results obtained from light scattering were compared more directly to zero shear viscosity. Similar to the results presented by Bothner and Wik (1987), zero shear viscosity was compared to the product of molecular weight and concentration (Figure 2-25).

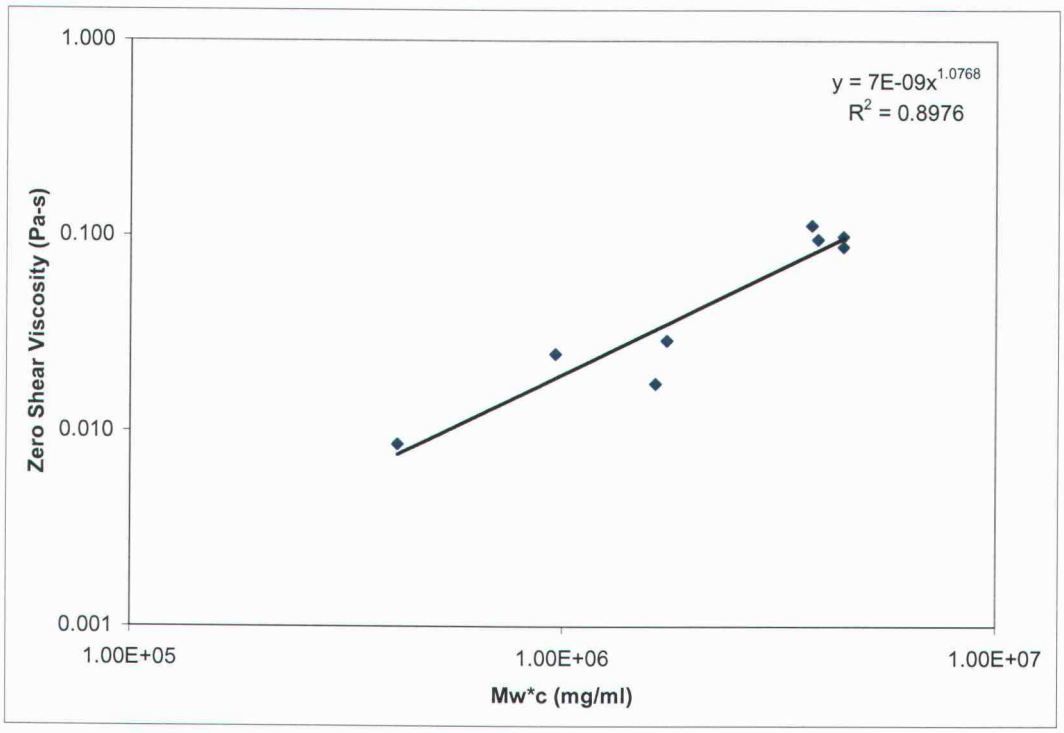


Figure 2- 25 Comparison of zero shear viscosity to molecular weight of HA samples

As exhibited in Figure 2-25, zero shear viscosity and molecular weight can be related by the following equation.

$$\eta_o \propto Mw^{1.08} \qquad \text{Eqn. 2- 42}$$

According to theory, zero shear viscosity should be proportional to molecular weight to the first power for a dilute system. While the samples studied are beyond the dilute region (c/c* ~ 2-8), they appear to not be too highly entangled, so the viscosity versus molecular weight relationship that holds best is for the dilute regime. This is consistent with the observed viscoelastic behavior in that the

solutions do not appear to be highly entangled from the dynamic oscillatory shear moduli versus frequency curves (Figures 2-23 and 2-24).

2.6 Summary of HA Characterization Results

This chapter discussed the molecular and rheological characterization of ten samples of sodium hyaluronate, a biologically important polymer. The molecular characterization of HA was completed using intrinsic viscosity measurements and SEC-MALLS to determine molecular weight. The intrinsic viscosity was measured two different ways; manually with a Cannon-Ubbelohde capillary viscometer, and using an in-line viscometer with the SEC-MALLS system. The two methods for measuring intrinsic viscosity did not agree (possibly due to shear thinning in the capillary viscometer method) so the results using SEC-MALLS with an in-line viscometer were taken to be correct. A Mark-Houwink-Sakurada relationship in PBS was developed yielding an "a" value of 0.68, which agrees well with literature values.

The rheological characterization of the same ten HA samples was performed. Both steady shear and dynamic oscillatory shear tests were done on the samples at 2.5 mg/ml. In the steady shear results, most samples exhibited a Newtonian plateau at low shear rates followed by a shear thinning region at higher shear rates. Two samples isolated from umbilical cords (HA2NaF and HA1NaL) showed a slight upturn at low shear rates, indicative of associations. This may be the result

of unique processing for these samples. The dynamic oscillatory shear tests found all HA samples except one (Hyvisc) to behave more viscously than elastic at the frequencies tested. All rheology results including steady shear viscosity and dynamic elastic and viscous moduli agreed with molecular weight results, in that the higher molecular weight samples exhibited the highest viscosity and moduli values, while the lowest molecular weight samples had low rheological properties.

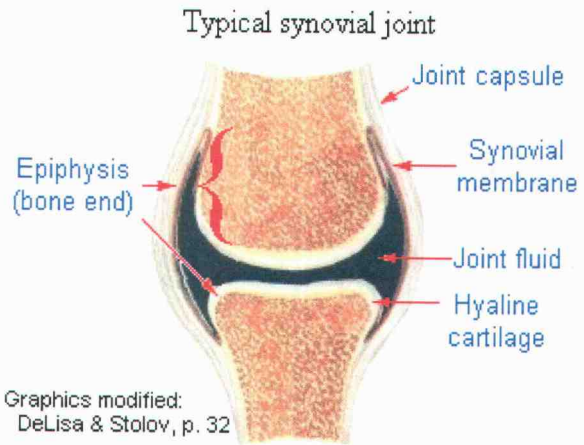
The molecular and rheological results correlate well; at the same concentration larger molecular weight molecules will exhibit larger viscoelastic properties. This conclusion agrees with polymer theory, and the MHS relationship developed agrees with literature values, therefore these characterization techniques were deemed successful. Both rheological and molecular characterization techniques can be applied to the more complex task of measuring HA in equine synovial fluid.

CHAPTER 3

SYNOVIAL FLUID CHARACTERIZATION

3.1 INTRODUCTION

Synovial fluid is found in all articular joints and is responsible for lubrication during low impact joint movement and shock-absorption during high impact activities. Sodium hyaluronate is the largest component of synovial fluid and is responsible for the viscoelastic properties necessary to protect and maintain healthy joints. In the joints, HA is produced and secreted by cells called type A phagocytes located in the synovial lining. Mechanical pressure from the movement of the limbs pushes HA out of the cartilage and into the joint cavity, a process known as weeping lubrication.



http://webschoolsolutions.com/patts/systems/capsule.gif

Figure 3- 1 Diagram of a typical synovial joint; HA is produced and secreted from the synovial membrane

The viscoelastic properties of HA in synovial fluid are dependent on molecular weight and concentration as demonstrated in Chapter 2. Some types of joint disease are known to degrade HA, thus decreasing its mechanical and protective properties which can lead to cartilage damage, further propagating the disease. Therefore it is of interest to characterize the HA in synovial fluid. Synovial fluid characterization could be used as a disease marker. Establishing a range of "normal" values for rheological and molecular properties can be extremely valuable in the diagnosis of disease. Comparing the properties of normal and unhealthy synovial fluid has been of great interest and has received much attention. In addition to a marker for disease, measuring the necessary viscoelastic properties of synovial fluid could lead to development of artificial supplements for joint lubrication.

This chapter will apply the characterization techniques developed in Chapter 2 to synovial fluid. It will include a complete rheological and molecular characterization of not just the HA in synovial fluid, but of synovial fluid itself which includes components that may complex with HA. The objective of this chapter is to use the molecular and rheological techniques to establish "normal" values of hocks and stifles (different joints may have slightly different HA and protein compositions). The properties of some diseased joints will also be compared to the healthy joints to establish differences between normal and abnormal joints.

3.2 Literature Review

Because the characterization of HA in synovial fluid is a very clinically relevant goal, many researchers have focused on this topic. The first to complete very thorough studies on synovial fluid, joint functions and all other topics relating to joint health was E.A. Balazs (Balazs' HA work began in 1942). Balazs et. al. (1967) completed a study on 503 human knee synovial fluid samples in which protein concentration, HA concentration, HA molecular weight and viscosity properties were measured. Molecular weight was measured using light scattering (dn/dc of 0.17) and found to range from 2.7 to 4.5×10^6 Da in arthritic joints. The average HA in healthy joints was 6×10^6 Da. Sodium hyaluronate concentration was measured using a hyaluronidase degradation reaction and sample concentration ranged from 1.45-3.42 mg/ml in normal and 1.09-1.2 mg/ml in arthritic joints. Balazs was perhaps the first to recognize that the HA of diseased joints has a lower molecular weight and a lower concentration than HA in healthy joints. Rowley et. al. (1982) also used the hyalruonidase method and found an average HA concentration in synovial fluid of healthy horses of 1.27 mg/ml.

Hyaluronidase (an enzyme that breaks down hyaluronate) assays and light scattering are two of the major methods used to measure the properties of HA in synovial fluid. Synovial fluid samples are incubated with hyaluronidase for a particular time period. The reaction is stopped (often by changing pH) and additional reagents are often added that will react with the products of the

hyaluronidase reaction. Many hyaluronidase reactions are measured using a spectrophotometer; therefore these reagents should produce a product with a measurable absorbance at a specific wavelength. The absorbance is compared to samples with known concentrations of HA that underwent the same reaction. This creates a standard absorbance versus concentration curve, thus the concentration of an unknown sample can be found (Rowley et. al. 1982). Other methods of quantifying hyaluronidase assays include chromatography, fluorimetry, zymography and capillary zone electrophoresis (Muckenschnabel et. al. 1998).

In Dahl et. al. (1985) published a study based on diseased human synovial fluid. Rather than measuring the HA in the fluid straight out of the body, the HA was extracted from synovial fluid prior to all tests. A method using radio-labels was used to determine concentration (about 2.03 +/- 0.4 mg/ml) and gel chromatography (calibrated with HA standards) to determine molecular weight ($2.9\text{-}1.9\text{x}10^6$ Da). Purifying the HA before analysis requires extra work, but may make analysis easier. There are a lot of proteins and other moderately-sized molecules in synovial fluid which could potentially interfere with measurements that involve size exclusion methods. If the size separation is poor, large molecules and moderately sized molecules could elute from the system at the same time, or their separation could overlap.

High pressure liquid chromatography (HPLC) was used by others to determine both the concentration and molecular weight of HA in synovial fluid. This method requires the calibration of columns with known molecular weight standards which makes it a relative method of molecular weight determination rather than an absolute one (such as light scattering). It allows one to compare hydrodynamic volume (the product of intrinsic viscosity and molecular weight) to elution time. In 1989 Saari et. al. used this method and reported finding HA concentrations around 0.47 mg/ml in synovial fluid.

R. M. Tulamo and coworkers (1991 and 1994) completed multiple studies regarding the molecular characterization of HA in equine synovial fluid. In 1991 she compared the HPLC method to a radiometric assay in which she bound a labeled protein to HA. She found the concentration of HA in synovial fluid from tarsocrural joints obtained using HPLC to be double the radiometric method. The HPLC results yielded a concentration around 0.35 +/- 0.19 mg/ml. Unlike earlier studies, Tulamo did not treat the synovial fluid prior to injection in HPLC. In another HPLC study (1994) she found the molecular weight in normal joints to range from $2\text{-}3\text{x}10^6$ Da and $1.5\text{-}3\text{x}10^6$ in arthritic joints. She also found a small difference in concentration between normal and arthritic joints ranging from 0.26-0.56 mg/ml in healthy joints and 0.11-0.41 mg/ml in samples from joints with traumatic arthritis. The lower concentration of HA in arthritic joints agrees with Balazs et. al. (1967). However, the molecular weight of HA measured by Tulamo

et. al. in normal and arthritic joints was not significantly different, unlike the findings of Balazs. Due to natural variations in individuals, inconclusive results are not uncommon. Perhaps the measurements made by Tulamo had a high standard deviation making her results for normal and arthritic synovial fluid very similar.

In addition to HPLC, some authors have used SEC/MALLS to measure the molecular weight of HA in synovial fluid. Kvam et. al. (1993) utilized low angle laser light scattering (LALLS, detection of scattered light is done at only one or two low angles rather multiple angles like MALLS) to measure HA molecular weight and concentration in human knee synovial fluid. Arguing that untreated samples caused overlap between the protein and HA peaks, she purified the HA by removing lipids and proteins. Once the HA was purified and analyzed, HPLC curves were compared to known molecular weight standards to determine concentration. She found HA sizes ranging from $1x10^4$ to $2x10^6$, the low end being much smaller than other reported values. The concentrations were also low ranging from 0.025 to 0.35 mg/ml. She also examined the effects of freezing synovial fluid samples on molecular weight and hypothesized that radicals (particularly in diseased joints) can cause further HA damage once removed from the body.

Adam and Ghosh (2001) also used SEC/MALLS to measure molecular weight, however they did not purify the HA from the ovine fluid prior to analysis. They found a range of $1x10^6$ to $7x10^6$ Da, although they reported that most samples have a molecular weight around $2 x10^6$ to $3 x10^6$ Da. They also determined that freezing synovial fluid samples tends to decrease molecular weight of HA.

Matsuno et. al. (1993) used an enzymatic reaction to characterize HA similar to the one previously discussed but did not report concentrations, rather change in concentration after injections of HA. He was one of the few to combine rheological properties and molecular characterization. His group measured extensional viscosity ("stringyness"), rheology and intrinsic viscosity before and after supplementation. His conclusions will be discussed in Section 4.2.

Additional rheological studies on synovial fluid were performed by Gomez and Thurston (1993). They compared the properties of normal, inflammatory (arthritis) and non-inflammatory (degenerative joint disease, DJD) fluids. Sodium hyaluronate concentration, complex and intrinsic viscosity were reported. They suggested that rheological properties would be a more useful diagnostic tool for determining disease than molecular characterization. Schurz and Ribitsch (1987) concurred with Gomez and Thurston when they compared the steady shear rheology of healthy and unhealthy synovial fluid samples and determined the shear

thinning behavior and viscosity range created a distinction between healthy, inflammatory and non-inflammatory synovial fluid.

