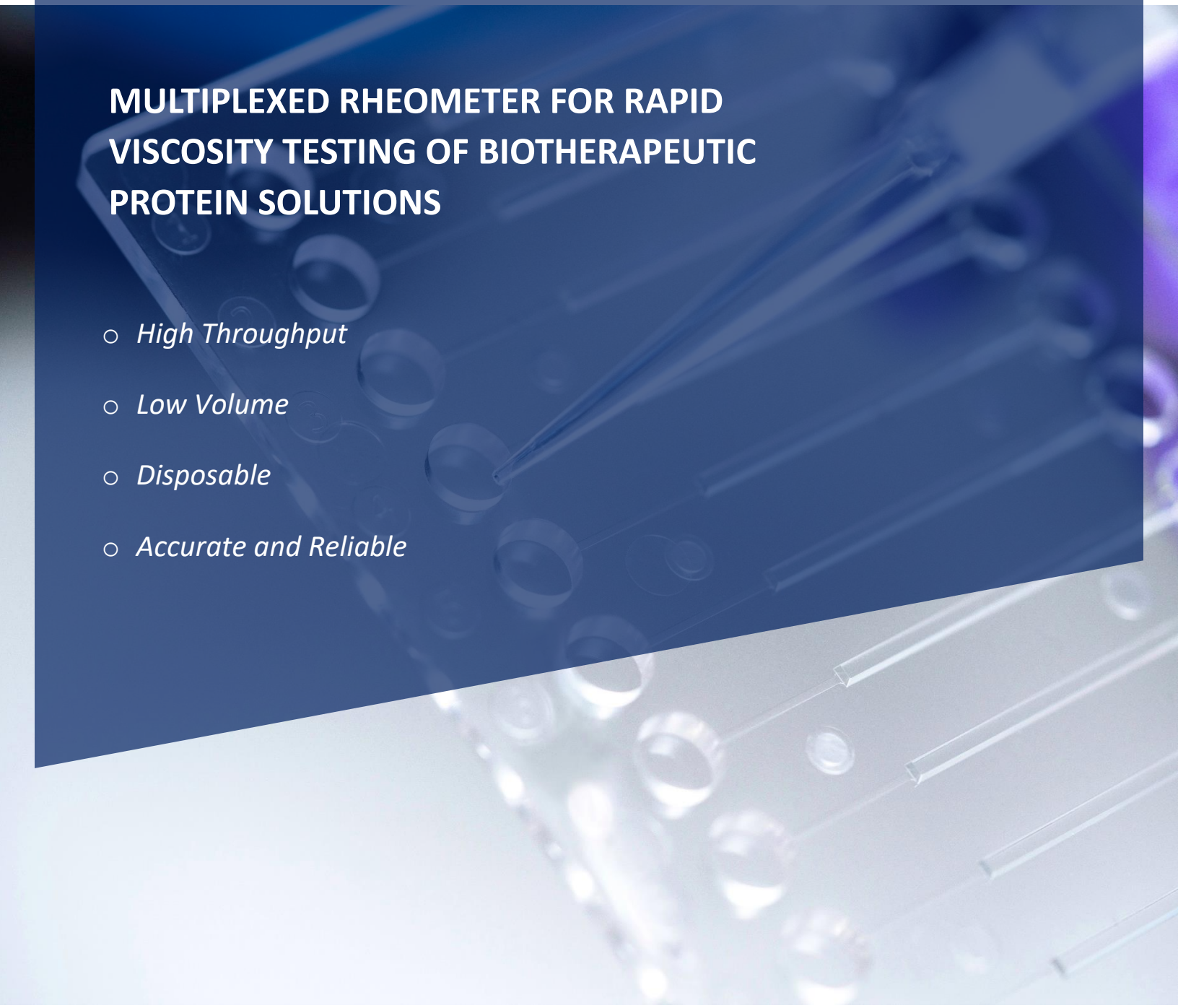
NEOFLUIDICS

# NeoVISC™

*Microfluidic Rheometer*

## MULTIPLEXED RHEOMETER FOR RAPID VISCOSITY TESTING OF BIOTHERAPEUTIC PROTEIN SOLUTIONS

- *High Throughput*
- *Low Volume*
- *Disposable*
- *Accurate and Reliable*



# Contents

The importance of viscosity measurements spans many applications including but not limited to pharmaceuticals, biotherapeutics, electrolyte solutions, and lubricants. Understanding how these substances resist motion under applied force enables users to predict their performance for any given application. Many macroscale rheometers on the market provide the necessary viscosity measurements for common applications, however, they lack throughput, require high volumes, and are costly to the user. Despite their significant value, viscosity measurements can become infeasible for certain applications due to these shortcomings. Here we present a microfluidic rheometer capable of highly accurate, reproducible, low volume and multiplexed viscosity measurements of both Newtonian and non-Newtonian fluids. In addition, we highlight its applicability to protein therapeutics processing through a case study using the model protein bovine serum albumin (BSA). We report the observed change in viscosity as a result of the prolonged exposure to high temperatures that is possible during typical industrial purification protocols.

## INTRODUCTION

Since their introduction almost 40 years ago, biotherapeutics have become an exciting alternative to small molecule drugs due to their versatility, biocompatibility, and site-specific delivery. In the field of protein biotherapeutics, viscosity measurements provide critical information about protein quality, processability, and injectability. After secretion/extraction, the protein of interest must be isolated from other components. Such isolation requires extensive purification and other post-processing steps that can be difficult to carry out with standard manufacturing processes if solutions are highly viscous[1,2]. Additionally, these processes can stress the protein significantly, causing deformation and aggregation that can increase solution viscosity and immunogenicity and decrease efficacy[2–4]. These solutions can also aggregate at high concentrations, causing viscosity to increase dramatically[2,5]. Since efficacious therapeutic concentrations may exhibit viscosities higher than what is allowable for intravenous administration, a thorough understanding of their rheological properties is required.

