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ARTICLE

The Role of Protein Content on the Steady and Oscillatory Shear Rheology of Model Synovial Fluids

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Z. Zhang, S. Barman and G.F. Christopher ^a,Received 1st April 2014,
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Recent studies have debated the role of protein content on the bulk rheology of synovial fluid; in particular, it has been questioned if proteins aggregate or interact with hyaluronic acid in synovial fluid to enhance bulk rheology, or if observed effects were due to systematic measurement error caused by interfacial rheology, stemming from protein adsorption to the interface. Utilizing several techniques to ensure results reflect only bulk rheology, an examination of the role of bovine serum albumin and γ -globulin on model synovial fluid rheology has been undertaken. When interfacial rheology caused by protein adsorption to the interface is abrogated, the bulk rheology of a model synovial fluid composed of bovine serum albumin, γ -globulin, and hyaluronic acid is found to be dominated solely by the hyaluronic acid over a wide range of shear rates, strains and frequencies. These results show that the previously reported enhanced rheological properties of model synovial fluids are solely due to interfacial rheology and not from any type of protein aggregation/interaction in bulk solution.

Introduction

Synovial joints, like the knee, are encapsulated systems that are the bearings responsible for mobility in the skeletal system. These joints are lubricated through several mechanisms that depend on synovial fluid to varying degrees.¹ Therefore, characterizing synovial fluid rheology is important to understanding the mechanical function of joints, treating the effects of diseases such as arthritis, and developing effective joint replacements.²⁻⁵ Due to the difficulty of obtaining large quantities of human synovial fluid, recent studies⁶⁻⁹ have used solutions composed of hyaluronic acid and several proteins as model synovial fluid. However, there is ongoing debate as to the importance of these proteins on synovial fluid rheology. In this work, an examination of the role of protein content on both steady and oscillatory rheology of a model synovial fluid is undertaken.

Synovial joints have developed with a hierarchical structure that enables lubrication. Bones in synovial joints are capped with a porous, soft, viscoelastic cartilage shell that allows the introduction of proteins into the synovial cavity. Between cartilage surfaces, encapsulated by the synovial membrane, is a thin film of synovial fluid. This film can be as thick as 50 μm , depending on factors such as disease, weight, motion speed and time.¹⁰⁻¹³ Synovial fluid is viscoelastic and primarily composed of a polyelectrolyte polysaccharide, hyaluronic acid, which under normal physiological conditions can vary in length from 0.8 mega-Daltons up to 10 mega-Daltons.⁵ In addition, there are a number of proteins in synovial fluid, primarily albumin, γ -globulin and the glycoprotein lubricin.

Disease and injury affects concentration of these various components and molecular weight of the hyaluronic acid. In general older/diseased patients' synovial fluid has lower concentrations of lower molecular weight hyaluronic acid as well as increased concentration of proteins.¹⁴ The rheological properties are strong functions of hyaluronic acid molecular weight and concentration.^{5, 6, 15}

Depending on motion speed, duration and type, synovial joints lubricate using a combination of synovial fluid, individual proteins and cartilage.¹³ The majority of joint lubrication research has focused on boundary lubrication, which occurs when cartilage surfaces are in direct contact after long periods of motion. Boundary lubrication is primarily affected by the properties of cartilage and free hyaluronic acid and lubricin within synovial fluid.¹⁶⁻¹⁹ Neither free hyaluronic acid nor lubricin alone are effective boundary lubricants.^{20, 21} Synovial joints that lack lubricin, and hence rely solely on hyaluronic acid for boundary lubrication, exhibit increased joint damage due to wear in comparison to joints with both lubricin and hyaluronic acid.^{22, 23} Individual lubricin molecules can bind to cartilage surfaces, forming a somewhat effective boundary lubricant.^{24, 25} However, boundary lubrication is greatly improved when the bound lubricin traps hyaluronic acid to the cartilage surface, forming a complex that is extremely effective boundary lubricant.^{1, 19}

Protein interaction between hyaluronic acid and lubricin plays a significant role in the establishment of low friction and wear surfaces in synovial joints during boundary lubrication. However, the role of protein interaction on early joint lubrication is not clear. During the first 30 minutes of motion when cartilage surfaces are separated by $\sim 50 \mu\text{m}$ thick synovial fluid film, lubrication is either hydrodynamic

^aDepartment of Mechanical Engineering, Texas Tech University, Lubbock, TX, 79409 USA. EMAIL: gordon.christopher@ttu.edu

or elasto-hydrodynamic, relying heavily on synovial fluid rheology. In these regimes, synovial fluid's high viscosity and strong shear thinning effectively lubricate joints during flexion, separating cartilage surfaces and reducing friction as speed increases.^{5, 10-12} A number of recent papers have concluded that hyaluronic acid is the only functional component in determining the rheology of synovial fluid relevant to these regimes;^{6, 9} however, the importance of interactions between proteins and hyaluronic acid on synovial fluid rheology is still actively debated.

The steady shear rheology of hyaluronic acid solutions and model synovial fluid composed of hyaluronic acid, γ -globulins and bovine serum albumin have been widely considered. Synovial fluid and hyaluronic acid solutions are both shear thinning;⁶ however, steady shear rheology of model synovial fluid deviates from pure hyaluronic acid solutions. In particular, model synovial fluid solutions exhibit a shear history dependence of viscosity; measured viscosity during a down ramp increase significantly with decreasing shear rate and is larger than corresponding shear rates in the up ramp. These behaviors have not been observed for pure hyaluronic acid solutions.^{7, 8} In the model synovial fluids, these behaviors are theorized to appear due to a tenuous protein network of bovine serum albumin and γ -globulin that was interpenetrated by hyaluronic acid. In extensional flows, extensional thickening, extensional viscosity and relaxation times of model synovial fluids are greater than that of pure hyaluronic acid solutions.^{6, 9} Micro-rheology of synovial fluids has demonstrated significant elasticity which is not observed in synovial fluid without lubricin, indicating that there may be some interaction between free lubricin and hyaluronic acid.²³ It could be concluded from these studies that interaction between hyaluronic acid, bovine serum albumin, γ -globulin and lubricin affect synovial fluid rheology.