Balazs (with Gibb, 1970 and Denlinger, 1985) also studied the dynamic oscillatory shear properties of human synovial fluid. He reported a distinct difference in the viscoelastic behavior between samples from young, old, and arthritic individuals. He also showed that although samples from younger joints had higher moduli than older joints, they were both far more elastic than arthritic samples.

In general, any attempt to average results among a number of synovial fluid samples have resulted in extremely high standard deviations due to the nature of biological systems. Reported values of concentration can be below 0.1 mg/ml (Kvam et al. 1993) to almost 4 mg/ml (Balazs et. al. 1967). The concentration may appear to be slightly lower in arthritic joints than healthy joints, however this remains inconclusive. Molecular weights have been reported between $1x10^4$ Da (Kvam et al. 1993) and $7x10^6$ Da (Adam and Ghosh, 2001). Due to the wide variation of conclusions surrounding the molecular characterization of HA in synovial fluid, rheology may be a more useful tool to distinguish between healthy and arthritic joints.

To summarize, a wide variety of techniques have been used to measure HA molecular weight and concentration in synovial fluid. Although the hyaluronidase

method is very straightforward, it measures concentration only. The SEC-MALLS method allows the measurement of both molecular weight and concentration in one test which is quite appealing. However, the fact that synovial fluid is made of a lot of different components may make clean light scattering readings of HA difficult to measure. For this reason purification may enhance SEC-MALLS readings. SEC-MALLS also requires only the calibration of the instruments whereas the hyaluronidase and HPLC methods both require a comparison to known concentration or molecular weight standards with every set of experiments. For this reason SEC-MALLS may be a more practical method as long as HA purification issues are addressed.

3.3 Materials and Methods

3.3.1 Samples

Equine synovial fluid samples were obtained from the OSU College of Veterinary Medicine under the direction of Dr. Jill Parker, DVM, with the help of Sara Tracy and Erica Zaworski. All horses had been euthanized for reasons not related to this study. Following collection all samples were centrifuged for 40 minutes to remove any cells or debris. The supernatant was removed and stored at 4° C at all times until use.

Sample names were given at random. The naming system consists of two parts; the first is DH, VH, or abDH standing for deceased horse, live horse and abnormal

deceased horse followed by a number. This identifies which horse the sample came from. The second part of the name refers to the joint, R and L stand for right or left, S is stifle and H is hock. For example, DH3 RH and DH3 RS are the right hock and right stifle of deceased horse number three. Three abnormal samples were diagnosed with ocsteochondrosis dissecans (OCD), abDH4, abDH5 and abDH8. Sample abDH6 LH was lame for unknown reasons.

Table 3- 1 Description of equine synovial fluid samples

	Sample	Comments
Stifles	DH1 LS	Euthanized, left stifle
	DH2 RS	Euthanized, right stifle
	DH2 LS	Euthanized, left stifle
	DH3 RS	Euthanized, right stifle
	DH3 LS	Euthanized, left stifle
Hocks	DH3 RH	Euthanized, right hock
	DH3 LH	Euthanized, left hock
	DH1 RH	Euthanized, right hock
	VH1 LH	Live, left hock
	VH3 RH	Live, right hock
Abnormal	abDH4 RS	Live, right stifle, OCD
	abDH5 LH	Live, left hock, OCD
	abDH6 LH	Euthanized, left hock, lame
	abDH7 RS	Live, right stifle, OA
	abDH8 RH	Live, right hock, OCD

3.3.2 SEC Light Scattering

The same instruments were used for the light scattering measurements of synovial fluid as the pure sodium hyaluronate samples (Section 1.4.4). The carrier phase

was phosphate buffers solution pumped at 0.2 ml/min. Synovial fluid samples were diluted in PBS (1:10 dilution) prior to filtration with a 0.45 μm filter. Following filtration samples were injected into the system in the same manner as pure HA samples.

3.3.3 Rheology

All rheological experiments were performed using the Rheometrics Fluids Spectrometer II (RFS II) with a 10 g-cm transducer and a 1 degree, 50 mm cone geometry (1° 50 mm C&P) at room temperature. After samples were loaded the outside edge of the cone was covered in a thin layer of 20 cst Dow Corning Silicone Fluid to prevent sample evaporation. Steady shear tests were performed at shear rates from 0.1–1000 s^{-1}. Dynamic tests were performed at frequencies from 0.01–15 Hz at 75-100% strain. For some samples at low shear rates and low frequencies, measured torque values fell below the limits of the torque transducer, thus that data has been omitted.

3.4 Results and Discussion

3.4.1 Molecular Characterization: SEC-MALLS

Since a large number of samples were analyzed using light scattering, only the raw data from one synovial fluid sample will be shown as an example. The sample chosen for demonstration is case abDH4 RS. This was the only synovial fluid

sample for which data was collected at Wyatt Technology Corporation. Although all the molecular weight and concentration values reported at the end of the chapter were from data collected at OSU, the additional data supplied by the viscometer is quite useful and will be used for demonstration. It should also be noted that the following synovial fluid data taken at Wyatt Technology Corporation was done at a flow rate of 0.5 ml/min while all other synovial fluid samples were measured at 0.2 ml/min. These two flow rates do not change results, only elution time. This sample was prepared as all other samples; diluted by a factor of 10 and filtered with a 0.45 μm filter; injection volume was 100 μL. Figure 3-2 and Figure 3-3 were both compiled using Astra 5.

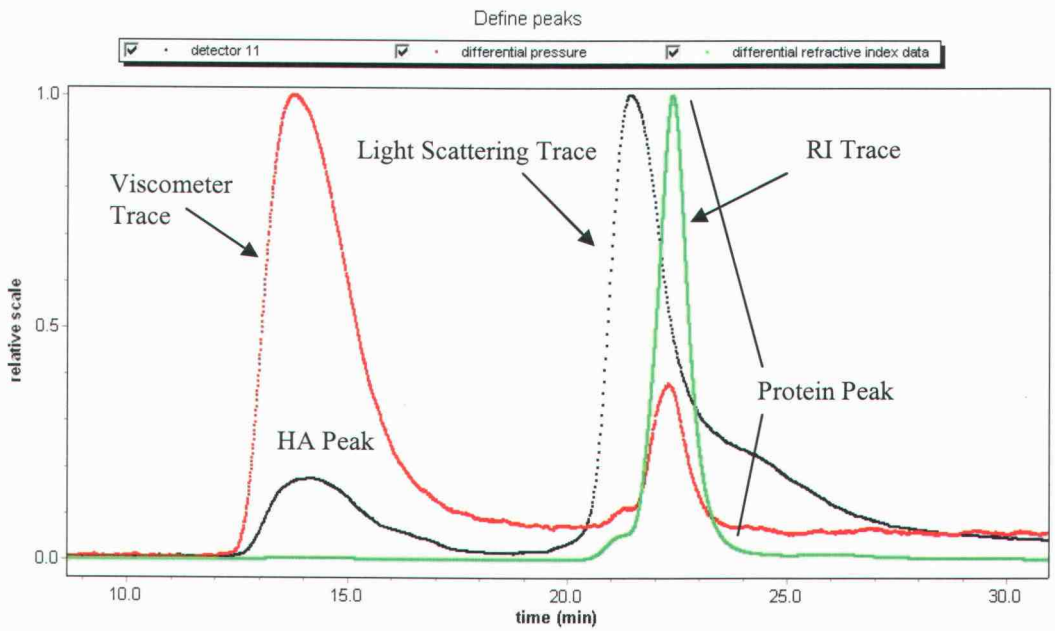


Figure 3- 2 Trace of light scattering, refractive index and viscometer data for synovial fluid, sample abDH4 RS (90^{o} detector)

Unlike the HA data, synovial fluid shows two peaks at distinct elution times. The first peak elutes in the range of 12-17 minutes after injection and the second peak comes out at about 20-24 minutes. The first peak is much smaller in magnitude than the second peak for the light scattering and RI detectors. Since the RI detector peak is so small, this must be due to a low concentration. The smaller peak elutes first and therefore must be a higher molecular weight than the second peak. In addition, the viscometer signal of the first peak is much greater than the second peak indicating much higher viscosity for this component of synovial fluid. Kvam et. al. (1993), who ran HPLC studies on synovial fluid, reported a protein peak that slightly overlapped with the HA peak. Because of these reasons, the first peak was determined to be an HA peak while the second peak is most likely some type of protein.

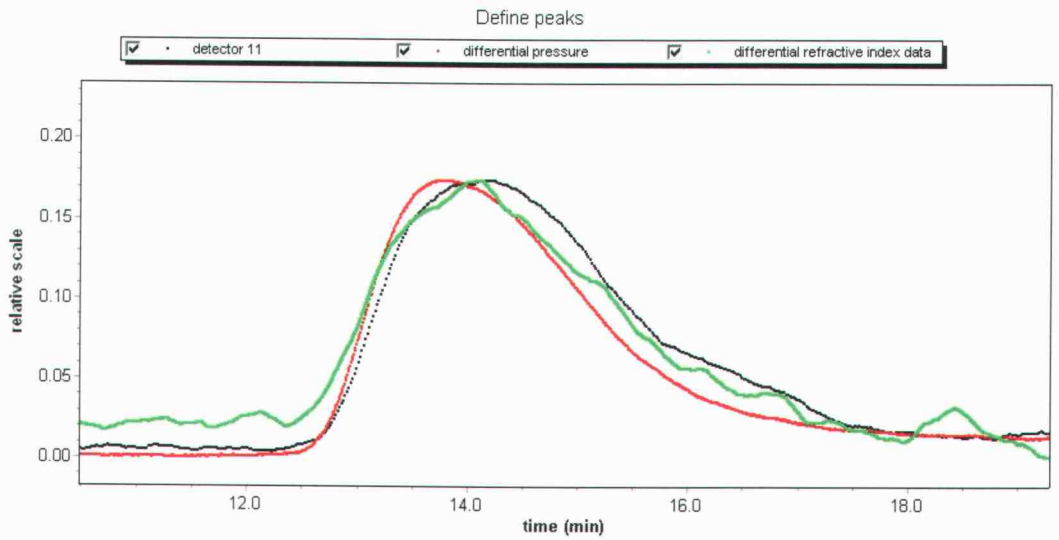


Figure 3- 3 Enlargement of HA peak in synovial fluid (abDH4 RS)

Figure 3-3 is a closer look at the HA peak (with the 90 degree detector data). The peak appears to be very monodisperse. Notice the scale on the y-axis is relative to include all data on the same plot. The RI data is more rough than the light scattering data, this is due to the very small concentration of HA being detected. The synovial samples were diluted by 10 before injection into the system, and only about 100 µl was injected, so a total HA mass of $1x10^{-5}$ to $1x10^{-6}$ g is being shown here.

Below (Figure 3-4) is the molar mass vs. time analysis for a different sample, VH3 LH. This data was collected at OSU and analyzed using Astra IV. The y-axis refers to the dots and lines only and the traces are from the RI detector.

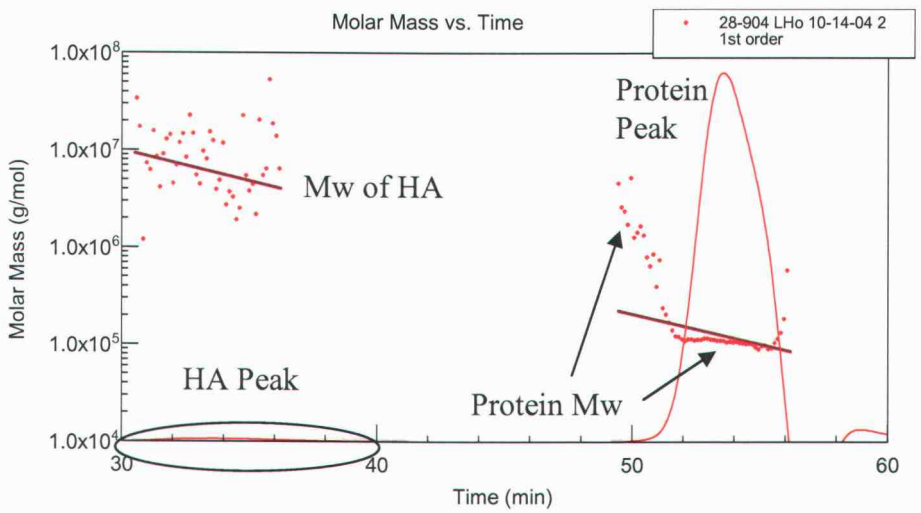


Figure 3- 4 Molecular weight and time of synovial fluid sample VH3 LH

Notice how much higher the molecular weight of the HA peak is than the protein peak. For all samples, the beginning of the protein peak has a high molecular weight in the beginning which flattens out to a much lower molecular weight. The sample shown in Figure 3-4 was analyzed using Astra 4 which does not allow the user to analyze peaks at differing dn/dc values. Therefore the molecular weight shown in the peak was calculated using the dn/dc for HA, 0.167 which is not correct. Although the numbers are not accurate, the trend of high to low molecular weight is the same for all the protein peaks that have been measured.

Astra 5 does however allow the user to change the dn/dc value, so a complete analysis of the second peak for sample abDH4 RS was done. The average molecular weight in this region is reported as about 10,000 Da (using a dn/dc value of 0.185 which is typical for proteins). The concentration in this peak is about 10 mg/ml. But, using the dn/dc value of 0.185 relies on the assumption that this peak is purely protein, which may not be entirely accurate.

An attempt was made to digest the synovial fluid samples with a protease and then perform SEC-MALLS to observe changes in the protein peak. Diluted synovial fluid samples (diluted by a factor of 10) were incubated at 37° C for one hour with P-6141 (a general protease) from Sigma. They were filtered with a 0.45 μm filter and run as normal. No change was observed in the peak after one trial and the tests were stopped in the interest of time. This would be an important next step in

synovial fluid studies for many reasons. First, determining the composition of synovial fluid is key to understanding it. Second, proteins may form aggregations with HA, and breaking this aggregation down chemically should be visible using SEC-MALLS and may alter the rheological properties. It is recommended that further work in this area be done following the procedure and protease described by Kvam et. al. (1993). Since these molecules were not the focus of this research and probably do not make as much of a significant contribution to rheological properties as HA, they will be ignored for the rest of the thesis.

Table 3-2 includes a summary of the molecular properties of HA in synovial fluid. The standard deviation (expressed here as a percent of the average) of each measurement was based on three or four trials and the algorithm used was the built-in Excel standard deviation function.