Protein viscosity measurements can be performed on conventional rheometers; however, these systems are often low-throughput, high-volume, laborious, and expensive. Their extensive cleaning protocols severely reduce measurement efficiency and are not ideal for biological samples, where cross-contamination can be detrimental to the measurement. Furthermore, with conventional methods, obtaining viscosity across a range of shear rates requires long measurement times and large sample volumes. Volume requirements are critical during both the initial formulation and subsequent manufacturing steps, as a multitude of variables for each active pharmaceutical ingredient (API) must be tested, including temperature, API concentration, storage conditions, route of administration, and possible excipients. Recently, microfluidic alternatives have been able to address some of the limitations of conventional measurement techniques. However, even these microfluidic viscometers have limitations, most notably their time-intensive cleaning protocols and limited throughput.

To address the limits of current viscometers, we have developed an integrated rheometer platform that utilizes microfluidic technology to generate low-volume, multiplexed viscosity measurements to rapidly assess protein quality at various stages of formulation and manufacturing. A cartridge

with ten microfluidic channels holds sample fluid that is driven with a known pressure. An overhead camera then captures the fluid-air interface of the sample as it travels through the channel, and an image processing algorithm uses the measured velocity to determine viscosity as a function of shear rate. In our system, the flow rate sensor is optical, allowing for the measurement of all ten samples simultaneously. In this way, viscosity sampling of a range of protein concentrations can be performed at one time. Additionally, viscosity measurements are taken across a broad range of shear rates, which can be important when evaluating proteins for therapeutic use. For example, injectability and processability can be determined by measuring viscosity at shear rates typically experienced during administration and manufacturing. In addition, Newtonian protein solutions can exhibit shear-thickening non-Newtonian behavior at high concentrations as a result of increased protein-protein interactions. Some non-Newtonian behavior can also be observed in these solutions at very low shear rates[6]. Therefore, it is of great importance to quantify viscosity over a large shear rate range to obtain a complete understanding of protein characteristics and behavior.