However, a number of studies have found little to no effect of proteins on the rheology of synovial fluid in steady shear. In particular, Bingol and coworkers⁶ did an extensive study of the steady shear rheology of hyaluronic acid solutions, hyaluronic acid combined with bovine serum albumin and γ -globulin, and human synovial fluid. They saw no difference between pure hyaluronic acid solutions and synovial fluids when concentration and molecular weight of hyaluronic acid were equivalent, indicating that hyaluronic acid alone was contributing to steady shear behavior. These results have been corroborated by other studies,⁹ which have theorized that the difference in steady shear response is due to different rheometer geometries being affected differently by interfacial rheology.

Utilizing a number of methods to ensure rheological results are accurately reflecting the bulk rheology, an examination of the role of bovine serum albumin and γ -globulin on model synovial fluid rheology has been undertaken. These tests are designed to examine the role of protein interaction on both steady and oscillatory shear response of model synovial fluid, and illuminate the role of protein content on the rheology of synovial fluid.

Experimental

Solution Preparation

Based on characterizations of healthy human synovial fluid,^{5, 26, 27} a model solution is used to simulate synovial fluid composed of hyaluronic acid sodium salt (Sigma-Aldrich 53747), bovine serum albumin (Sigma-Aldrich A3059), and γ -globulin (Sigma-Aldrich G5009) at concentrations shown in Table 1. The hyaluronic acid has a molecular weight of 1.6 mega-Daltons synthesized from streptococcus equi bacteria with protein content less than 1%. All

Solution	HA	BSA	γ -Globulin
Synovial Fluid	3.4	10	0.5
Hyaluronic Acid	3.4	0	0
Bovine Serum Albumin	0	10	0

Table 1. Solution compositions. All units are mg/ml.

solutions are dissolved in a 10 mM phosphate buffered saline solution with pH 7.4, and are stored in refrigerator and used within several days of preparation in order to avoid bacterial contamination or sample degradation. Due to the difficulty in obtaining Lubricin in quantities adequate for testing, it was not included in the model synovial fluid.

The solution used in this work is similar to that of other recent publications,⁶⁻⁸ however, the concentrations used in this study are different, but in line with published values of the composition of synovial fluid, which is highly variable. The main difference between this solution and that of other papers is the concentration of γ -globulin, which in previous studies was 7 mg/ml.^{7, 8} As the following work will show, this change has little impact on model synovial fluid rheology.

Interfacial Rheology

All interfacial rheology measurements were done using a double wall ring geometry attached to an AR-G2 stress controlled TA instruments rheometer. Samples of the bulk solution were added to the double wall ring base, creating an air/water interface to which molecules adsorb. This air/water interface was allowed to sit 1 hour before measurement, and then measured subsequently 5 hours later. The applied frequencies and strains were set to match those of bulk experiments. Measurements of both viscosity and viscoelastic moduli were corrected using code supplied by Dr. Jan Vermant to account for bulk shear effects that may occur at small Boussinesq numbers.²⁸ This code takes into account deviations from the linear interfacial velocity profile that may occur due to low surface viscosities and moduli through an approach that uses analytical solutions of both the bulk and interfacial flow to calculate torque on the double wall ring. Interfacial viscosities or moduli are iterated until calculated torque matches experimentally measured value. Using this methodology, the reported interfacial viscosities and moduli represent the effects of the interfacial properties alone, and are devoid of any bulk contribution.

Bulk Rheology

Bulk rheology measurements of all solutions were done using an AR-G2 stress controlled TA instruments rheometer. Experiments were conducted with a parallel plate (60mm diameter), single gap couette cell (bob diameter 28mm, bob length 42mm), and double gap couette cell (bob inner diameter 32mm, bob outer diameter 35mm, rotor height 53mm). Solutions were characterized in both steady and oscillatory shear over a range of applied rates, strains and frequencies. In order to ensure accurate measurements, precise rotational mappings identical to experiments were used to negate machine noise/error. Furthermore, best practices were used to attempt to ensure precise alignment of geometries and accurate filling of volumes.

Results and Discussion

Effects of Interfacial Rheology and Surface Tension on Bulk Rheology Measurements

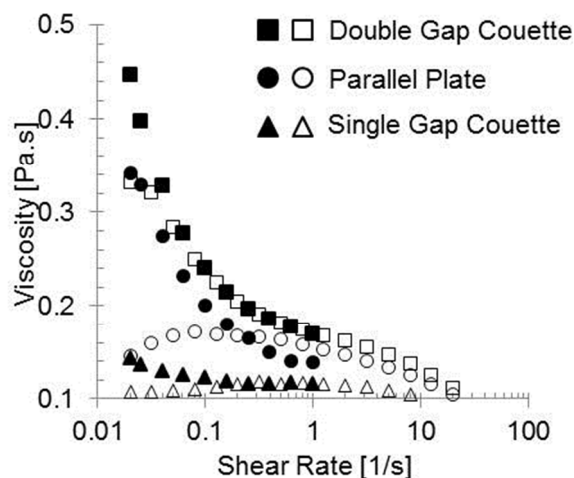


Fig. 1 Steady shear viscosities of model synovial fluid measured using 3 different geometries on a rotational rheometer. All tests involved a 300s 60 s⁻¹ preshear, followed by an up ramp from 0.01 s⁻¹ to 100 s⁻¹ (hollow symbols), which was followed by a down ramps from 1 s⁻¹ to 0.01 s⁻¹ (solid symbols). Each geometry exhibits similar qualitative behavior and approach the same values at high shear rates. However due to the effects of both interfacial rheology, each geometry exhibits varying shear history dependence and apparent viscosity at low shear rates.

Previous examination of the role proteins on rheology of synovial fluid have not considered interfacial rheology explicitly, but a number of recent studies have shown significant effects of interfacial rheology on bulk shear rheology measurements of globular protein solutions, in particular pure bovine serum albumin solutions, done using rotational rheometers.²⁹⁻³¹ These measurements have shown that the bulk yielding of low concentration globular protein solutions can be almost completely attributed to interfacial viscoelasticity caused by proteins adsorbed to the interface. When measuring steady shear viscosity in microfluidic rheometers that do not have interfacial area or subtracting interfacial effects from bulk measurements, there is no evidence of bulk yielding for bovine serum albumin solutions.³¹ However, bulk aggregation can affect bulk rheology once interfacial effects are removed for some globular proteins, but this effect is typically small and depends on the protein studied.²⁹

To examine if this phenomenon affects model synovial fluid solutions, bulk steady shear tests identical to those described by Oates and coworkers were conducted.⁷ In brief, a constant shear rate of 60 s⁻¹ was applied for 300 seconds, followed by a shear rate up ramp and down ramp. In original study using this protocol,⁷ a measured viscosity of the down ramp was larger than the corresponding shear rates in the up ramp, indicating a shear history dependence of the solution. Using the double gap couette cell, shear history dependent viscosities were observed similar to earlier studies,^{7, 8} despite the difference in γ -globulin concentrations between this and previous works (Figure 1). This indicates that if bulk aggregation or interaction between proteins and hyaluronic acid is occurring, the γ -globulin is not playing an important role since results show identical behavior trends and magnitudes when γ -globulin concentration decreases by an order of magnitude.