The molecular weight values range from 1.56 $x10^6$ to 6.54$x10^6$ Da, and the concentration of HA ranges from 0.11-0.84 mg/ml. Unfortunately, the standard deviation of molecular weight is very high, ranging from 13.2% to almost 40%. The standard deviation of the concentration is a bit lower (except abDH8 RH and DH3 LS), ranging from about 4% to 30%. This means that the lack of repeatability could be coming from the light scattering data. It could be because of noise, perhaps more filtering is necessary. Another possibility is that the synovial fluid is non-uniform and that any individual injection into the SEC-MALLS

system is unique. Perhaps treating synovial fluid with a protease will clean up the HA peak. Or maybe simply mixing the sample before injection would increase repeatability. These studies will be continued to try and achieve greater accuracy and reproducibility.

Table 3- 2 Summary of synovial fluid light scattering results

	Sample	Mw	*Mw St Dev	Mw/Mn	Mw/Mn St Dev	c (mg/ml)	c St Dev
St	DH1 LS	2.64E+06	*12.7%*	1.11	*6.88%*	0.84	*8.1%*
	DH2 RS	5.46E+06	*36.2%*	1.01	*0.57%*	0.48	*7.2%*
	DH2 LS	3.31E+06	*13.8%*	1.01	*1.14%*	0.48	*3.9%*
	DH3 RS	4.81E+06	*18.6%*	1.14	*2.32%*	0.34	*14.5%*
	DH3 LS	2.64E+06	*15.2%*	1.06	*7.12%*	0.17	*37.1%*
Ho	DH3 RH	2.73E+06	*39.8%*	1.33	*14.68%*	0.15	*25.6%*
	DH3 LH	6.54E+06	*16.3%*	1.16	*14.43%*	0.13	*28.0%*
	DH1 RH	3.00E+06	*17.2%*	1.02	*1.27%*	0.13	*13.0%*
	VH1 LH	4.57E+06	*23.2%*	1.14	*2.63%*	0.31	*12.9%*
	VH3 RH	3.06E+06	*37.6%*	1.03	*0.97%*	0.22	*18.2%*
Ab	abDH4 RS	1.56E+06	*38.8%*	1.66	*8.08%*	0.18	*6.7%*
	abDH5 LH	2.20E+06	*16.9%*	1.27	*8.96%*	0.27	*16.7%*
	abDH6 LH	1.88E+06	*31.0%*	1.27	*7.06%*	0.18	*22.2%*
	abDH7 RS	4.93E+06	*13.2%*	1.01	*0.99%*	0.32	*9.7%*
	abDH8 RH	5.14E+06	*23.3%*	1.26	*21.72%*	0.11	*76.7%*

*Note: As a comparison, the narrow distribution polyethylene oxide (PEO) standards (Toso Bioscience) used to calibrate the SEC-MALLS system typically have standard deviations of 1-10%

The molecular weight is definitely in the range of values reported in the literature. The concentration is also, but it is lower than most, similar to those reported by

Kvam et. al. (1993). It is possible that the some HA is filtered out of solution prior to injection into the SEC system. Reed et. al. (1989) reported that approximately 10% of HA was removed with after filtration with a 0.45μm filter. An easy test of this is to use a larger filter to see if the concentration changes, this will be done in the continuing studies.

Unfortunately, the three categories of synovial fluid (stifle, hock and abnormal) are indistinguishable according to molecular weight and concentration. However, there is a significant difference in polydispersity between abnormal samples and normal hocks and stifles. The increase in polydispersity of HA in abnormal joints could be indicative of the breakdown of HA in the diseased joints and warrants further examination.

3.4.2 RHEOLOGY

Steady shear and dynamic oscillatory shear results will be presented separately. Within each of these sections, synovial fluid samples will be separated into three categories: healthy stifle joints, healthy hock joints and abnormal or diseased joints. This is to look for similarities among the same joint from different horses. Additionally, the steady shear results from three horses that donated multiple joint samples, DH1, DH2 and DH3, will be presented together to compare different joints from the same horse.

3.4.1.2 Steady Shear

3.4.1.2.1 Stifle Joints

Figure 3-5 and Figure 3-6 show the steady shear and complex viscosity results for five healthy stifle joints. The results are similar to the pure HA sample in that the synovial fluid samples typically exhibit a zero shear viscosity at low shear rates (except DH1 LS) and a shear thinning region at higher shear rates. Both right and left stifles are presented for two horses; DH2 and DH3. The viscosity behavior of left and right stifle samples from DH2 are nearly the same and are about ten times higher than the viscosity of the other samples. The left and right stifles from DH3 are not as similar to each other as DH2 and are much lower in viscosity than DH2. This shows that differences between right and left sides may vary from horse to horse; some horses may have very similar properties in the left and right joints while some may not. DH1 LS does not approach a zero shear viscosity.

The complex viscosity resembles the steady shear viscosity. Left and right samples from DH2 have very high viscosity and approach Newtonian plateaus. DH1 LS exhibits a slight upturn at low frequencies.

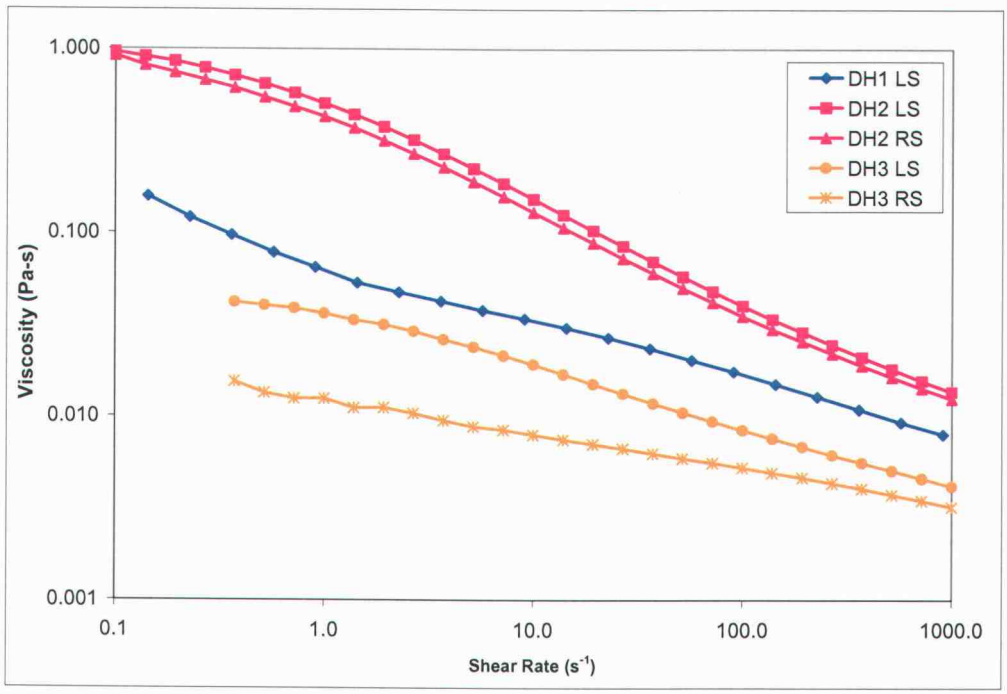


Figure 3- 5 Steady shear results for equine synovial fluid from healthy stifle joints

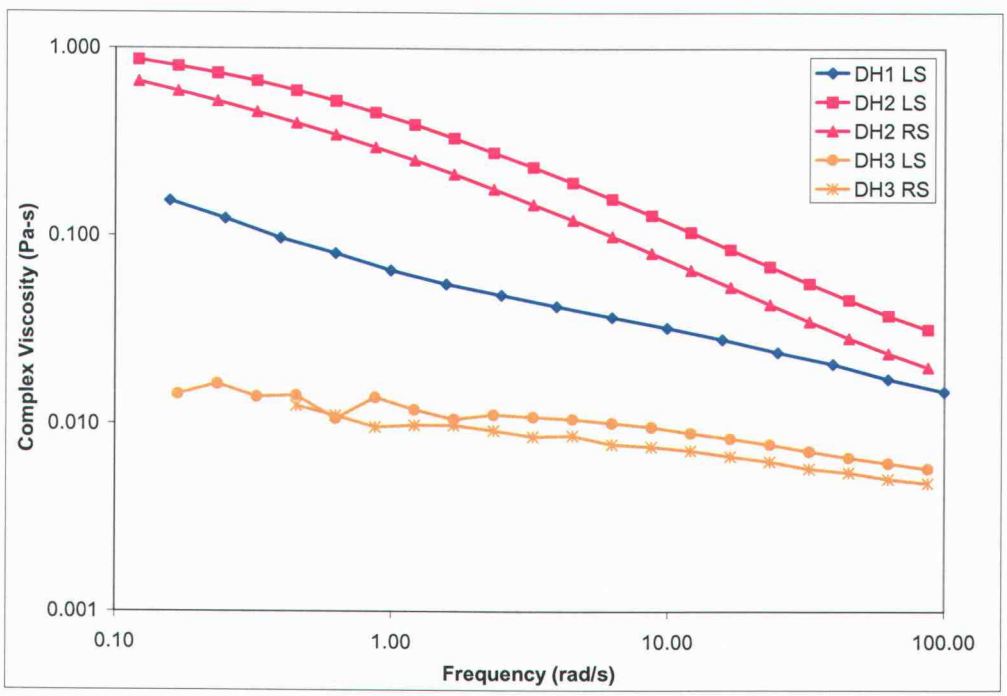


Figure 3- 6 Complex viscosity of healthy equine synovial fluid, stifle joints

3.4.1.2.2 Hock Joints

Figure 3-7 and Figure 3-8 show the viscosity and complex viscosity of five hock joints as a function of shear rate and frequency. Below are the results for the hock joints. The right and left hock of DH3 are very similar to each other, although the viscosity is very different than the stifle joints from the same horse. This suggests that there may be a significant difference between joints within a single horse. The hock samples from dead horses do not exhibit very strong Newtonian plateaus in the rate range that was measured, and no samples have viscosity as high as the stifles of DH2. The complex viscosity exaggerates the upturn of DH1 RH, but the samples from DH3 are very flat. The samples from live horses lie between the samples from DH3 and DH1 RH. They are also much less shear thinning and approach Newtonian plateaus at low shear rates, but exhibit some upturn in complex viscosity at low frequencies.

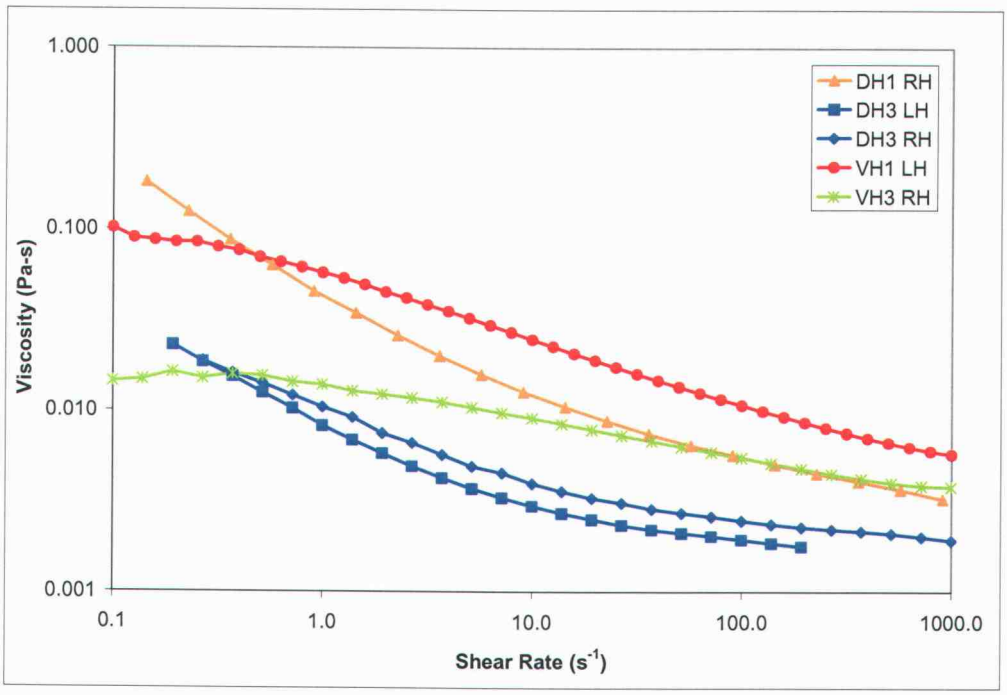


Figure 3- 7 Steady shear results for synovial fluid from healthy hock joints

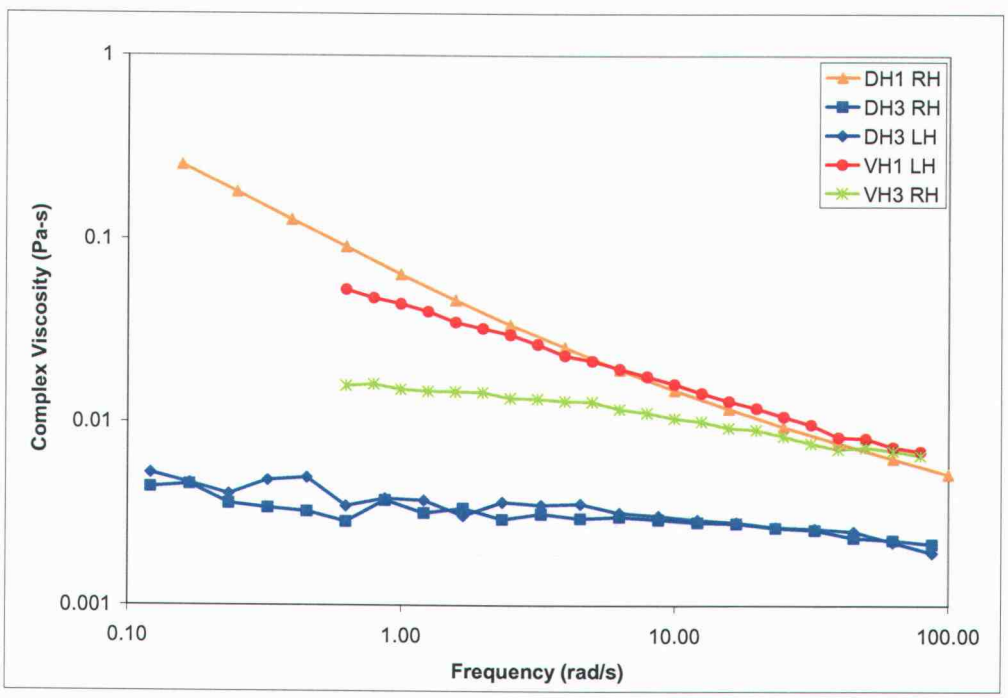


Figure 3- 8 Complex viscosity of healthy equine hock joints

3.4.1.2.3 Abnormal Joints

Of the three categories of joints tested, the abnormal joints appear to group most closely to each other. The samples from abDH4, abDH5 and abDH8 have osteochondrosis dissecans (OCD), a developmental joint disease. The samples from abDH6 and abDH7 were both lame for unknown reasons. None of these samples approach zero shear viscosity; all exhibit an upturn in both viscosity and complex viscosity. They also appear to have a slightly steeper shear thinning slope, an indication of the break-up of aggregates.