From dynamic viscosity, one can also find a protein's intrinsic viscosity, which is a concentration-independent quantitation of the inherent viscosity of the protein in a particular solvent[7]. Intrinsic viscosity is a reliable indicator of the effective size, or hydrodynamic radius, of a protein, which is dependent on its specific higher order structures[5,8]. Thus, a protein's characteristic hydrodynamic radius should remain constant for the protein to effectively carry out its intended biotherapeutic function[9]. During the development, purification, and storage process, protein conformation and stability can be assessed by finding intrinsic viscosity and using well-investigated equations to evaluate hydrodynamic radius.

In the present study, we aim to use our microfluidic viscometer platform to demonstrate the relationship between viscosity and protein quality to ensure reliable formulations for biologic drug therapy.

## MATERIALS AND METHODS

### *Cartridge Design and Functionality*

A multi-height microfluidic viscometer cartridge was fabricated in cyclic olefin copolymer (COC) using injection molding techniques. The cartridge contains ten independent microfluidic channels, each of which consist of a small channel connected in series with a larger channel. The hydrodynamic resistance of the small channel is designed to have a much greater resistance than the large channel. A reservoir at the inlet of the small channel holds the sample, which is then pushed into the microfluidic channel using an integrated pressure source. As the fluid plugs travel through the small channel, they experience a high degree of resistance based on channel geometry and fluid viscosity. As they move into the large channel, a camera and integrated image processing software track the fluid velocities, and subsequently calculate the viscosities for each fluid.

### *Measurement Automation*

All fluid channels are driven with independent ramping pressure profiles, where the input pressure continuously increases in a linear fashion for the length of the test. At the beginning of each measurement, the software tracks fluid movement under a known pressure to approximate fluid viscosity. This information is then used to apply a pressure ramp that is appropriate for the fluid at hand. In this way, a full range of fluid viscosities can be processed in a single cartridge

simultaneously. As the fluid is moving, the camera and image processing software track the distance traveled by the fluid-air interface in the large channel. The software automatically fits a curve to the distance vs. time data and differentiates to obtain velocity at each pressure value. Shear stress is calculated for each input pressure and plotted against shear rate, which is determined from the velocity data. Finally, the data for shear stress, true shear rate, and true viscosity are displayed for 20 evenly spaced pressure points along the curve.


**Figure 1. NeoVISC™ workflow.** To perform a NeoVISC™ measurement, user pipettes samples into the cartridge, places the cartridge in the instrument, and begins the test via the proprietary user interface.

## *Measurement Principle*

Viscosity is determined through the relationship between pressure drop and flow rate across a microfluidic channel of known resistance. Viscosities of both Newtonian and non-Newtonian fluids were found using the following equation:

$$\mu = \frac{\tau_w}{\dot{\gamma}} \quad (1)$$

where $\dot{\gamma}$ is the shear rate and $\tau_w$ is the shear stress[10], given by:

$$\tau_w = \frac{\Delta P\, wh}{2L(w+h)} \quad (2)$$

Here, $\Delta P$ is the pressure drop applied by the integrated pressure controller, and $w$, $h$, and $L$ are the width, height, and length of the small channels, respectively. As a result of the resistance disparity between the large and small channels, only the dimensions of the small channel need to be considered for the shear stress calculation. Additionally, in the case of the proposed cartridge, $w>>h$ such that the $w+h$ term simplifies to $w$.

For the present microfluidic channels, the apparent shear rate, $\dot{\gamma}_0$, is given by:

$$\dot{\gamma}_0 = \frac{6Q}{wh^2} \quad (3)$$

where $Q$ is the flow rate of the fluid in the channel[10]. Since the small and large channels are in series, the flow rate in the large channel is equal to the flow rate in the small channel. The flow rate is determined from the product of the fluid velocity in the large channel and the cross-sectional area of the large channel.