If results were due to bulk aggregation of proteins or interaction between the hyaluronic acid and bovine serum albumin, changes in geometry should not affect measured viscosity. However, changing geometry to a 60 mm parallel plate reduces this behavior somewhat, and using a single gap cup and bob geometry nearly negates it completely as shown in Figure 1. Furthermore, changes in geometry

also affect the zero shear viscosity. This indicates that this behavior is due to a systematic measurement error, which is likely the interfacial rheology of these solutions affecting bulk measurement.

To directly gauge the effects of interfacial adsorption of the various proteins, the interfacial rheology of the synovial fluid system has been measured and compared to both bovine serum albumin and pure hyaluronic acid.

Bovine serum albumin is known to be interfacially active.^{30, 31} In interfacial steady shear tests, a significant interfacial viscosity is observed for bovine serum albumin solutions (Figure 2a). In small amplitude oscillatory interfacial shear tests shown in Figures 2b and c, bovine serum albumin solutions exhibit significant interfacial elasticity with moduli on the order of 0.01 Pa m in both strain

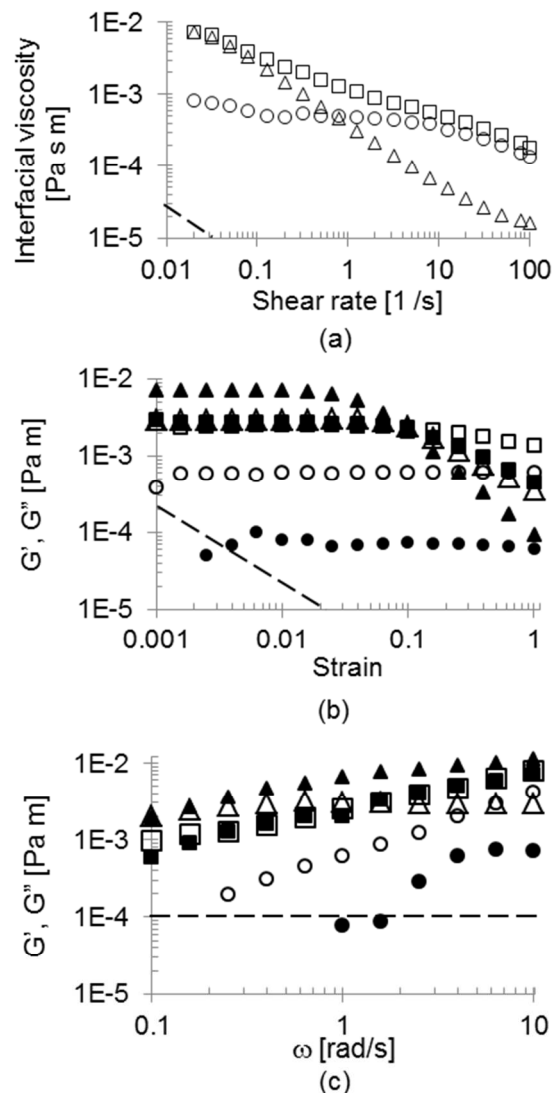


Fig. 2 (a) Interfacial steady shear ramp from 0.1 s⁻¹ to 100 s⁻¹ for synovial fluid (\square), hyaluronic acid (\circ) and bovine serum albumin (Δ). The combined rheology of the model synovial fluid appears to be a combination of the low shear rate behavior of the bovine serum albumin and the high shear rate behavior of the hyaluronic acid. (b) Interfacial strain amplitude sweep at 1 rad/s and (c) frequency sweep at a strain of 0.004 of same solutions with G' (solid symbols) and G'' (hollow symbols). The interfacial linear viscoelastic moduli of the model synovial fluid exhibits elastic properties similar to pure bovine serum albumin with reduced magnitude, and viscous properties of hyaluronic acid with increased magnitude. Dashed lines represent minimum measurable moduli using the double wall ring.

amplitude and frequency sweeps. In amplitude sweeps, the bovine serum albumin interface yields at a strain of 0.03, and in frequency sweeps it exhibits a crossover frequency of 0.1 rad/s. The data of the interfacial rheology agrees well with previous studies of the interfacial viscosity of BSA. In particular, when a yield stress model, $\eta_s(\dot{\gamma}) \sim \tau_Y/\dot{\gamma}$, the yield stress is found to be 4×10^{-4} Pa m. This value agrees well with the estimate of 3×10^{-4} Pa m inferred from Figure 2b, and agrees with the work of Sharma and coworkers,³¹ and was within an order of magnitude of the work of Castellanos and coworkers.²⁹

To our knowledge, interfacial rheology of hyaluronic acid sodium salt has not been previously reported. Hyaluronic acid slightly reduces surface tension of an air/water interface, indicating a limited amount of interfacial adsorption.³² In interfacial steady shear tests, a significant interfacial viscosity is observed for pure hyaluronic acid solutions (Figure 2a). The hyaluronic acid presents very little elasticity in both strain and frequency sweeps, primarily exhibiting a viscous response (Figure 2b and c). In frequency sweeps, measurements of the interfacial elasticity were too small to measure below 1 rad/s, and hence no crossover frequency is observed; at high frequencies, the loss modulus of the hyaluronic acid actually exceeds the bovine serum albumin (Figure 2c). The interfacial rheology of the hyaluronic acid solution could stem from interfacially adsorbed hyaluronic acid, which due to its long length could provide resistance to interfacial flow. It is also likely partially due to the residual proteins in the purchased hyaluronic acid adsorbing to the interface. The mechanism for the observed interfacial viscosity is not of great importance to this study, but its existence is.