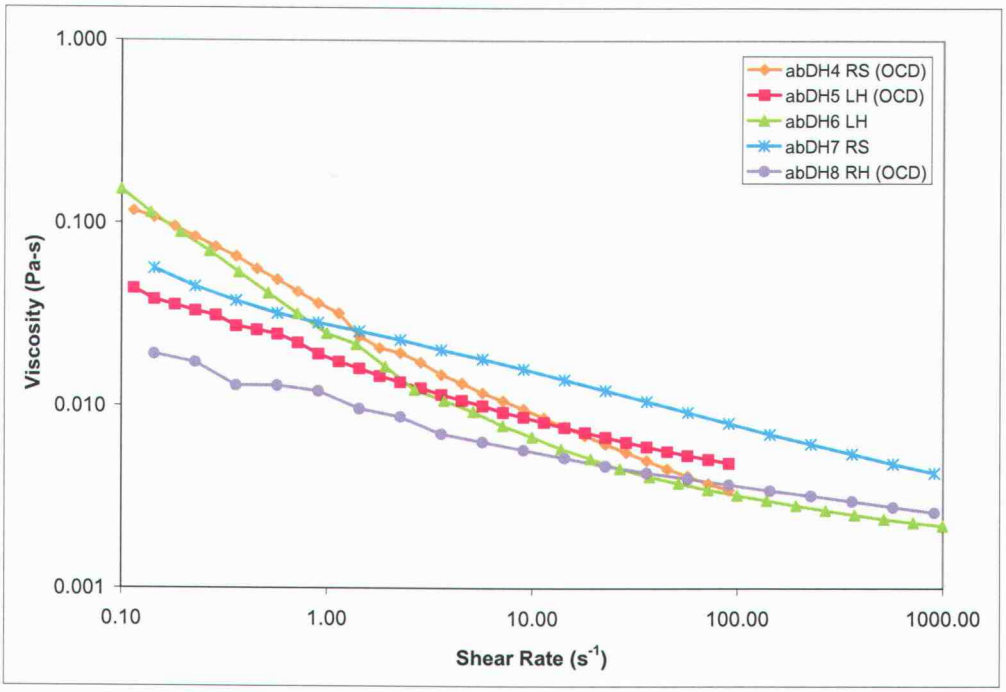


Figure 3- 9 Steady shear results for synovial fluid from abnormal joints

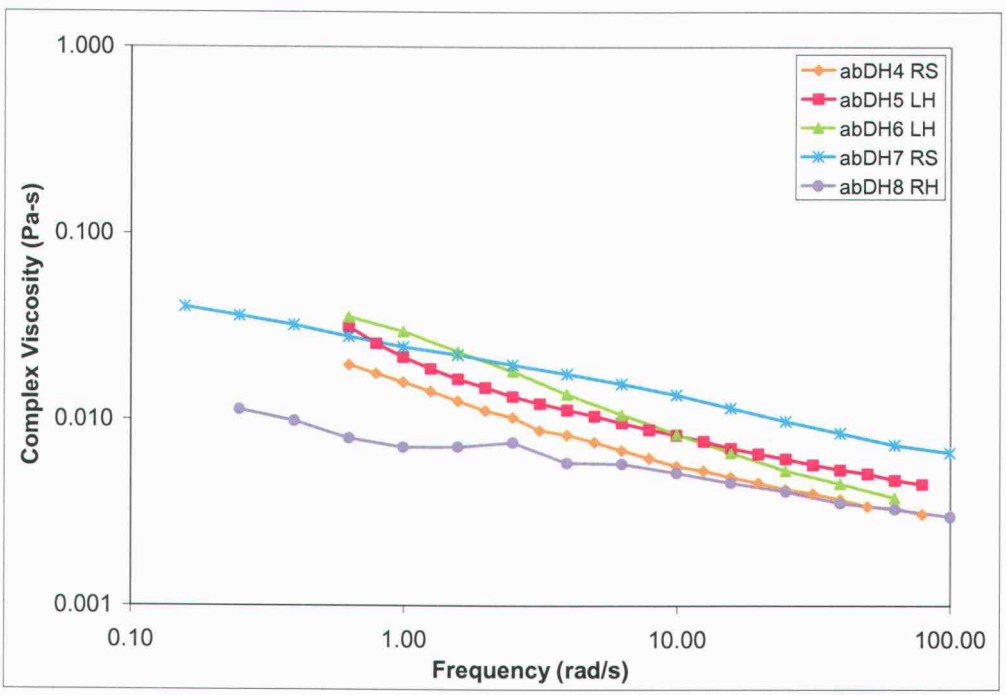


Figure 3- 10 Complex viscosity of abnormal equine synovial fluid

3.4.1.2.4 Steady Shear Results by Horse

This section will allow examination of multiple joints for horses DH1, DH2, and DH3.

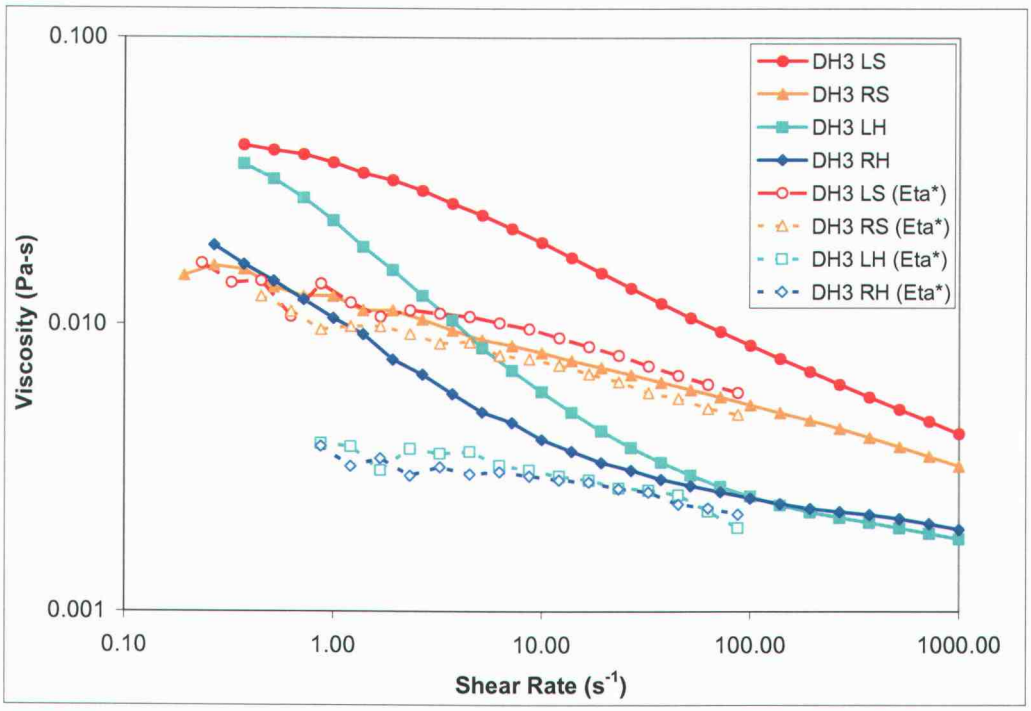


Figure 3- 11 Horse DH3- comparison of steady shear and complex viscosity data for all joints

Figure 3-11 groups all the viscosity results (steady shear and complex) of all joints from horse DH3. There appears to be a significant difference between the hock and stifle behavior for this horse. However, left and right stifles and left and right hocks have similar steady shear viscosity behavior and nearly identical complex viscosity behavior.

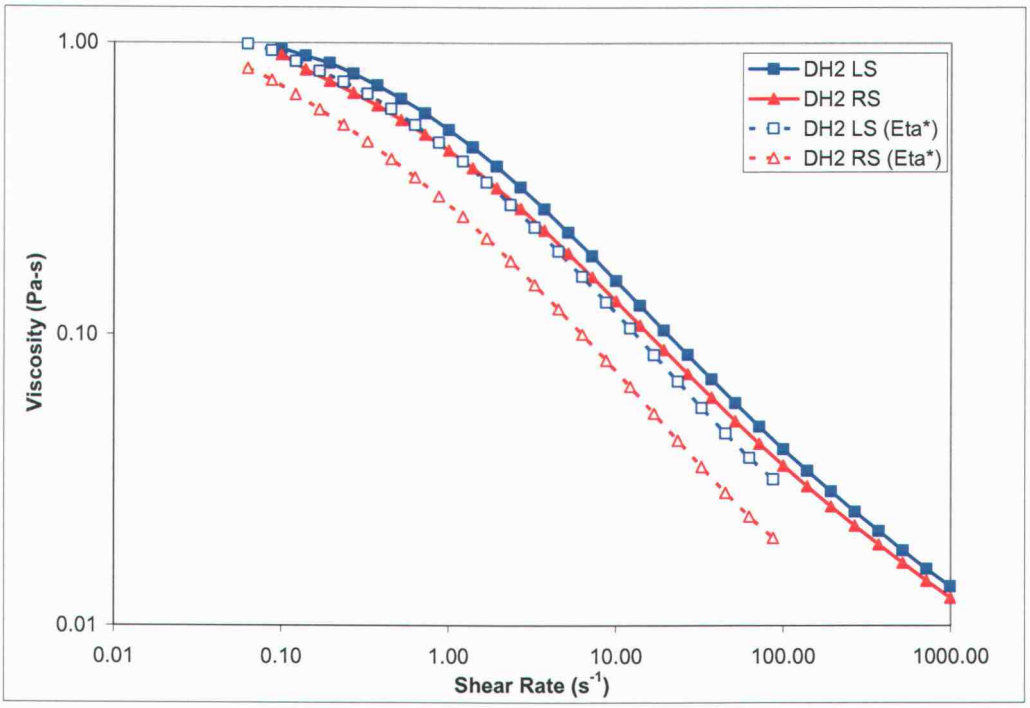


Figure 3- 12 Steady shear viscosity and complex viscosity comparison of right and left stifles of DH2

The stifles of DH2 exhibit the highest viscosity of all synovial fluid samples reported. The left and right stifles are almost identical in steady viscosity, but there is a more pronounced difference in complex viscosity. Both stifles approach a zero-shear viscosity, which appears to be uncharacteristic of most synovial fluid samples. The complex viscosity closely follows the steady shear viscosity and provides a good example of the Cox-Merz rule (where complex viscosity and steady shear viscosity approach the same value at low shear rates).

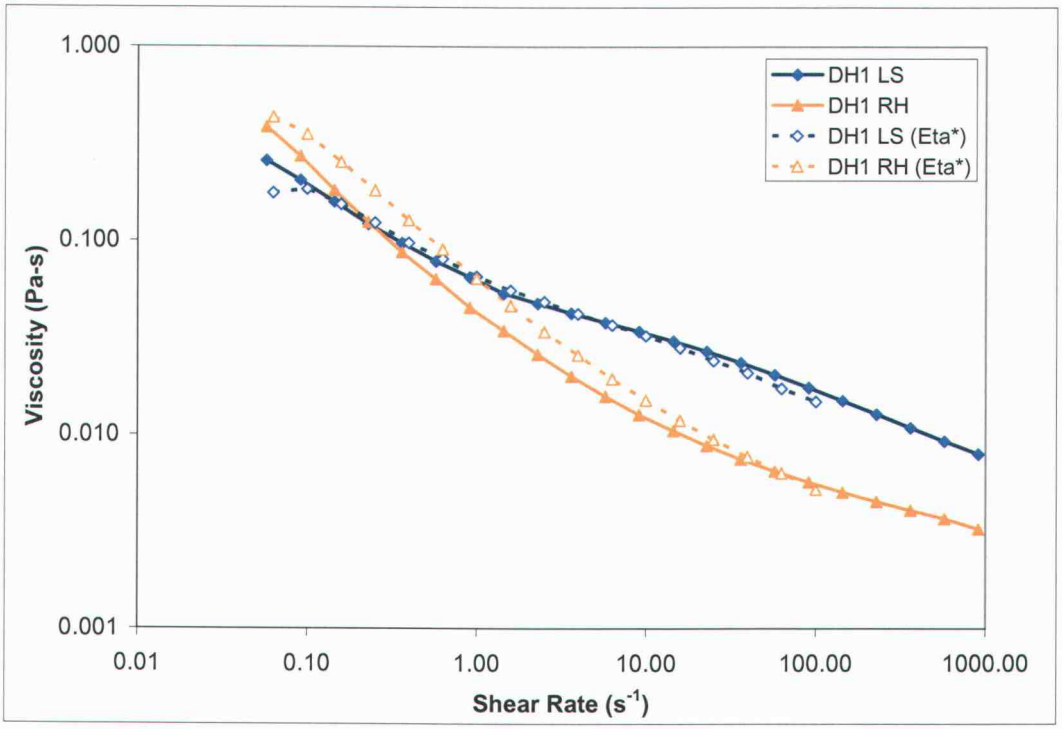


Figure 3- 13 Steady shear and complex viscosity comparison of the left stifle and right hock of DH1

The right hock and left stifle of DH1 allow a close comparison of two different joint types of the same horse. The viscosity behavior is not as different as the viscosity behavior between hocks and stifles of DH3. Unlike DH2, the samples from DH1 do not exhibit zero shear viscosities; instead they have an upturn at low shear rates, an indication of aggregates in these synovial fluid samples.

3.4.2.2 Dynamic Oscillatory Shear

Now the dynamic oscillatory shear results will be presented for each joints studied.

3.4.2.2.1 Stifle Joints

Figure 3-14 shows the dynamic oscillatory shear results for the stifle joints.