For Newtonian fluids, where the fluid experiences a perfectly parabolic flow profile across the width of the channel, the apparent shear rate is equal to the true shear rate. However, for a non-Newtonian fluid, which experiences a variable flow profile through the channel, the apparent shear rate must be multiplied by the Weissenberg-Rabinowitch[11] correction factor to obtain the true shear rate, $\dot{\gamma}$:

$$\dot{\gamma} = \frac{\dot{\gamma_0}}{3}\left(2 + \frac{d(\ln \dot{\gamma_0})}{d(\ln \tau_w)}\right) \quad (4)$$

The system software automatically approximates the term $d(\ln \dot{\gamma_0})/d(\ln \tau_w)$ in Equation 4 by varying input pressure to subject the fluid to a range of shear rates, plotting apparent shear rate versus shear stress at each pressure, fitting an appropriate polynomial to the points, and differentiating. Then, the true shear rate, and subsequently the viscosity, are automatically found for each pressure drop. For Newtonian fluids, the $d(\ln \dot{\gamma_0})/d(\ln \tau_w)$ term is equal to 1, and the value in parentheses cancels with the value in the denominator, leaving $\dot{\gamma} = \dot{\gamma_0}$.

## *Calculating Hydrodynamic Radius*

The intrinsic viscosity of native and denatured BSA was determined using the following equation:

$$[\eta] = \lim_{c \to 0} \frac{\eta - \eta_0}{\eta_0 \, c} \quad (5)$$

where $\eta$ is the sample viscosity at a particular concentration $c$, and $\eta_0$ is the solvent viscosity[8,12]. In equation (5), the $(\eta-\eta_0)/\eta_0$ term is known as the inherent viscosity, and intrinsic viscosity is found by extrapolating inherent viscosity to a protein concentration of zero. From intrinsic viscosity, hydrodynamic radius was determined using the Einstein viscosity relation:

$$V = \frac{5[\eta]M}{2N} \quad (6)$$

where $M$ is the molecular weight of the protein, $N$ is Avogadro's number, and $V$ is the effective diameter of a protein particle assuming the particle is spherical[8,12]. Past research has shown that at concentrations below 100mg/mL, BSA, a globular protein, can successfully be approximated as a spherical particle[13,14]. Hydrodynamic radius was then determined from the equation for the volume of sphere:

$$R_h = \left(\frac{3}{4\pi} V\right)^{1/3} \quad (7)$$

The hydrodynamic radii of native and denatured protein were subsequently compared to quantify the conformational change caused by heat-induced protein deformation.

## *Measuring Viscosity Using the NeoVISC™*

To begin the viscosity measurement, 40 µL of sample was pipetted into the inlet reservoir. Up to ten different samples were loaded into the ten reservoirs on the microfluidic cartridge. The cartridge was placed into the instrument and a pressure manifold was lowered onto the surface of the cartridge to seal the inlet ports. Using proprietary software, all loaded channels were selected, temperature was set, and the measurement was started. After the fluids reached the ends of their respective channels, the test automatically stopped, and data was output in the form of a chart containing the raw shear stress, shear rate, and viscosity values, as well as shear rate vs. viscosity plots for each channel.

### *Measuring Viscosity Using a Cone-and-Plate-Rheometer*

Values obtained using the NeoVISC™ were compared to a conventional cone-and-plate rheometer (AMETEK Brookfield DV2T, Middleboro, MA). Measurements were performed using 500 µL of sample pipetted into the sample cup. The appropriate cone spindle for the relevant viscosity range was attached. Shear rate was dictated by spindle rotation speed.