The interfacial rheology of the model synovial fluid systems appears to be a direct result of both bovine serum albumin and hyaluronic acid on the interface. The model synovial fluid has the largest interfacial viscosity, and appears to be dominated by the bovine serum albumin response at low shear rates and the hyaluronic acid at high shear rates. In strain and amplitude sweeps, the model synovial fluid has nearly identical loss and storage moduli in both strain amplitude and frequency sweeps. G' is lower than pure bovine serum albumin interface due to some of albumin being displaced by

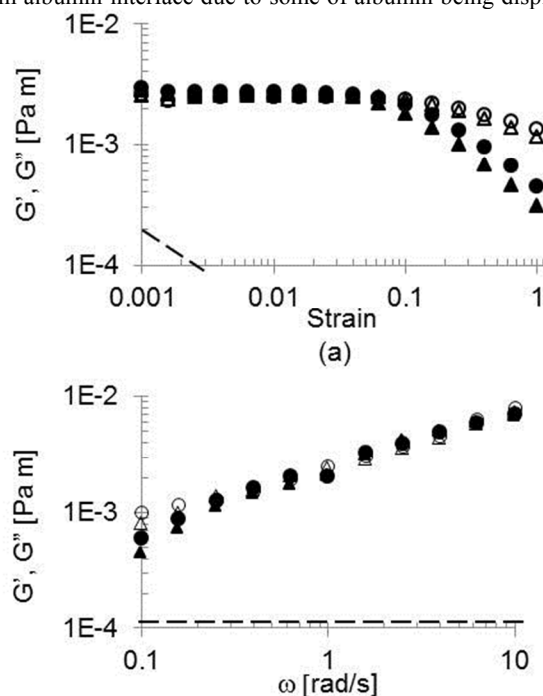


Fig. 3 Effects of aging on interfacial linear viscoelastic moduli of model synovial fluid in (a) strain amplitude sweeps at 1 rad/s and (b) frequency sweeps at a strain of 0.004. G'' (hollow symbols) and G' (solid symbols) for a 1 hour old interface (\blacktriangle , \triangle) and a 6 hour old interface (\bullet , \circ). Small increases in elasticity and viscosity are observed at large strains and low frequencies. Dashed lines represent minimum measurable moduli using the double gap couette cell.

hyaluronic acid, but the presence of hyaluronic acid increases G'' in comparison to bovine serum albumin. The values of interfacial viscosity and linear viscoelastic moduli of synovial fluid are large enough to affect bulk measurements.^{29, 31}

Bovine serum albumin interfaces are known to exhibit age dependent behavior;³³ the model synovial fluid interfaces were tested to see if there were any interfacial aging effects that occur. As shown in Figure 3 a and b, both interfacial storage modulus and loss modulus slightly increased after 5 hours of aging. However, these effects are relatively small in comparison to the magnitude of the moduli.

It is clear from both the bulk measurement and the interfacial rheology tests that interfacial rheology of synovial fluid is not trivial, and its impact on bulk rheology cannot be ignored. To remove interfacial effects from bulk measurements, two techniques are considered. The interfacial rheology measurements can be used to calculate the true bulk values of viscosity and moduli using the following formulas:^{27, 30}

$$\eta_{bulk} = \eta_{measured} - \frac{\eta_{surface}}{K} \quad (1)$$

$$G'_{bulk} = G'_{measured} - \frac{G'_{surface}}{K} \quad (2)$$

$$G''_{bulk} = G''_{measured} - \frac{G''_{surface}}{K} \quad (3)$$

Where η is viscosity, G' is storage modulus and G'' is loss modulus. The subscripts *bulk*, *measured*, and *surface* refer to true bulk property, the result from the rheometer, and interfacial measurements respectively. K is a geometric parameter, which in the case of the double gap couette cell geometry is height. Equations (1) – (3) can be used when all measurements are made with identical strains, frequencies and shear rates.

Alternatively, experiments using bulk geometries can remove the effects of interfacial rheology by applying a surfactant to the air/water interface.^{29, 34} The surfactant can displace the protein on the interface by initially residing in gaps between proteins and forcing proteins to compress into a thick film, which at high enough surface pressures/surfactant concentrations will force the proteins off the interface.³⁵ Using a surfactant also alleviates a secondary problem that may be affecting measurements at low torque values; small misalignment in the geometry rotational axis with the rotational spindle axis, coupled with contact line migration on the geometry can result in appearances of enhanced elasticity in oscillatory measurements and viscosity in steady measurements due to surface tension induced torque.³⁶ This problem is significant with large contact lines, which the double gap couette cell provides. It can be mitigated through proper geometry filling and alignment, and by minimizing surface tension magnitude. The surfactant reduces the surface tension of the air/water interface, and therefore reduces extraneous torque.

For this work, a small amount of the surfactant sodium dodecyl sulfate (SDS), was applied to the interface to displace the bovine serum albumin and reduce interfacial viscoelasticity.²⁹ SDS is more interfacially active than bovine serum albumin, and will displace them from the interface.³⁵ 0.2 ml of 2 wt% SDS/DI water solution was added to the air/water interface. This amount represents 1.7% volume of total tested solution, or 1.2 mMol/L of SDS; this well below the SDS' CMC of 8 mMol/L in water at 25°C.³⁷ Typically, low molecular weight surfactants like SDS do not have significant shear interfacial rheology;³⁸ below the CMC, SDS forms Newtonian solutions with viscosity on the order of 1 mPa s.³⁹ SDS can affect the

viscoelasticity of polymer solutions at concentrations lower than the CMC. This effect is not observed in our experiments due to the drastic decrease in both elasticity and viscosity of tested solutions as outlined below, the use of bovine serum albumin rather than a polymer and the low concentration of SDS.^{40, 41} It has been shown that films of SDS and β -lactoglobulin, another globular protein, have interfacial shear viscosities lower than films made of the individual components.³⁵

The double gap couette cell, although clearly affected by interfacial rheology, is typically used for low viscosity fluids due to its ability to measure low torques providing greater sensitivity to low bulk viscosities. Because the solutions without interfacial effects have much lower viscosities and moduli, we choose to continue to use this geometry to measure bulk properties with the addition of SDS to negate the effects of interfacial rheology. Results of steady shear measurements using the double gap cell with SDS are compared to corrected measurements using equation (1) in Figure 4a. The SDS measurements exhibit no shear history dependence. The corrected measurements show less shear history dependence than uncorrected. In both amplitude and frequency sweeps (Figure 4b and c), loss moduli between the corrected and SDS measurements are nearly identical; however, in both tests, corrected measurements exhibit larger elasticity than SDS measurements.