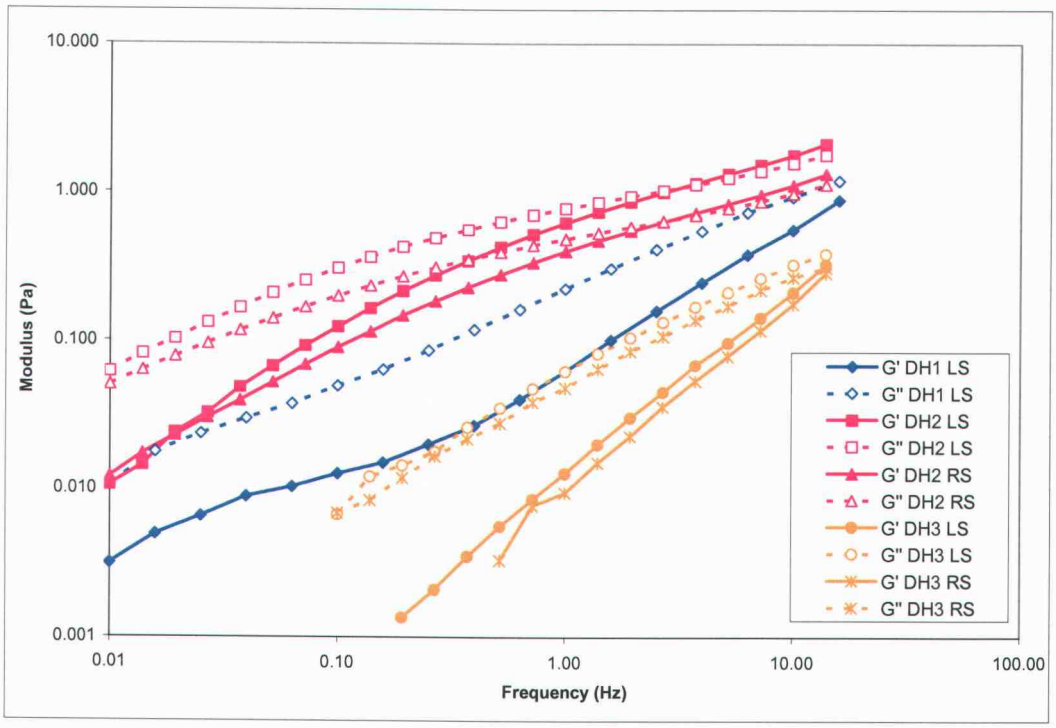


Figure 3- 14 Dynamic oscillatory shear results of synovial fluid from healthy stifle joints

Again the presentation will begin with the dynamic results of the stifles (Figure 3-14). As observed in the steady shear results, the right and left stifles for DH2 are very similar to each other. And, unlike the steady shear graph, the right and left stifle of DH3 are also very similar. The complex viscosity of DH3 LS and DH3 RS were also similar to each other, perhaps the contribution of elastic behavior demonstrates the similarity between these two samples. DH2 shows the highest modulus, and both samples exhibit a cross-over point between 1-10 Hz, which no

samples thus far have shown. This must mean that there is something extraordinary about the HA content of these two samples. Also of note is the fact that the samples lie in the same order for highest modulus as they do for highest viscosity. This agreement between the two tests is a good verification of the results.

3.4.2.2.2 Hock Joints

Below are the dynamic oscillatory shear results for hock joints.

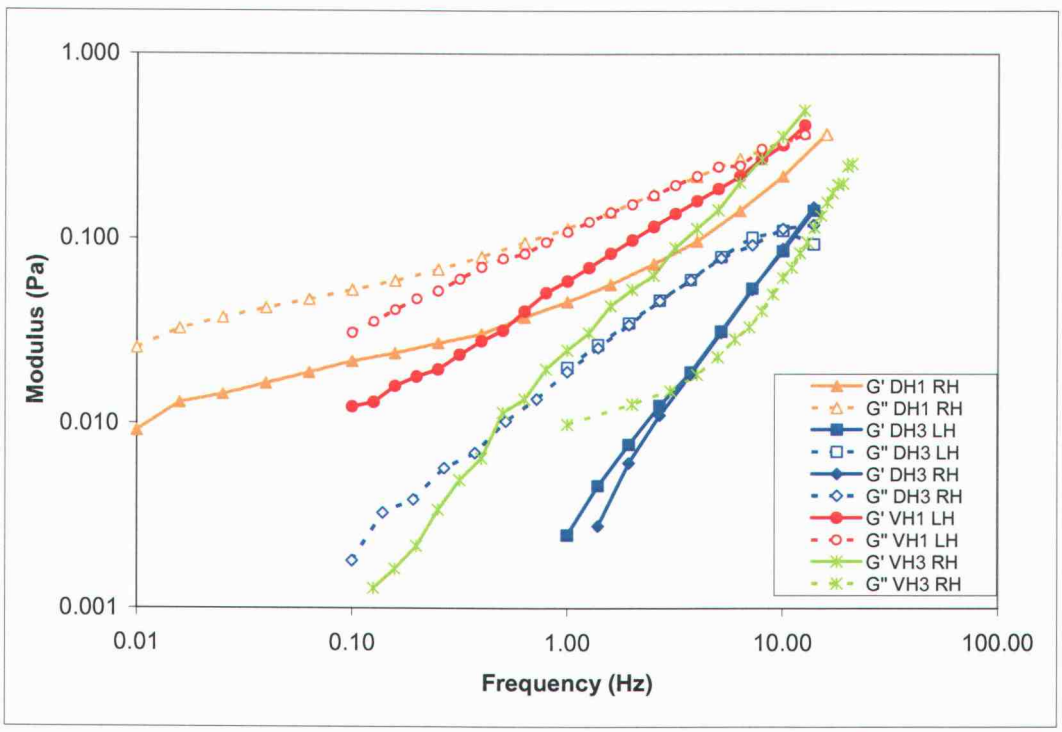


Figure 3- 15 Dynamic oscillatory shear results for synovial fluid from healthy hock joints

The dynamic results for the hocks (Figure 3-15) correlate well with the steady shear results also. Both hocks for DH3 are similar to each other and have low modulus values, just as the viscosity results. They appear to cross over near 10 Hz, but the flat shape of G" is not typical for viscous modulus results. Therefore these are probably not actual cross over points, rather a product of the instrument. The moduli for DH1 RH are much higher, and this cross over point appears to be a genuine one. The modulus from the live horses, VH1 LH and VH3 RH lie in between the DH3 samples and DH1 RH. Both VH1 LH and VH3 RH exhibit cross over points at a high frequency. The range of G' and G" values are similar to the stifles.

3.4.2.2.3 Abnormal Joints

Figure 3-16 shows the dynamic results for the abnormal samples.

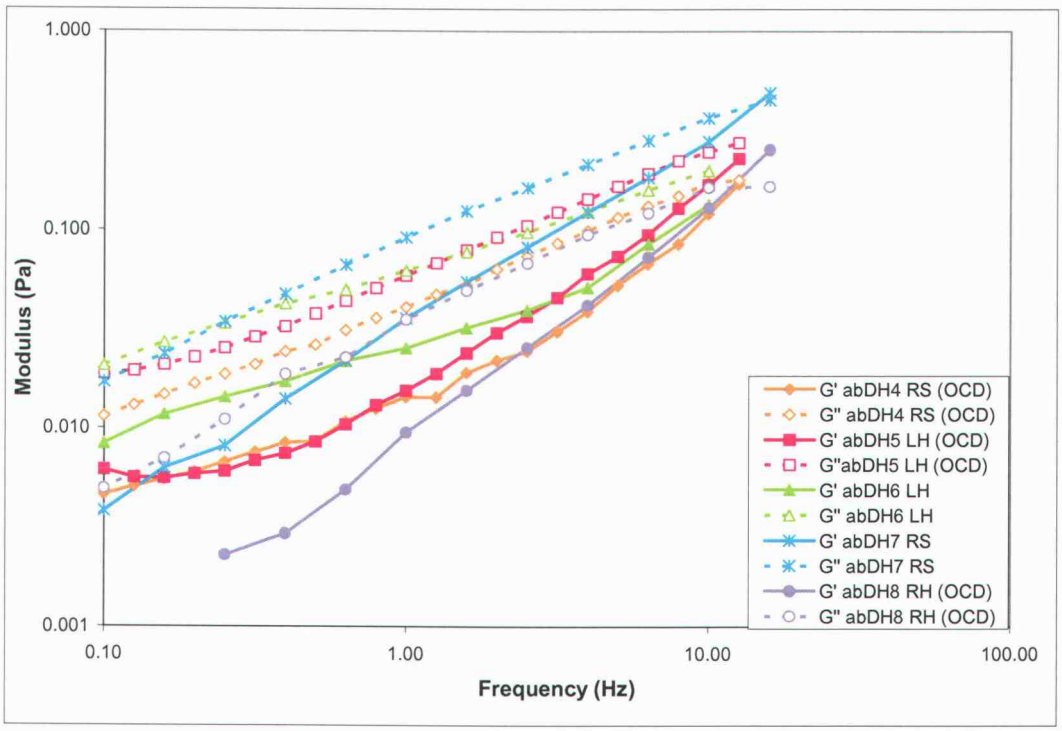


Figure 3- 16 Dynamic oscillatory shear results for synovial fluid from abnormal joints

These are very tightly grouped together just as the viscosity values were. It is also notable that most of these samples either approach or exhibit a cross over point within the measured frequency range. Notice also that the G' values for the three OCD samples are very similar to each other, especially at higher frequencies.

3.6 Summary of Synovial Fluid Results

In this chapter the molecular and rheological characteristics of synovial fluid were examined. The SEC-MALLS technique developed for HA was applied to synovial fluid samples. Molecular weight measurements ranged from about $1.5x10^6$ Da to

$6.5x10^6$ Da and concentration ranged from 0.11 to 0.84 mg/ml. These values are both reasonable according to literature.

Rheology was used to characterize the flow properties of synovial fluid. Samples were shear thinning and in the same viscosity range as HA at 2.5 mg/ml. Steady shear behavior is summarized in Table 3-3, which also includes some molecular characterization information (refer to Table 3-2 for complete results).

Table 3- 3 Summary steady shear viscosity and power-law index for synovial fluid samples

	Sample	Mw	Mw/Mn	c (mg/ml)	η_0 (Pas)	*n
Stifles	DH1 LS	2.64E+06	1.11	0.84	N/A	0.67
	DH2 RS	5.46E+06	1.01	0.48	0.916	0.49
	DH2 LS	3.31E+06	1.01	0.48	0.960	0.48
	DH3 RS	4.81E+06	1.14	0.34	0.016	0.80
	DH3 LS	2.64E+06	1.06	0.17	0.042	0.68
Hocks	DH3 RH	2.73E+06	1.33	0.15	N/A	0.56
	DH3 LH	6.54E+06	1.16	0.13	N/A	0.43
	DH1 RH	3.00E+06	1.02	0.13	N/A	0.72
	VH1 LH	4.57E+06	1.14	0.31	0.110	0.65
	VH3 RH	3.06E+06	1.03	0.22	0.015	0.80
Abnormal	abDH4 RS	1.56E+06	1.66	0.18	N/A	0.67
	abDH5 LH	2.20E+06	1.27	0.27	N/A	0.52
	abDH6 LH	1.88E+06	1.27	0.18	N/A	0.29
	abDH7 RS	4.93E+06	1.01	0.32	N/A	0.72
	abDH8 RH	5.14E+06	1.26	0.11	N/A	0.83

*"n" is the power law index (discussed in Section 2.5.2.3)

The zero shear viscosity for the HA samples ranged from 0.01-0.1 Pa-s, and the shear thinning region began between 10-100 s^{-1} depending on the sample. Of the synovial fluid samples shown, zero shear viscosity values (where applicable) range from 0.01-1 Pa-s. Although the viscosity of most synovial fluid samples lie within the range of HA samples, a few such as the stifles from DH2 were much higher than the pure HA samples. In addition, the power law index of the synovial fluid samples range from 0.43 to 0.8 which is similar to the shear thinning behavior of the HA samples. The dynamic moduli for many of the samples (Figures 3-14, 3-15 and 3-16) indicate more of a viscoelastic nature to the synovial fluid samples as compared to the pure HA samples (Figues 2-23 and 2-24).

The HA concentrations of these samples measured by light scattering are presented in Table 3-3. The concentration used for HA characterization, 2.5 mg/ml, is much higher than the HA concentrations measured in synovial fluid. If the HA concentration in synovial fluid is lower than the pure HA samples, and the molecular weight is similar, one would expect the viscosity of the pure HA samples, those with a higher concentration, to be larger. However, this is not true. This means there must be something besides pure HA that contributes to the viscosity of synovial fluid.

Synovial fluid has many other components in addition to HA such as proteins and glycosaminoglycans (GAGs). It is highly possible that the HA in synovial fluid

forms a complex with proteins and other GAGs. This would explain the upturn at low shear rates that many samples exhibit. It is likely that the shear thinning region of synovial fluid is due to the disassociation of an HA aggregate; rather than the disentanglement of HA-HA interactions. The complexity of synovial fluid could be part of the reason that a direct comparison of rheological and molecular behavior was difficult. However, research efforts in this area will continue to identify the relationship between the molecular characteristics of the HA in synovial fluid and the resulting rheological behavior. The current research does highlight, as indicated by previous researchers (Gomez and Thurston (1983) and Schuz and Ribitsch (1987)), that rheological characterization of synovial fluid (both steady shear and dynamic) can provide useful information on the lubrication efficacy of the synovial fluid and potentially the general health of the joint.

CHAPTER 4

INTRA-ARTICULAR SODIUM HYALURONATE STUDY

4.1 INTRODUCTION

Osteoarthritis (OA) is a major contributor to equine lameness. This lameness can end the careers of racehorses, show horses, and personal pets. This disease is responsible for the loss of a lot of money invested in their treatment, breeding, and training. The intra-articular or intravenous administration of sodium hyaluronate (viscosupplementation) has been recognized as a treatment for the pain associated with OA. However, the mechanism of HA supplementation is not well understood and much of the evidence of its efficacy is clinical.

One destructive result of OA is the breakdown of HA within the joint (resulting in lower molecular weights), possibly due to the release of digestive enzymes or free radicals. These enzymes and radicals are released as part of the autoimmune response associated with OA, which breaks down HA molecules. Many believe that the concentration of HA is also decreased in patients with OA. It still remains unknown how HA is responsible for joint health. The importance of hyaluronate's contribution to mechanical properties versus its involvement in biochemical pathways has not yet been determined. Regardless of the biochemical involvement of HA, there is definitely a correlation between molecular weight, concentration, and viscoelastic properties.