### *Reagents*

Reagent grade deionized (DI) water was obtained from VWR (Radnor, PA). Glycerol and phosphate buffered saline (PBS) pH 7.4 were purchased from Thermo Fisher Scientific (Waltham, MA). Bovine serum albumin (BSA) 98% was purchased from Alfa Aesar (Haverhill, MA), and polyethylene oxide powder was sourced from Sigma Aldrich (St. Louis, MO).

### *Preparation of Newtonian and Non-Newtonian Fluids*

Glycerol solutions of 25, 60, and 80 weight percent were prepared by pipetting glycerol into the appropriate amount of water for a final volume of 10 mL and vortexing until fully mixed. Solutions were left for one hour at room temperature (25 °C) to allow for dissipation of any air bubbles. PEO solutions were prepared by adding 10 mL of DI water to 50 mg, 100 mg and 150 mg of PEO to make 0.5%, 1.0% and 1.5% solutions. PEO and water were added in a layered fashion to avoid large aggregate formation. The solution was covered in foil to prevent light degradation and stirred overnight with a magnetic stir bar at 25 °C.

### *BSA Solutions*

BSA was dissolved on an orbital shake table in PBS at 4 ºC for one hour and allowed to come to room temperature before measuring. For the comparison of BSA to a cone-and-plate rheometer, 4 mL of BSA was prepared at eight concentrations (10, 50, 100, 150, 200, 225, 250, and 300 mg/mL). For the comparison of native and denatured protein, 4 mL of each BSA concentration (10, 50, 100, and 200 mg/mL) was prepared and measured. 2 mL of each BSA solution was then denatured by heating to 60 ºC for 30 minutes and allowed to cool to room temperature prior to measuring viscosity. All measurements were performed in triplicate.

### *Monoclonal Antibodies*

Undisclosed monoclonal antibodies of unknown concentrations in proprietary storage buffer were provided by Just Biotherapeutics (Seattle, WA). Protein concentrations were disclosed after determination of sample viscosity.

## RESULTS

### *Newtonian Fluids*

To validate system performance, the NeoVISC™ was first compared against a conventional cone-and-plate rheometer using Newtonian solutions. DI water and glycerol solutions were chosen, as they are commonly used as viscosity standards. First, viscosity of DI water was characterized at temperatures ranging from 10-60 ºC and compared to viscosity values obtained on a cone-and-plate rheometer (Figure 2A). DI water and high concentration glycerol solutions were measured on the NeoVISC™ at room temperature and showed high fidelity compared to literature values (Figure 2B). The viscosities shown are averages along a shear rate range for n=12, n=6, and n=4 for water, 60% glycerol, and 80% glycerol, respectively. To demonstrate the automatic shear rate sweep generated by the NeoVISC™ and verify the expected Newtonian behavior of DI water and

glycerol solutions, we report the viscosity as a function of shear rate at 25 °C for water, 25%, and 60% glycerol (Figure 2C). The software's ability to automatically produce a wide shear rate sweep is evident in Figure 2C. If more focused shear-dependent viscosity information is desired, the user can also apply a custom shear rate range. The data presented here highlights the system's ability to measure accurate viscosities for Newtonian fluids over a wide range of fluid viscosities, temperatures, and shear rates.