The increased elasticity in the corrected measurements at low strains, frequencies and shear rates could be attributed to several factors. The age of the interface in bulk measurements and the age of the interface in interfacial rheology measurements were not rigorously matched; therefore, the interfacial moduli used to correct bulk measurements may not have been reflective of the true interfacial rheology in the bulk experiment. However, based on interfacial aging effects in Figure 3, this is a minor problem. The corrected measurements likely show increased elasticity due to surface tension effects as described earlier. Hence, even when correcting for interfacial rheology, these extraneous torques due to surface tension result in increased elasticities. Based on results in Figure 4, the SDS is able to completely remove any effects of both interfacial rheology and surface tension, which the correction method is unable to do.

In order to ensure that the bulk rheology measurements are not affected by both interfacial rheology and surface tension, the most effective choice based on the above results is to use SDS to displace the bovine serum albumin and hyaluronic acid and then conduct bulk measurements using a double gap couette cell which will be most sensitive to bulk rheology. The SDS could interact with the hyaluronic acid, bovine serum albumin, or γ -globulin in the bulk; we will address this concern in the subsequent sections.

Steady Bulk Rheology

Based on the above results it is clear that the interface has been significantly affecting rheology measurements of model synovial fluid solutions, causing the perceived effect of proteins in published measurements of steady shear. Using the methods described above, the Oates test^{7, 8} was re-conducted using the double gap couette cell and SDS on the interface for both a model synovial fluid and pure hyaluronic acid.

In Figure 5, we note first that when comparing the hyaluronic acid solution with SDS to one without SDS, we see the solutions without SDS still exhibit a small shear history effect and have slightly higher viscosities at low shear rates in comparison to the solution with SDS. The shear history effect confirms that there is some interfacial rheology impact in the hyaluronic acid solution. When SDS is used with the hyaluronic acid, there are no shear history effects; this is consistent with the work of Bingol and coworkers⁶ who found no

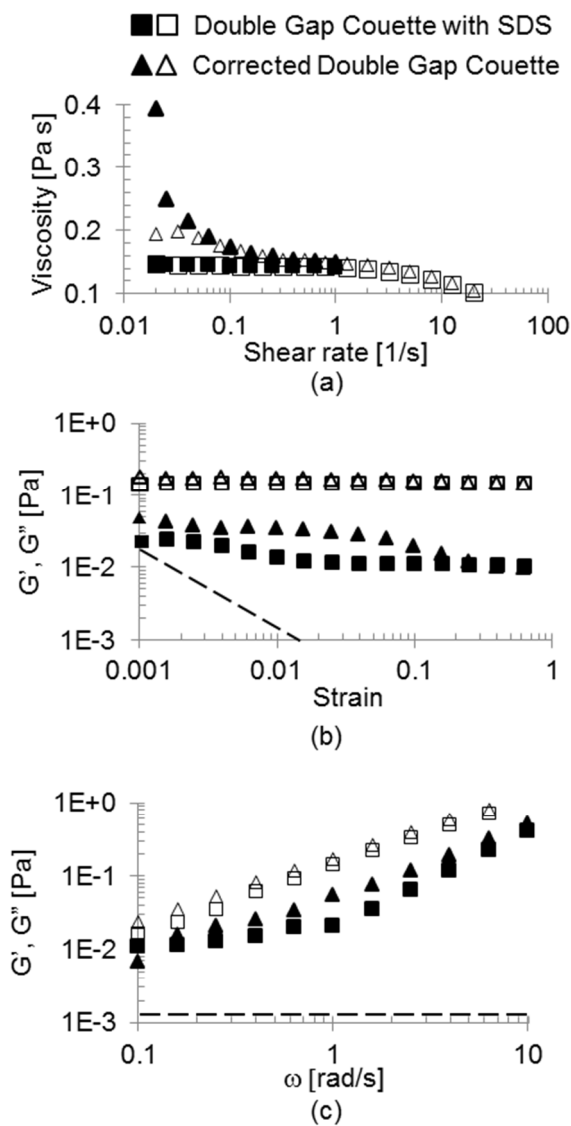


Fig. 4 (a) Steady shear viscosities of model synovial fluid measured using SDS on interface (\square , \blacksquare) and measurement corrected using interfacial rheology (\blacktriangle , \triangle). All tests involved a 300s 60 s⁻¹ preshear, followed by a up ramp from 0.01 s⁻¹ to 100 s⁻¹ (hollow symbols), which was followed by a down ramps from 1 s⁻¹ to 0.01 s⁻¹ (filled symbols). Both methods correct the interfacial rheology contribution of the torque, but using SDS also alleviates surface tension torques, resulting in no shear history effects. (b) Strain amplitude sweep at 1 rad/s and (c) frequency sweep at a strain of 0.01 with G' (solid symbols) and G'' (hollow symbols). The results are consistent with (a) in which corrected measurements exhibit larger elasticities than SDS measurements due to extraneous surface tension torque. Dashed lines represent minimum measurable moduli using the double gap couette cell. SDS bulk concentration for all tests 1.2 mMol/L.

shear history effects when examining hyaluronic acid on 60 mm plates. This indicates that any interaction between SDS and hyaluronic acid has a smaller impact on bulk rheology than interfacial rheology, because these interactions would increase viscosity, which is not observed.

Corrected for interfacial rheology and surface tension effects using the SDS, the hyaluronic acid and synovial fluid with SDS curves are identical in steady shear (Figure 5), indicating that there is no effect of protein aggregation or interaction with hyaluronic acid on the bulk steady shear viscosity. This result is further corroborated by looking

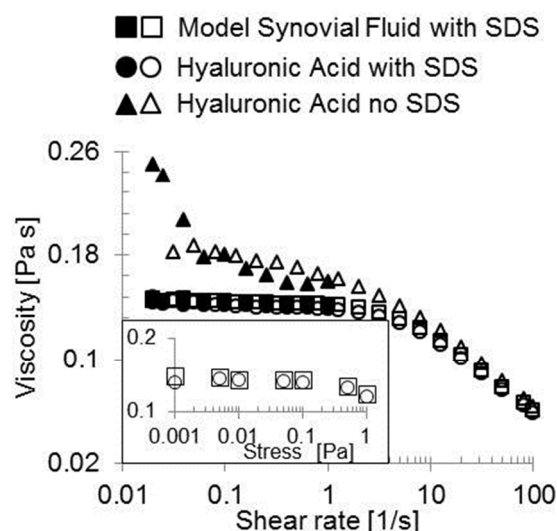


Fig. 5 Steady shear viscosities of model synovial fluid (\square , \blacksquare) and pure hyaluronic acid (\bullet , \circ) both with SDS on interface. All tests involved a 300s 60 s⁻¹ preshear, followed by an up ramp from 0.01 s⁻¹ to 100 s⁻¹ (hollow symbols), which was followed by a down ramps from 1 s⁻¹ to 0.01 s⁻¹. Behaviors of the solutions are identical with no indication of protein interaction or aggregation. (Inset) Similar results obtained using creep measurements to evaluate steady shear viscosities. SDS bulk concentration for all tests 1.2 mM/L.

at the results of creep tests done on the model synovial fluid and hyaluronic acid solutions with SDS. Tabulated results of viscosity found in the steady state region from creep tests at stresses ranging from 0.0001 Pa to 1 Pa are shown in the inset of Figure 5. As can be seen, the 2 curves are identical, and display results consistent with the shear rate ramp experiments.