Viscosupplementation is a treatment involving the injection of sodium hyaluronate supplements into diseased joints. This treatment was originally devised to boost the mechanical properties of unhealthy synovial fluid, but it was later concluded that injected HA also plays a biochemical role in joint health. For horses, viscosupplementation treatments are available in both intravenous and intra-articular form. Oral HA supplements are also available but have not been studied clinically and are not considered an FDA approved treatment for joint disease.

This chapter summarizes a preliminary study performed in collaboration with the OSU College of Veterinary Medicine in which the properties of synovial fluid of five horses were analyzed before and after intra-articular HA injection or placebo treatment. The objectives of this study were to establish techniques to measure the rheological properties of equine synovial fluid and determine whether these methods could be used to determine the differences (if any) in synovial fluid before and after intra-articular injection of HA. In addition, changes in rheology were compared to nucleated cell and total protein counts in attempt to understand the change in synovial fluid after treatment.

4.2 Literature Review

As described by Moskowitz and Lewallen (2004), osteoarthritis (OA) is an imbalance between production and destruction of cartilage. Normal cartilage is

marked by a cartilage-HA matrix surrounding cells. In addition to cartilage destruction, the HA in arthritic joints is also broken down by enzymes or radicals. This reduction in the protective pocket of HA is one source of cartilage damage. OA is also associated with inflammatory response.

Viscosupplementation was originally conceptualized by E.A. Balazs in the late 1960's (Balazs, 1974; Blazas and Denlinger, 1985). He was the first to measure and publish the rheological properties of synovial fluid and the first to realize that the viscoelastic properties decrease with incidence of disease. He proposed that injecting pure HA into the joint would increase the mechanical properties of synovial fluid, thus protecting the joint. Viscosupplementation as a treatment for arthritis began in the early 1970's and has faced some criticism since then. Clinical studies are not always conclusive due to placebo effects, differences in frequencies and duration of treatment, and other variables. There remain discrepancies concerning the ideal molecular weight of HA in addition to the ideal frequency of and total number of treatments (Marshall, 1998).

While the original concept of viscosupplementation was a good one, Balazs' original explanation for its efficacy was incomplete. In a normal joint, the turnover of HA is very rapid, with a half life of less than 24 hours. For example, Brown et. al. (1991) suggested that HA is completely replaced every 28 hours in the joints of rabbits. Similar results were found by Robert et. al. (1981) who also

studied the HA turnover rate in rabbits. The lifetime of HA in horses has been reported as 96 hours (Stashak, 2002) with the lifetime in humans probably between that of rabbits and horses. This realization greatly conflicted with clinical reports of positive viscosupplementation effects for up to six months after treatment.

Some years later, Balazs and Denlinger (1993) proposed that the HA matrix in the joint cavity acts as a sieve that protects the cells. The breakdown of that sieve allows more damaging radicals and enzymes into the cavity further increasing HA damage. They argued in a normal joint, if HA is added (exogenous HA), the clearance rate of HA will increase to remove the extra HA. However, in an arthritic joint this added, or exogenous, HA brings the joint concentration closer to normal, thus increasing the lasting effects of treatments.

This evidence suggests that HA plays an important pharmacological role in synovial fluid, in addition to the obvious mechanical one. Ghosh (2002) published a literature review supporting this evidence, from which much of the below information was taken, however it has been supported by other sources. One such role of HA is the interaction with cell receptor CD44. These interactions may be molecular weight dependent, meaning low molecular weight HA may trigger a pathway that cannot be triggered by a high molecular weight HA molecule (George, 1985). This receptor and its interactions with HA may lead to immunoregulatory and anti-inflammatory activities in the joint. CD44 is also

important in pain perception, and HA may reduce the effects of the pain producing pathway. This could be part of its efficacy in OA treatment.

Hyaluronate may also protect chondrocytes (the cells that make up cartilage), although this phenomena appears to occur more frequently with treatment of high molecular weight HA (>$2x10^6$ Da). Conversely, low molecular weight HA seems to give better synovial cell protection. The difference in effectiveness could be due to increased mechanical properties of high molecular weight HA compared to higher penetration in the synovial cavity by low molecular weight HA, meaning smaller HA molecules will have greater access to the joint cavity. Exogenous HA may also reduce the activity of radicals and enzymes that break down HA (Ghosh, 2002). It also appears that HA protects cartilage, plays a role in the inflammatory process, reduces synovial cell proliferation and removes radicals and other damaging items from the joint cavity (George, 1985).

Moskowitz (2000) placed the major functions of HA in synovial fluid into three categories. First, the large hydrodynamic volume of HA has a great influence on the hydration properties of tissues. Second, the interactions of HA with other macromolecules in the matrix, potentially forming complexes. (This includes HA interactions with cell receptor CD44.) Third, exogenous HA may protect endogenous HA, in addition to stimulating production of more HA.

Many clinical studies of viscosupplementation have been performed in humans which has lead to great discussion as to whether the benefits of treatment are genuine or placebo. In a summary of the state of intra-articular HA supplement, George (1985) argued that although HA seems to have a positive effect on joints, there is also a reduction in pain associated with joint aspiration (standard procedure prior to injection) that interferes with measurements. In a similar summary, Cohen (1998) also agreed that HA injections may improve pain in patients with mild arthritis and frequent injections.

Peyron (1993) stated in his summary of clinical trials of intra-articular injection prior to 1993 that 60-75% of patients in HA groups felt improvement compared to 25-30% of the control group. There is also a low occurrence of side effects in all patients. Although viscosupplementation may not be a permanent fix for OA, it does not cause damage which justifies its continued use as a treatment for arthritis.

Animal studies may contribute more concrete findings because the evaluation of lameness is less subject to placebo effect than human evaluation of his or her own pain. Although there are numerous studies in existence, a few will be summarized here for example. In Kawcak et. al. (1997) studied intravenous effects of HA on horses. Twelve horses were treated and walked on a treadmill with synovial fluid samples taken up to 72 days after treatment. It was determined that the experimental group showed a significant reduction in lameness. Analysis of

pulled synovial fluid samples showed a decrease in production of inflammatory mediators.

Rydell and Balazs (1971) also noticed a decrease in further cartilage damage associated with HA injections. In addition to seeing a reduction in lameness in racehorses, Galley (1986) also noticed a change in disposition of treated horses in his study. He said horses receiving HA treatment did not seem to "dread" trips to the race track as they had prior to injections.

Although the clinical effects of HA injections have been well studied (even if they remain somewhat inconclusive), few have actually studied the properties of synovial fluid before and after treatment. Matsuno et. al. (1998) was one group who did. He measured HA concentration (using enzymatic degradation), molecular weight, fluid viscosity, extensional viscosity (reported as "stringyness") and glycosaminoglycan (GAG) and protein concentration before and after HA injection in humans. He found the content of proteins and GAGs increased after intra-articular (IA) injections of HA. The molecular weight of HA did not appear to change, but concentration (and consequently viscosity) increased after treatment. Unfortunately, these results showed a wide variation due to the nature of a biological study making a strong statistical argument difficult.

Goto et.al. (1993) also examined synovial fluid from individuals with rheumatoid arthritis before and after intra-articular injection of HA. They found the concentration HA, specific viscosity and "stringency" to increase greatly after injection of HA. However they did not see a significant difference in molecular weight, total synovial fluid volume or protein concentration. They also concluded that there was a significant difference in pain for the individuals who received treatment.

In general, most studies seem to agree that intra-articular HA injections appear to have a positive clinical effect for individuals who receive them. The debate involves the pharmacological pathways in which HA is involved and to what extent this is molecular weight dependent. A few studies have been completed in which the properties of synovial fluid are measured before and after treatment, but these were done using a very limited rheological study.

4.3 THEORY

All of the techniques used in these studies (molecular and rheological characterization) have been previously presented, with the exception of relative viscosity measurements. In Chapter 2 the determination of intrinsic viscosity was discussed. In order to measure the intrinsic viscosity of a solution the exact concentration of polymer must be known. At the time of this preliminary study SEC-MALLS procedures had not yet been developed, so a concrete value for HA

concentration was unobtainable for these samples. Instead of graphing specific viscosity against concentration, relative viscosity was graphed. Relative viscosity is the comparison of solution viscosity, η, to solvent viscosity, η_s, as follows where the subscript "s" denotes solvent. Viscosity is proportional to flow time, t.

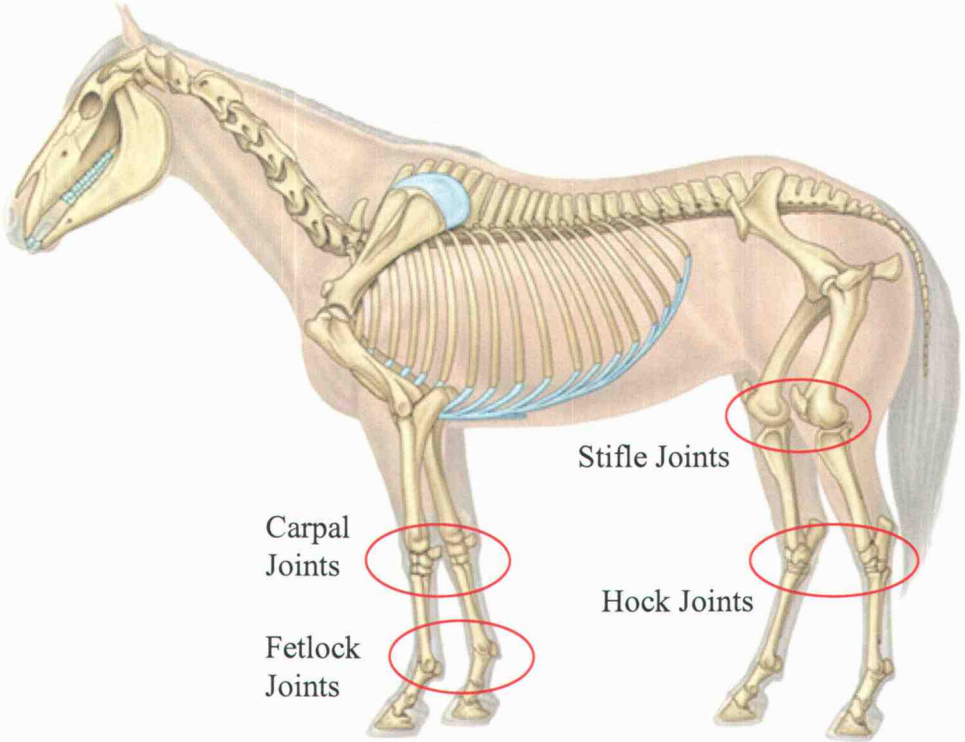
$$\eta_{rel} = \frac{\eta}{\eta_s} = \frac{t}{t_s} \qquad \text{Eqn. 4- 1}$$

In dilute regimes, relative viscosity, η_{rel}, versus concentration should be linear. Any nonlinearity would be due to polymer-polymer interactions. Higher values in relative viscosity are due either to larger molecules (high molecular weight) or more molecules (high concentration), just as in measurements for viscosity.

4.4 Materials and Methods

4.4.1 Experimental Design

Six middle-aged horses (two mares and four geldings) were evaluated for clinical lameness in the tarsocrural hock joints (Figure 4-1) prior to the study. Upon physical examination, the right and left tarsocrural hock joints of all horses were deemed "normal," meaning without disease. The six horses were divided evenly into a control and an experimental group. According to standard procedure of intra-articular injections, the synovial fluid was aspirated from the hocks of all horses prior to administration of treatment (this sample is referred to as the week zero sample). The week zero sample of synovial fluid was used as the baseline value to compare subsequent samples from each hock.



(www.antbits.co.uk/ant_pages/ bio-pages/bio-04.html)

Figure 4- 1 Diagram of joints in the horse

Following aspiration, each hock received one of three treatments at week zero. The experimental group received an intra-articular injection of 2 mL of Hyvisc^{TM} in each hock (a high molecular weight HA supplement). The control group was injected with 2 mL of sterile lactated ringers solution (simulates plasma) in one hock (positive control) and given no treatment to the other (negative control, also after removal of synovial fluid). (All samples from one experimental horse were

rejected due to blood contamination and inadequate quantity of fluid.) Joints were treated as outlined in Table 4-1.

Table 4- 1 Treatment for each hock

Joint	Injection	Experimental Group
VH1 RH	2 mL LRS	Positive Control
VH1 LH	None	Negative Control
VH2 RH	2 mL Hyvisc	Experimental
VH2 LH	2 mL Hyvisc	Experimental
VH3 RH	None	Negative Control
VH3 LH	2 mL LRS	Positive Control
VH4 RH	2 mL LRS	Positive Control
VH4 LH	None	Negative Control
VH5 RH	2 mL Hyvisc	Experimental
VH5 LH	2 mL Hyvisc	Experimental

Synovial fluid was aspirated one week and two weeks after treatment to evaluate the effects of each treatment. Between joint aspirations the horses were kept as normal with no restriction of movement or unusual boarding procedures.

Daily physical examinations of each horse were performed to monitor for swelling, lameness or other signs of infection. Cytology, which included the documentation of color and clarity, cell morphology, total protein, and nucleated cell counts, was performed on each sample prior to rheological characterization. These tests were performed to monitor inflammation levels of the joints and to correlate with viscosity and elasticity if changes occurred.

4.4.2 RHEOLOGICAL CHARACTERIZATION

Rheological characterization of each sample was performed to quantify any effects on the viscoelastic properties of synovial fluid. Three tests were run on each sample: steady shear tests, dynamic oscillatory shear tests, and dilute capillary viscometer tests. In accordance with rheological characterization of HA and other synovial fluid samples, both the steady shear and dynamic oscillatory tests were performed with the Rheometrics Fluids Spectrometer II (RFS II) using a one degree cone geometry and a gap setting of 0.05 mm. Dynamic tests recorded the elastic and viscous moduli at a frequency range of 0.1-15 Hz and steady shear tests measured viscosity between 0.1-100 s^{-1}.

The dilution tests were performed with a Cannon Ubeholde #75 micro-dilution capillary viscometer. Relative viscosity was measured beginning with a 50% synovial fluid, 50% phosphate buffer solution (PBS). Samples were sequentially diluted to a final dilution of 6.25% synovial fluid in PBS.