Based on the exact matching of steady shear viscosities in Figure 5, the alignment of the double gap couette geometry with the spindle of the rheometer was both accurate and consistent between tests, giving consistent results with little to no extraneous surface tension torque. It is safe to conclude that the steady shear rheology of model synovial fluid solutions is completely dictated by the presence of hyaluronic acid, and the chosen proteins do not affect the system at all. It is possible that lubricin may have some effect when added to the model solution, but that cannot be determined by this work.

Because the model synovial fluid matches the results of the hyaluronic acid, any interaction between the SDS with either the γ -globulin or the bovine serum albumin in the bulk or on the interface is minor, because any interaction or aggregation would increase synovial fluid viscosity in comparison to pure hyaluronic acid, which is not observed.

Since our initial tests of synovial fluid without SDS matched the work of Oates and coworkers^{7, 8} and removing interfacial rheology with SDS negated shear history behavior, we are confident that the shear history effect observed was entirely due to interfacial rheology and not aggregation or interaction between any of the proteins.

Oscillatory Bulk Rheology

Using the SDS on the interface, the effect of proteins were examined on oscillatory bulk rheology of model synovial fluid and compared to hyaluronic acid solutions tested on the double gap couette cell as shown in Figure 6a and b. In a strain amplitude sweep of the solutions (Figure 6a), both pure hyaluronic acid and model synovial fluid are primarily viscous for all strains. The model synovial fluid curve follows the hyaluronic acid response for strains

larger than 0.01. However, at strains lower than 0.01, an increase in elasticity of the model synovial fluid is observed that does not occur for the hyaluronic acid. This difference is clearer in the $\tan(\delta)$ data shown in the inset in Figure 6a. A similar effect is observed in the frequency sweep, with a divergence in the elasticity of the model synovial fluids at angular frequencies below 1 rad/s. The increase is small, about a factor of 2. At high frequencies, the response is clearly dictated by the hyaluronic acid, based on the overlapping curves. These results can also be seen in $\tan(\delta)$ inset, which shows a decrease in this value, indicating an increasing elasticity of the synovial fluids. These small but measurable elasticity increases in model synovial fluid solutions in comparison to the pure hyaluronic acid could occur due to surface tension effects. From steady shear viscosities matching in Figure 5, extraneous torque due to surface tension was nearly completely abrogated due to the use of SDS and rigorous procedure. In the strain amplitude sweep, if surface tension were the effect causing increased elasticity, decreases in elasticity would not be expected as strain increased; rather, elasticity would remain constant due to the surface tension torque until bulk effects

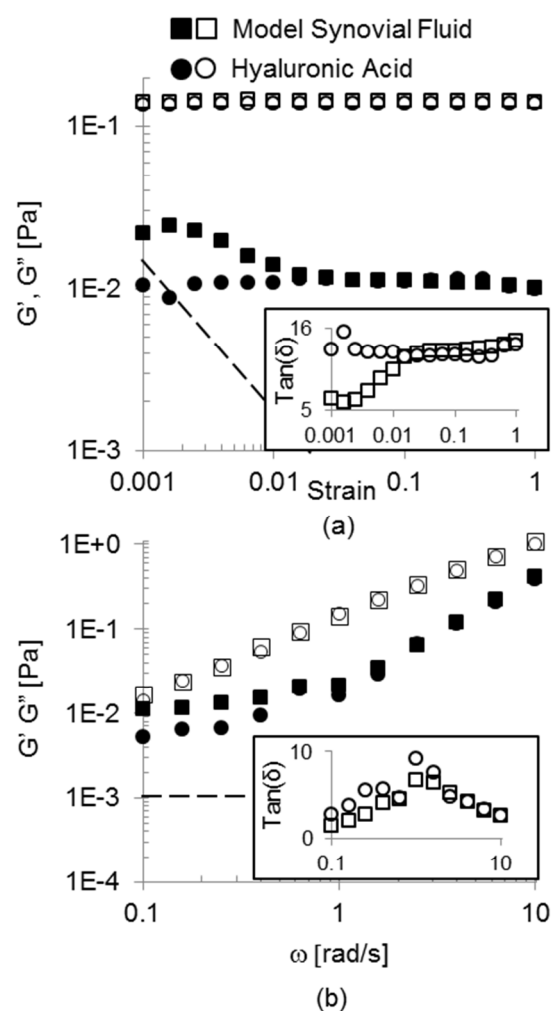


Fig. 6 (a) Strain amplitude sweep at 1 rad/s and (b) frequency sweep at a strain of 0.01 with G' (solid symbols) and G'' (hollow symbols) for model synovial fluid (\square , \blacksquare) and pure hyaluronic acid (\bullet , \circ) both with SDS on interface (bulk concentration for all tests 1.2 mM/L). Small increases in elasticity are seen at small strain (a) and low frequencies (b). Due to efforts to mitigate effects of surface tension and interfacial rheology, these increases are due to protein interaction or aggregation. Dashed lines represent minimum measurable moduli using the double gap couette cell.

were large enough to overcome this extraneous torque. Although this argument cannot be applied to the frequency sweeps, due to the pains taken to abrogate interfacial and surface tension effects and result of the strain amplitude and viscosity, the increase in elasticity at low frequencies is similarly concluded not to be due to surface tension.

The minimum measurable moduli of the AR-G2 using a double gap couette cell was calculated for each experiment using the minimum measurable torque (3 nN m) (dashed lines in Figure 6a and b).³⁶ As can be seen, the moduli at the low strain and frequencies are close to this minimum, but generally above these values. The resolutions of the measurements are on the order of 10^{-3} Pa or lower for all tests. The observed differences in elasticities are above both the measurement limit and resolution limit in these measurements. Furthermore, these effects were seen upon repeated testings and at several different strain values.