4.5 Results and Discussion

4.5.1 Data Analysis

The raw rheological data for a few representative joints will be presented in the following section. However, due to the large volume of samples (36 over the three weeks of the study) it would be impractical to present all the data in its original form. In order to present important results for all tests only one or two points from each test were chosen for analysis. For example, for the dynamic oscillatory tests, both the viscous modulus and the elastic modulus were recorded at a frequency of 1 Hz. For each hock the percent change from its own baseline values (week zero sample) of elastic and viscous modulus was calculated for one and two weeks post-treatment. These values were averaged according to test group (experimental, positive and negative control) and week. This same data analysis was performed with the steady shear tests (change in viscosity was averaged at shear rates of 0.1 and 10 s^{-1}) and capillary viscometer tests (change in relative viscosity was averaged at 50% synovial fluid and 50% PBS). The results were not analyzed statistically due to the small test population; this was designed as a preliminary study only.

4.5.2 Cytology Results

For all hocks each week, protein levels were below 2.5 g/dl, which is considered a normal level. Samples from week one and two also exhibited moderate to severe

blood contamination. Average nucleated cell (white blood cells) counts by group are reported in Figure 4-2.

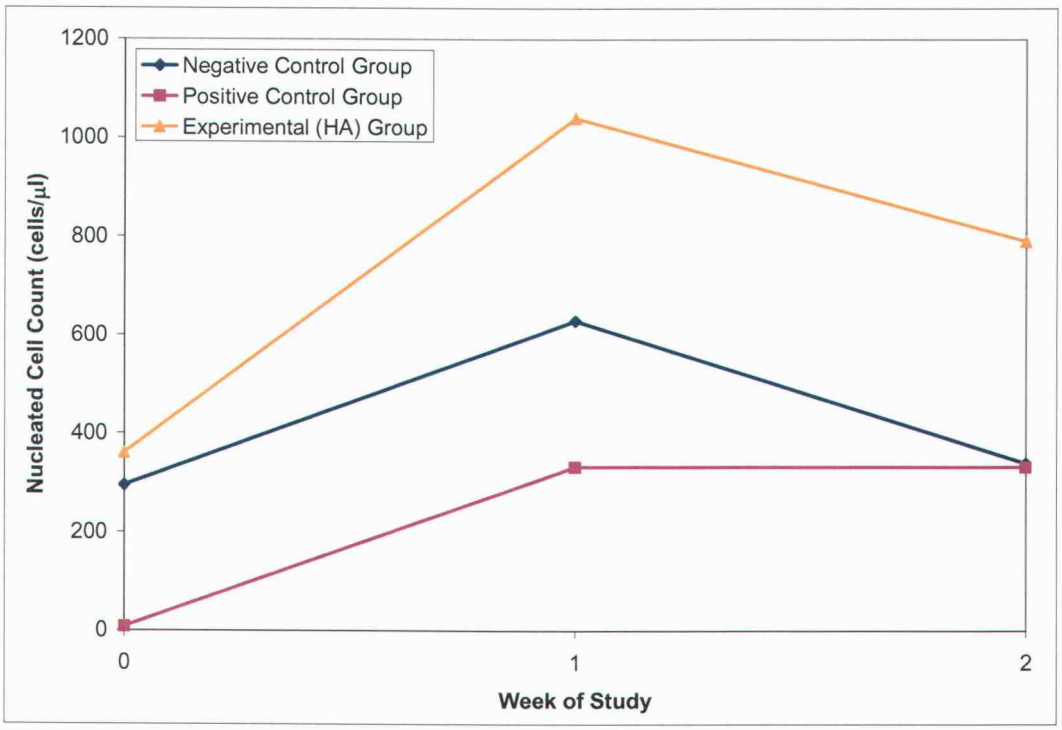


Figure 4- 2 Average nucleated cell count by week and group

Cell counts below 500 cells/µl are considered normal levels and a count above 500 cells/µl is normally considered inflamed. One week after treatment the experimental and positive control groups show a mild inflammation and the experimental group continues to exhibit above average cell counts two weeks after

treatment. The greatest increase in cell counts likely occurred in the experimental group because injecting a substance (HA) into the joint can cause inflammation.

The mild inflammatory response exhibited by most joints included in the study is to be expected as a response to insertion of a needle into the joint. No joints became infected as a result of this study (infection is usually indicated by cell counts above 30,000 cells/μl) and all hocks returned to pre-treatment condition upon completion of fluid collection.

4.5.3 Rheological and Molecular Characterization

The rheological study that was performed on the synovial fluid samples will now be presented. Figure 4-3 is an example of the steady shear baseline value of all hocks, which are all fairly similar.

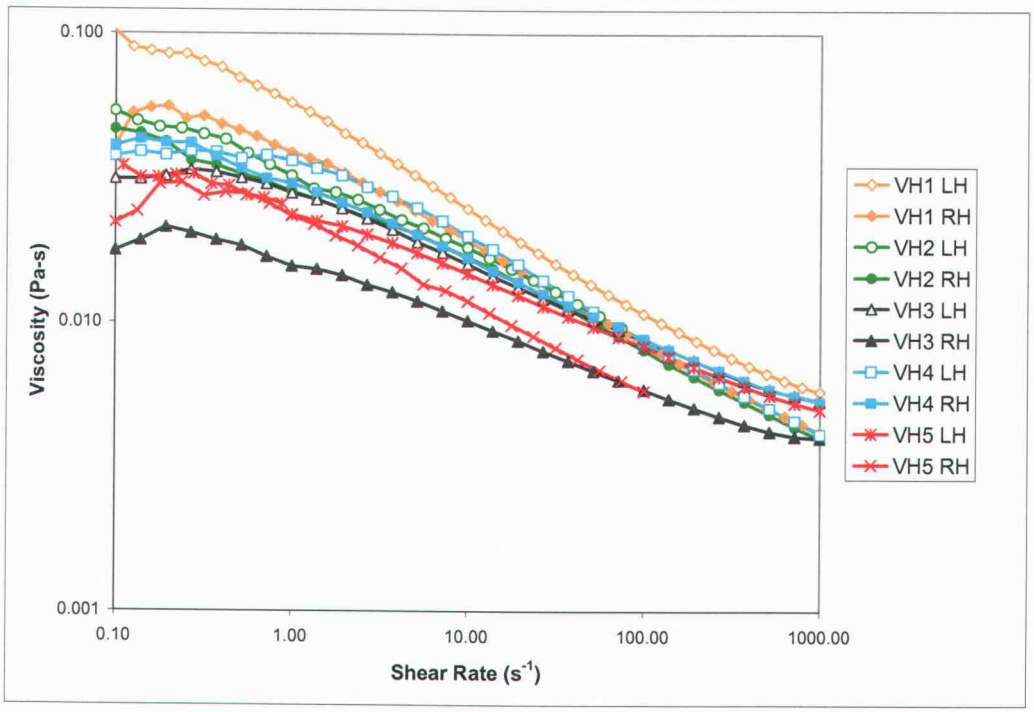


Figure 4- 3 Pre-treatment (week zero) synovial fluid steady shear test results

As with synovial fluid samples collected from dead horses, these samples were shear thinning. The viscosity range was typically 0.01-0.1 Pa-s, about 10-100 times the viscosity of water. Unlike synovial fluid samples from euthanized horses, all samples from live horses show a slight Newtonian plateau. With the exception of the samples with the highest and lowest viscosity, all ten hock samples group together in a tight band. The reason for this is currently unknown.

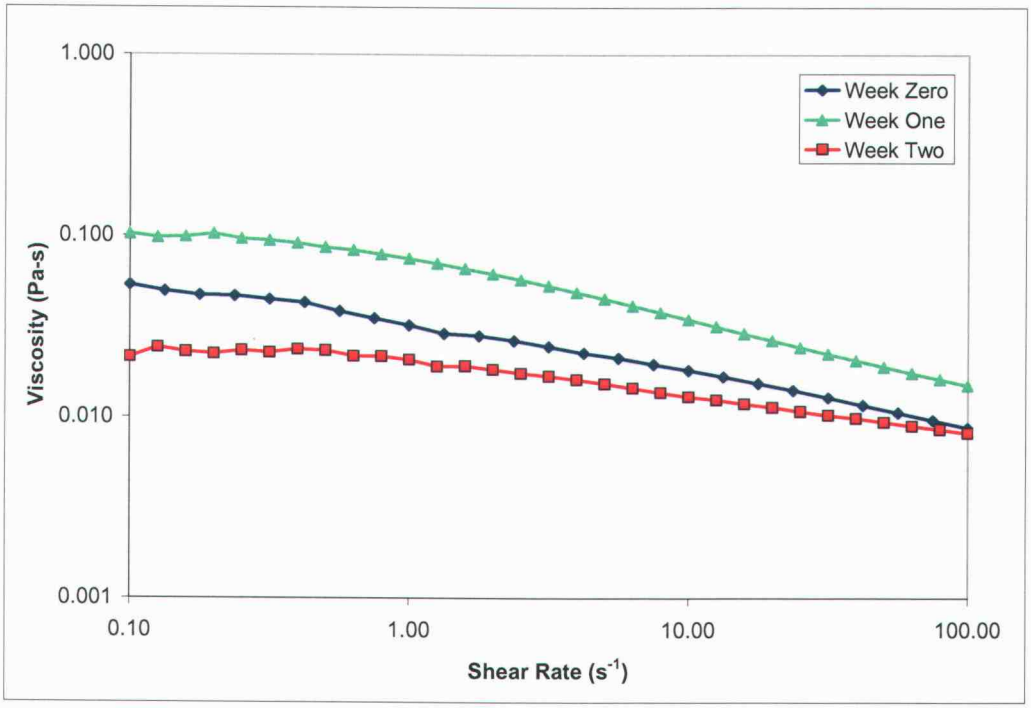


Figure 4- 4 Steady shear data for an experimental hock, VH2 LH (left hock) for all three weeks of the study

Figure 4-4 shows the results of one hock from horse VH2 of the experimental group. There is a substantial increase in viscosity the week after injection of HA, and a decrease in viscosity in week two. This pattern was very typical of hocks in the experimental group.

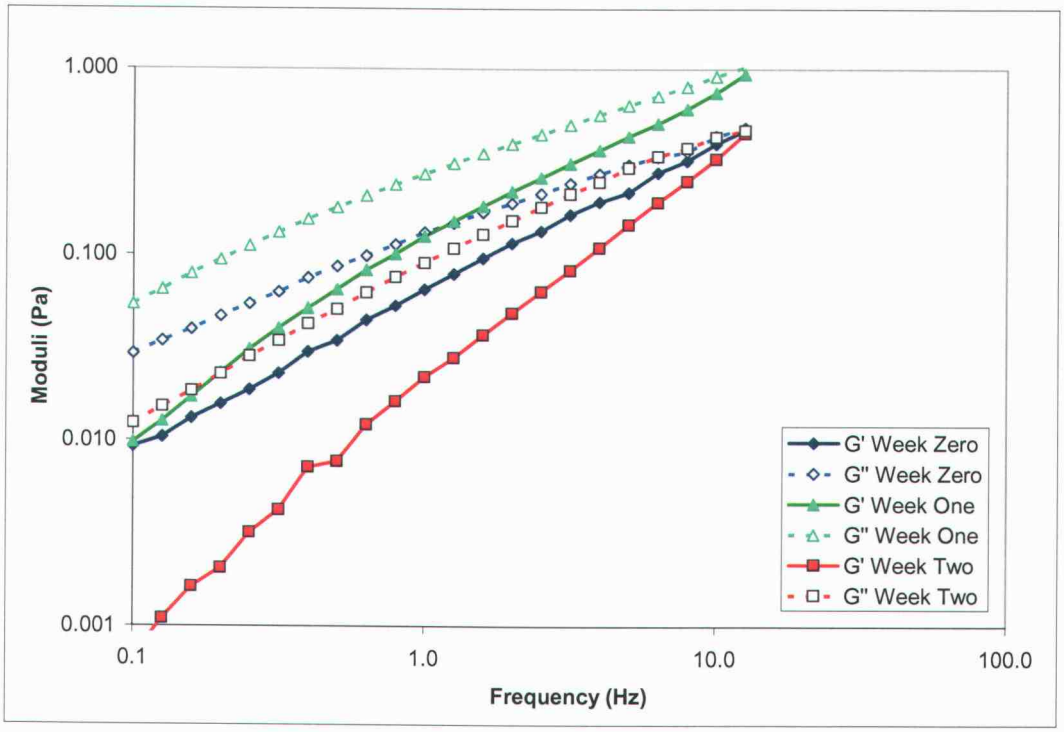


Figure 4- 5 Dynamic oscillatory shear data for an experimental hock, VH2 LH, for all three weeks of the study

Figure 4-5 is an example of the dynamic results from the same hock. Again there is an increase in elastic and viscous modulus after one week. The sample from week two is also below the baseline, which is consistent with the steady shear results. Note that in the frequency range tested these samples either exhibit or approach a crossover point near a frequency of 10 Hz. These samples are slightly more elastic than the dead horse samples presented in chapter 3.

A sample of capillary viscosity results is shown in Figure 4-6 (also the same experimental hock).

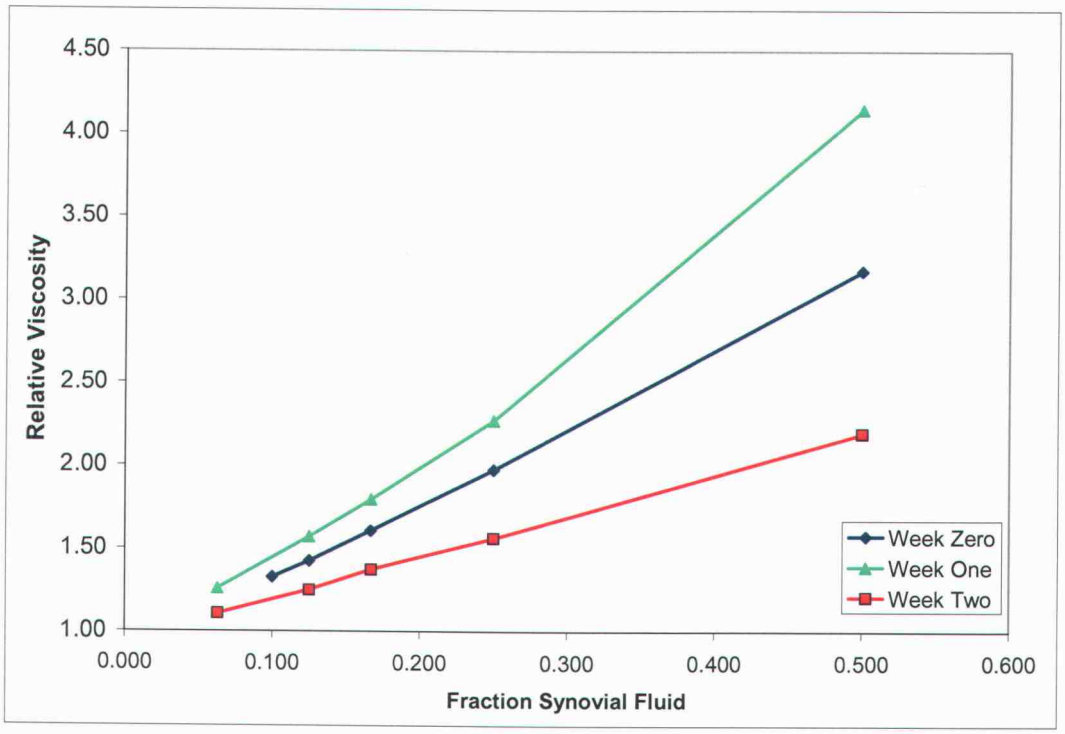


Figure 4- 6 Relative viscosity data for an experimental hock, VH2 LH, for all three weeks of the study

Notice that the pre-treatment and week one samples exhibit a linear relationship between relative viscosity and concentration. The sample from week one exhibits non-linear behavior at high concentrations which is an indication of inter-molecular interactions. This type of test was designed to analyze polymers in the dilute regime, and an exit from this regime into the concentrated regime will show this upturn at high concentrations. This means that the week two sample from this

hock has either a higher concentration of HA or higher molecular weight HA than the other samples.

Now that some individual hock results have been examined, the data will be analyzed as a whole. Figure 4-7 is the graph discussed in section 4.5.1, it reports the average percent change in viscoelastic properties for week one. It demonstrates that all of the rheological tests performed on synovial fluid from the experimental group showed an increase in viscosity and elasticity one week after treatment.

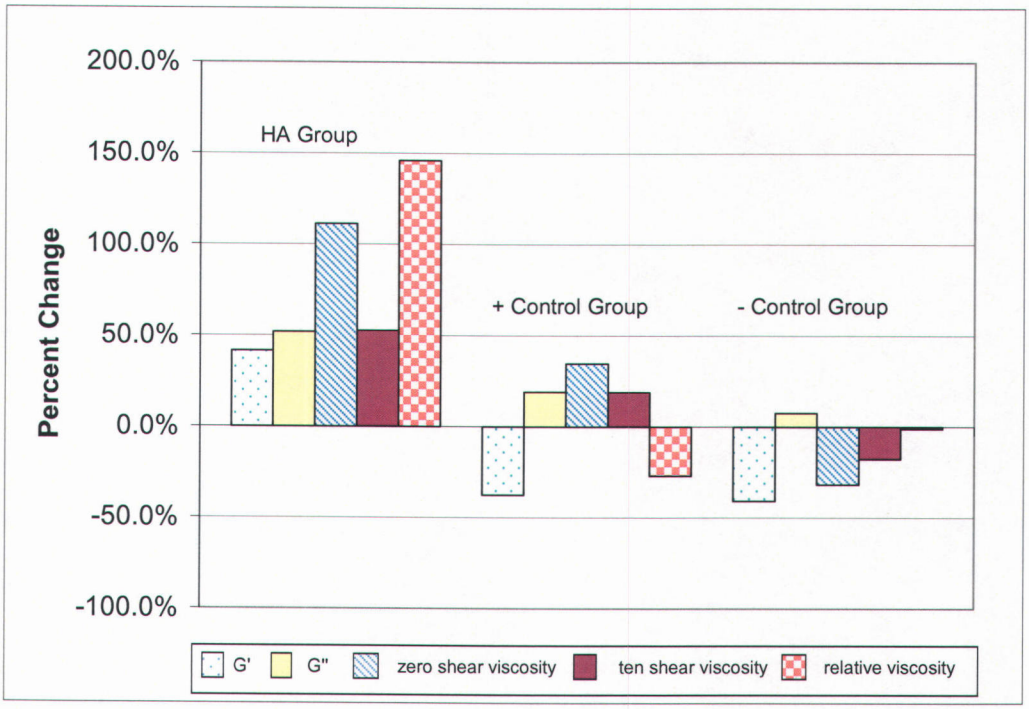


Figure 4- 7 Change in viscoelastic properties of synovial fluid one week after treatment

The increases in properties range between 50% and 150% of the week zero values; the relative viscosity values showing the largest change. Both control groups exhibit very minimal changes after one week. The positive control group exhibits a slight increase in viscous modulus, steady shear viscosity at 0.1 and 10 s^{-1} while the negative control group saw an increase in viscous modulus only. Since the changes for the control groups are both positive and negative, no concrete trend can be drawn from these results. It should also be noted that the positive increases in viscoelastic properties exhibited by the control groups are very small in magnitude compared to changes in the experimental group. For both the negative

control group and the positive control group the elastic modulus, G', decreases by about 30%.

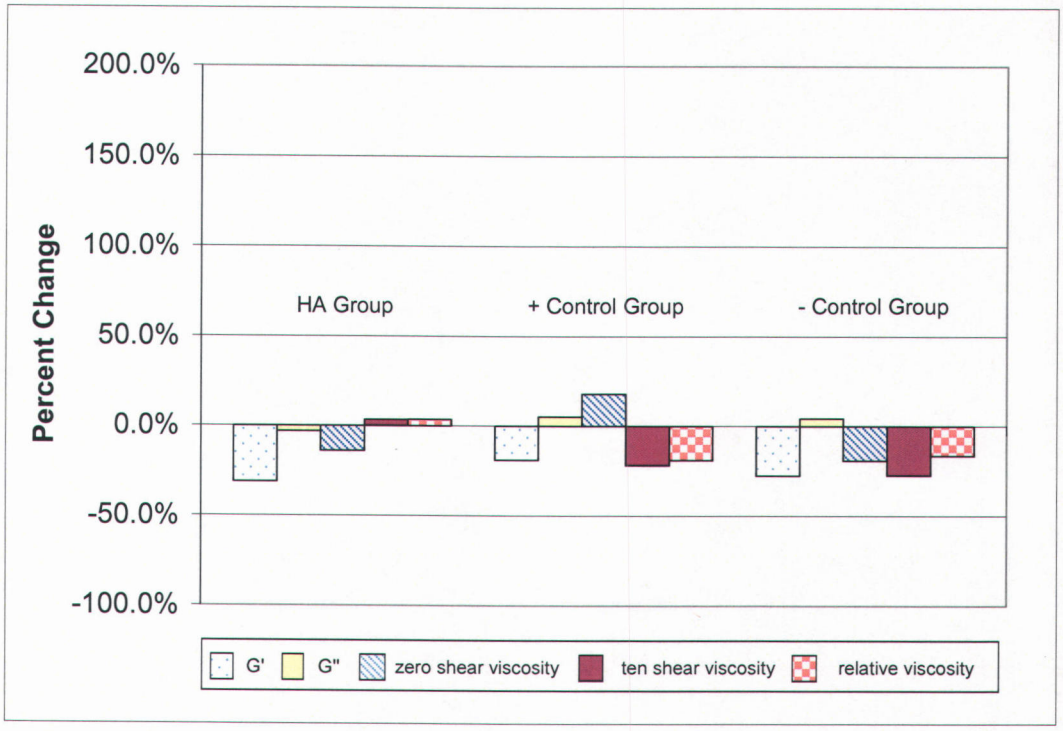


Figure 4- 8 Change in viscoelastic properties of synovial fluid two weeks after treatment

The second week after treatment (Figure 4-7) the viscoelastic properties of the experimental group have dropped to near the baseline values; they are no longer all positive. The range from -30% to 5%, a significant difference from the week one results. The control groups are also near the baseline value. No change in viscoelastic properties of any group is greater than 50% and each group exhibits both increase and decrease depending on test type.

4.6 Discussion

The increase in viscosity and elasticity one week after treatment of the experimental group seemed to be due to the addition of HA to the joint. The decrease in week two was likely the result of removal of the HA-enhanced synovial fluid in the experimental group by aspiration combined with the inflammatory effects of aspiration. This left the treated joints in the same position as both control groups. At this point all the joints (experimental groups and control groups) had the same ingredients to regenerate synovial fluid, and therefore there should not be any difference between all the groups; removing synovial fluid at week one essentially "zeroed the scales." In addition, the elastic modulus decreased for all groups in the third week of treatment. Perhaps aspiration of synovial fluid decreases its elastic properties significantly. This could be due to insufficient replacement of HA concentration following synovial fluid removal.

The enhanced viscoelastic properties of synovial fluid from the experimental group one week after treatment could be a result of multiple mechanisms. One possibility is that the actual injected (exogenous) HA was still present one week after its injection. In this case the concentration of HA in the fluid is greater than normal. The other theory is that exogenous HA stimulated an increase in productivity (concentration) or quality (molecular weight) of endogenous HA. The latter theory may be less likely since all joints used in this study were normal

based on clinical signs, and should have had normal sodium hyaluronate levels. However, they were middle aged horses and thus could have had joint fluid with rheological properties less than a younger horse would have.

4.7 Summary of Intra-articular Study

This experiment indicates that intra-articular injection of Hyvisc to normal joints results in an increase in viscosity and elasticity one week after treatment. Unfortunately no conclusions can be made about the synovial fluid two weeks after treatment due to a mistake in experimental design.

The increase in cell count after aspiration is consistent with inflammation associated with aspirating synovial fluid and/or injecting hyaluronate. That the rheological properties improved in week one in HA-treated horses despite mild inflammation, which would more likely decrease viscosity, further supports the beneficial effects of hyaluronic acid on rheological properties of synovial fluid

The change in these rheological properties one week post treatment is most likely a result of either the continuing presence of the exogenous HA (Hyvisc) in the joint or increased production of endogenous HA. Either possibility would lead to a higher than normal concentration of HA in the synovial fluid, which would cause an increase in the rheological properties. It is also possible that Hyvisc stimulates the production of higher molecular weight endogenous HA, which would also

result in higher viscoelastic properties. However, this may be a less accurate description as the joints in this study are normal and would have no reason to increase the molecular weight of HA. If the same study was conducted in osteoarthritic joints, which usually produce low molecular weight HA, it would be more reasonable to expect the molecular weight of endogenous HA produced to increase when the joint is treated with Hyvisc.

CHAPTER 5

CONCLUSIONS

Sodium hyaluronate (HA) is a polysaccharide found in all parts of the body. Although it performs important functions in the eye, the coagulation process and other parts of the body, its contribution to synovial fluid is particularly important. As the major component of synovial fluid, HA is responsible for the viscoelastic properties important in joint lubrication and cartilage protection. In this thesis, molecular and rheological characterization techniques were used to study; i) commercial HA materials and HA synovial fluid supplements; ii) equine synovial fluid from different joints of both live and deceased horses; iii) equine synovial fluid from a clinical study of intra-articular HA supplementation in the hock joints of a group of six horses.

In the study of pure sodium hyaluronate samples, size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) and in-line capillary viscometry, and steady shear and dynamic oscillatory shear rheology were all used to characterize ten HA samples sold either for laboratory use or as supplements. The molecular weight range of these was 2.88×10^5 to 1.96×10^6 Da. The molecular weight and intrinsic viscosity were correlated and a Mark-Houwink-Sakurada equation for HA in phosphate buffer solution (PBS) was found to be $[\eta] = 0.17 Mw^{0.68}$. The "a" value of 0.68 indicates HA behaves as a random coil in PBS

which is consistent with values reported in literature. Zero shear viscosities of the samples at a concentration of 2.5 mg/ml ranged from approximately 0.06 to 0.5 Pa-s and were found to have a nearly linear relationship with the product of molecular weight and concentration ($\eta_o \propto cMw^{1.08}$). All samples exhibited both viscous and elastic properties in the dynamic oscillatory shear tests. The correlation between molecular weight and rheological properties of pure HA indicates that these techniques may be used in the future to characterize HA materials and possibly to discern the connection between molecular properties of HA and their lubrication properties in synovial fluid.

Once the characterization techniques for HA were developed, similar techniques were applied to equine synovial fluid. Sodium hyaluronate plays an important role in the health of joints, thus the ability to explore the size and properties of HA in joints would be of medical interest. SEC-MALLS and rheology were both used to explore the molecular weight, concentration, and flow behavior of HA in synovial fluid. Molecular weights were found to be in the range of $1.5x10^6$ to $6.5x10^6$ Da and concentrations ranged from 0.11 to 0.84 mg/ml. These values are similar to those found in the literature, although repeatable light scattering was difficult due to the complexity of the fluid. This warrants further investigation, possibly treatment of synovial fluid with a protease to remove proteins or a more extensive HA extraction process such as the one outlined by Kvam et. al. (1993). The steady shear viscosity of synovial fluid was in a similar range to pure HA samples at 2.5

mg/ml, ranging from about 0.001 to 1 Pa-s. Many samples exhibited an upturn in viscosity at low shear rates, indicating possible aggregations. Although each samples exhibits unique rheological properties, it is difficult at this time to correlate changes in rheology to either a change in concentration or molecular weight of HA in the synovial fluid.

Finally a preliminary clinical study was performed on six horses to determine the effects of intra-articular injection of high molecular weight HA on equine tarsocrural hock joints. Synovial fluid samples were taken from all hock joints prior to treatment (this sample was treated as the baseline for each hock). Following aspiration, hock joints received one of three treatments: injection of 2 ml Hyvisc, an HA supplement (experimental group); a 2 ml injection of Lactated Ringers Solution (positive control group); or no injection (negative control group). Rheological properties of synovial fluid were measured before treatment, one and two weeks after treatment for all test groups.

It was found that the intra-articular application of HA increases the rheological properties of synovial fluid one week after treatment compared to the control group. This is presumably due to exogenous HA remaining in the joint one week after injection; some of the additional 22 mg of HA that were injected had not yet been cleared from the joint one week after treatment. The second week after treatment all test groups showed no change in rheological properties from the pre-

treatment state. Upon aspiration one week after treatment all hocks essentially became negative controls. This is likely the reason that there was no significant change in the rheological properties of synovial fluid from any test group two weeks after treatment. In future studies, a larger test group will be used and all joints would be sampled only once after treatment to avoid similar problems.

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