Although it is possible the higher elasticity at low strains and frequencies are coming from some other systematic error or noise, based on our accounting of interfacial effects and the repeatability of the measurement, we believe that these values could be an indicator of some form of protein interaction or aggregation in the model synovial fluid that does not occur in the hyaluronic acid. Although not tested in this study, lubricin has been shown to have similar effects on the low strain behavior of synovial fluid when tested using micro-rheology techniques, where lubricin free synovial fluid exhibited decreased elasticity in comparison to healthy synovial fluid.²³ Moreover, these results are consistent with the results of Castellanos and coworkers,²⁹ which showed that some globular proteins could exhibit bulk aggregation that affects bulk rheology. In that study, bovine serum albumin was found to have a small bulk effect; however, the combination of proteins in this work may result in other bulk aggregation or protein interaction not evidenced for pure solutions.

However, due to the small increases in the elasticity observed, and the wide range of possible mechanisms not related to protein content including machine measurement limits, residual interfacial rheology of SDS, or surface tension effects, we cannot explicitly conclude that the observed elasticity is from protein interaction in the bulk.

Conclusion

The interfacial rheology of the model synovial fluid system in steady and oscillatory interfacial shear has novel and interesting behaviors. These appear to be a combination of the effects of both the hyaluronic acid and the bovine serum albumin on the interface. The hyaluronic acid primarily appears to provide the viscous response where the bovine serum albumin adds interfacial elasticity. Despite the interesting behavior, due to the encapsulated nature of a synovial joint, interfacial rheology plays little role in effective lubrication of the knee. However, the results do indicate that this interfacial behavior is in fact quite important in measuring bulk rheology.

The steady shear history of model synovial fluids observed in previous studies appears to be entirely an artifact of interfacial rheology and surface tension affecting bulk rotational rheometer measurements. These results are consistent with recent studies that have found appearance of yield in bovine serum albumin is attributable to interfacial rheology primarily and very little due to bulk aggregation of bovine serum albumin. In both steady shear rate and creep measurements viscosity of model synovial fluid solutions is identical to the behavior of hyaluronic acid solutions once interfacial rheology is accounted for.

However in oscillatory experiments in which the effect of interface has been satisfactorily removed from measurement, a slight increase in elasticity of synovial fluid at low strains and frequencies is observed. This behavior may be attributable to a number of mechanisms, but could be occurring due to interaction of included proteins with each other or hyaluronic acid. It is possible that lubricin may have some effect when added to the model solution, but that cannot be determined by this work. Further testing of the low strain and frequency behavior of model synovial fluids needs to be done in order to definitively conclude where increased elasticity stems from.

Proteins, in particular lubricin, have been found to play an important role in boundary lubrication regimes in previous studies due to their ability to adhere to cartilage surfaces.^{10, 16-18, 42, 43} The results of this work indicate that proteins may play a small role in non-boundary thin film lubrication regimes when synovial fluid rheology plays a significant role in lubrication; however, this role is clearly quite minor and only effect very slow small strain movements. The very low strains and frequencies required to see these effects limit their usefulness in synovial joint lubrication to very small movements at low speeds, which are akin to small shifts of position rather than full motion such as walking or running. This indicates that elasticity may come into play only in joint stiffness after inactivity, before full hydrodynamic or elasto-hydrodynamic lubrication take effect. In these scenarios, some elasticity would allow knee joints to stay separated avoiding joint damage; once larger movements occurred the high rate/high frequency shear thinning behavior of these solutions would dominate providing more effective lubrication. This low strain low frequency elasticity might also have importance in the compression response of the knee over long times, as the knee slowly settles.

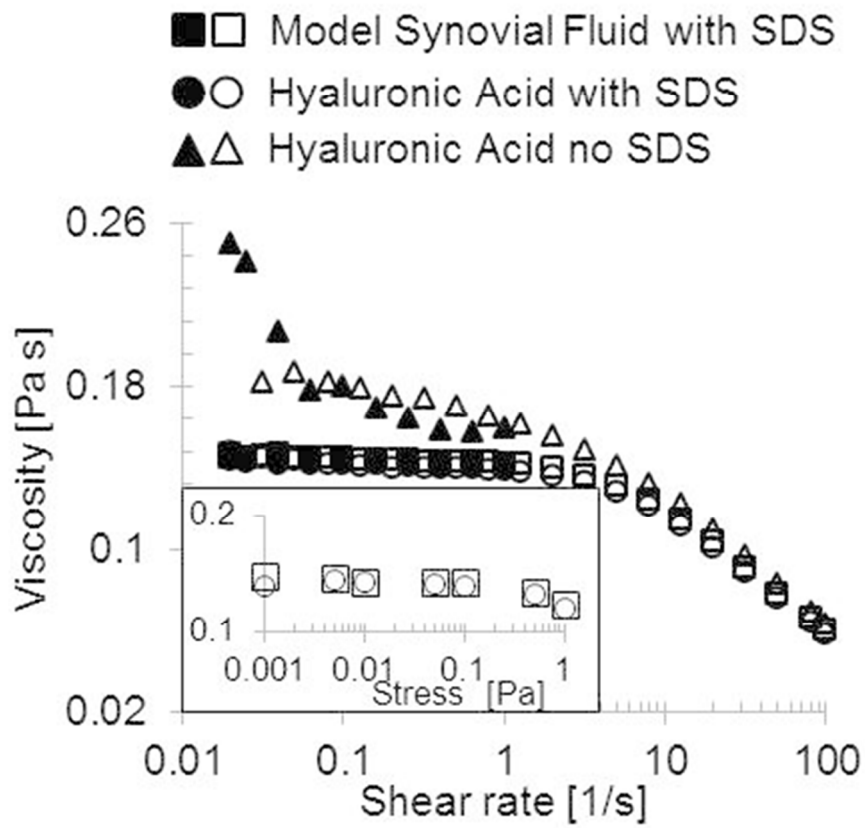
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References

1. G. W. Greene, X. Banquy, D. W. Lee, D. D. Lowrey, J. Yu and J. N. Israelachvili, *Proceedings of the National Academy of Sciences of the United States of America*, 2011, **108**, 5255-5259.
2. H. Birnbaum, C. Pike, R. Kaufman, M. Marynchenko, Y. Kidolezi and M. Cifaldi, *Curr Med Res Opin*, 2010, **26**, 77-90.
3. P. R. Scudder, W. McMurray, A. G. White and T. L. Dormandy, *Annals of the Rheumatic Diseases*, 1978, **37**, 71-72.
4. M. M. Smith, M. A. Cake, P. Ghosh, A. Schiavinato, R. A. Read and C. B. Little, *Rheumatology*, 2008, **47**, 1172-1178.
5. H. Fam, J. T. Bryant and M. Kontopoulou, *Biorheology*, 2007, **44**, 59-74.
6. A. O. Bingol, D. Lohmann, K. Puschel and W. M. Kulicke, *Biorheology*, 2010, **47**, 205-224.
7. K. M. N. Oates, W. E. Krause and R. H. Colby, in *Advanced Biomaterials-Characterization, Tissue Engineering and Complexity*, ed. S. C. Moss, 2002, vol. 711, pp. 53-58.
8. K. M. N. Oates, W. E. Krause, R. L. Jones and R. H. Colby, *Journal of the Royal Society Interface*, 2006, **3**, 167-174.
9. S. J. Haward, A. Jaishankar, M. S. N. Oliveira, M. A. Alves and G. H. McKinley, *Biomicrofluidics*, 2013, **7**, 044108-044114.

10. J. P. Bao, W. P. Chen and L. D. Wu, *Mol. Biol. Rep.*, 2011, **38**, 2879-2885.
11. L. Han, A. J. Grodzinsky and C. Ortiz, in *Annual Review of Materials Research, Vol 41*, ed. D. R. F. P. Clarke, 2011, vol. 41, pp. 133-168.
12. S. M. McNary, K. A. Athanasiou and A. H. Reddi, *Tissue Engineering Part B-Reviews*, 2012, **18**, 88-100.
13. A. Neville, A. Morina, T. Liskiewicz and Y. Yan, *Proceedings of the Institution of Mechanical Engineers, Part C: Journal of Mechanical Engineering Science*, 2007, **221**, 1223-1230.
14. T. B. Goudoulas, E. G. Kastrinakis, S. G. Nychas, L. G. Papazoglou, G. M. Kazakos and P. V. Kosmas, *Annals of Biomedical Engineering*, 2010, **38**, 57-65.
15. V. Tirtaatmadja, D. V. Boger and J. R. E. Fraser, *Rheologica Acta*, 1984, **23**, 311-321.
16. R. Crockett, *Tribology Letters*, 2009, **35**, 77-84.
17. J. P. Gleghorn, A. R. C. Jones, C. R. Flannery and L. J. Bonassar, *Journal of Orthopaedic Research*, 2009, **27**, 771-777.
18. T. A. Schmidt and R. L. Sah, *Osteoarthritis Cartilage*, 2007, **15**, 35-47.
19. J. Yu, X. Banquy, G. W. Greene, D. D. Lowrey and J. N. Israelachvili, *Langmuir*, 2012, **28**, 2244-2250.
20. R. Tadmor, N. H. Chen and J. N. Israelachvili, *Journal of Biomedical Materials Research*, 2002, **61**, 514-523.
21. R. Tadmor, N. H. Chen and J. N. Israelachvili, *Biophysical Journal*, 2002, **82**, 163A-164A.
22. G. D. Jay, J. R. Torres, D. K. Rhee, H. J. Helminen, M. M. Hytinen, C.-J. Cha, K. Elsaid, K.-S. Kim, Y. Cui and M. L. Warman, *Arthritis and Rheumatism*, 2007, **56**, 3662-3669.
23. G. D. Jay, J. R. Torres, M. L. Warman, M. C. Laderer and K. S. Breuer, *Proceedings of the National Academy of Sciences of the United States of America*, 2007, **104**, 6194-6199.
24. B. Zappone, M. Ruths, G. W. Greene, G. D. Jay and J. N. Israelachvili, *Biophysical Journal*, 2007, **92**, 1693-1708.
25. D. A. Swann, F. H. Silver, H. S. Slayter, W. Stafford and E. Shore, *Biochem. J.*, 1985, **225**, 195-190.
26. M. W. Ropes, E. C. Rossmeisl and W. Bauer, *Journal of Clinical Investigation*, 1940, **19**, 795.
27. B. Decker, W. F. McGuckin, B. F. McKenzie and C. H. Slocumb, *Clinical Chemistry*, 1959, **5**, 465-469.
28. S. Vandebriel, A. Franck, G. G. Fuller, P. Moldenaers and J. Vermant, *Rheologica Acta*, 2010, **49**, 131-144.
29. M. M. Castellanos, J. A. Pathak and R. H. Colby, *Soft Matter*, 2014, **10**, 122-131.
30. A. Jaishankar, V. Sharma and G. H. McKinley, *Soft Matter*, 2011, **7**, 7623-7634.
31. V. Sharma, A. Jaishankar, Y. C. Wang and G. H. McKinley, *Soft Matter*, 2011, **7**, 5150-5160.
32. K. Vorvolakos, J. C. Coburn and D. M. Saylor, *Soft Matter*, 2014, **10**, 2304-2312.
33. E. M. Freer, K. S. Yim, G. G. Fuller and C. J. Radke, *Langmuir*, 2004, **20**, 10159-10167.
34. T. W. Patapoff and O. Esue, *Pharmaceutical development and technology*, 2009, **14**, 659-664.
35. A. R. Mackie, A. P. Gunning, P. J. Wilde and V. J. Morris, *Langmuir*, 2000, **16**, 8176-8181.
36. M. T. Johnston and R. H. Ewoldt, *Journal of Rheology*, 2013, **57**, 1515-1532.
37. A. Dominguez, A. Fernandez, N. Gonzalez, E. Iglesias and L. Montenegro, *Journal of Chemical Education*, 1997, **74**, 1227.
38. M. A. Bos and T. van Vliet, *Advances in Colloid and Interface Science*, 2001, **91**, 437-471.
39. W. H. Jiang and S. J. Han, *Journal of Colloid and Interface Science*, 2000, **229**, 1-5.
40. D. P. Acharya, T. Sato, M. Kaneko, Y. Singh and H. Kunieda, *The Journal of Physical Chemistry B*, 2006, **110**, 754-760.
41. *European Polymer Journal*, 1998, **34**, 225-228.
42. C. Clasen, H. P. Kavehpour and G. H. McKinley, *Appl. Rheol.*, 2010, **20**.
43. A. Mavraki and P. M. Cann, *Tribology International*, 2011, **44**, 550-556.



Model synovial fluid steady shear viscosity to hyaluronic acid solution are identical when interfacial rheology effects are removed.

78x71mm (150 x 150 DPI)