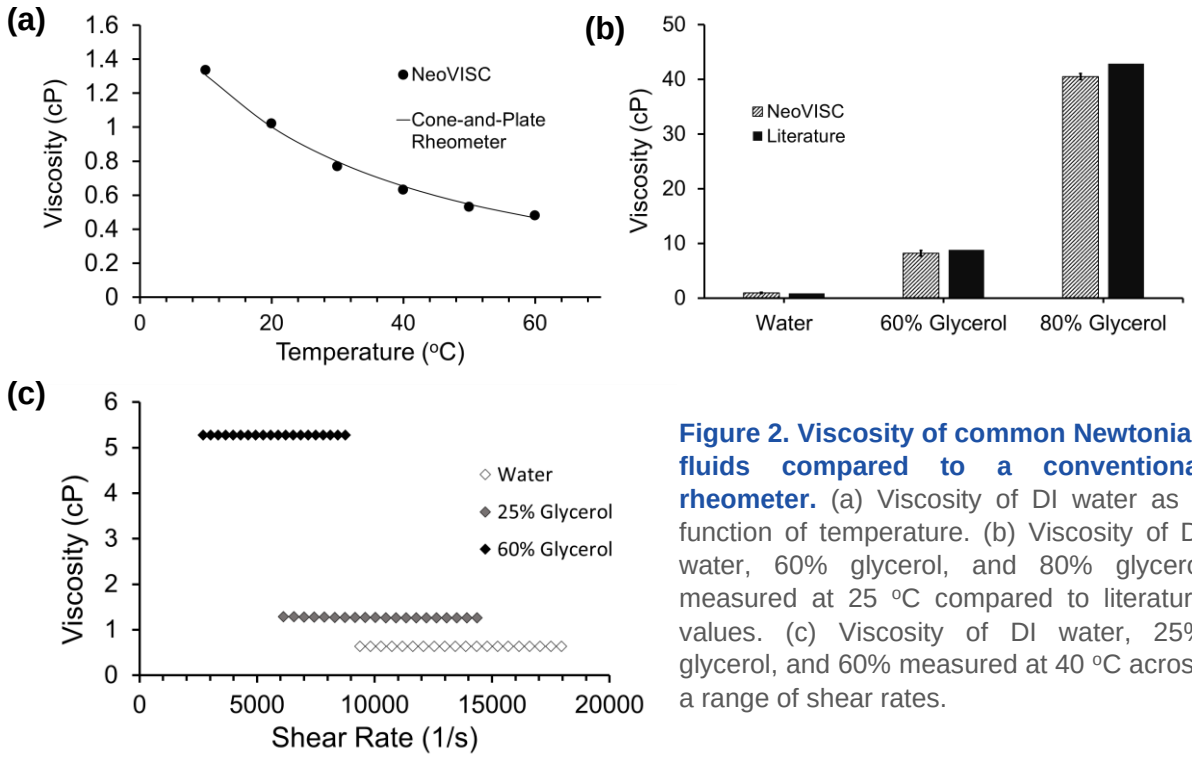



**Figure 2. Viscosity of common Newtonian fluids compared to a conventional rheometer.** (a) Viscosity of DI water as a function of temperature. (b) Viscosity of DI water, 60% glycerol, and 80% glycerol measured at 25 °C compared to literature values. (c) Viscosity of DI water, 25% glycerol, and 60% measured at 40 °C across a range of shear rates.

## *Non-Newtonian Fluids*

The NeoVISC™ was then evaluated using a non-Newtonian, or shear-rate-dependent, polymeric solution of PEO. Unlike Newtonian solutions, the viscosity of a non-Newtonian fluid changes as a function of shear rate. PEO is known to be a shear-thinning fluid, meaning that as shear rate increases, the fluid viscosity decreases. In agreement with a conventional cone-and-plate rheometer, the NeoVISC™ is able to accurately quantify the shear-thinning behavior for 1% PEO (Figure 3A). All points shown in Figure 3A were collected in under five minutes with just 40 µL of sample. Conversely, the cone-and-plate measurement required over 12 times the volume and continous adjustment of instrument spindle rotation settings, resulting in a roughly five times as long measurement time and a narrower shear rate range. Similar shear rate sweeps were perfomed for 0.5% and 1.5% PEO solutions (Figure 3B). Increasing PEO concentration elucidated more apparent shear-thinning behavior.

## *Protein Solutions*

The NeoVISC™ can also successfully measure the viscosity of complex biological solutions. First, well understood solutions of BSA were measured on both the NeoVISC™ and a cone-and-plate rheometer. A close correlation can be observed across the tested concentration range (Figure 4A). A limited volume (10 µL) of blind-coded monoclonal antibody solutions of unknown

concentration were also provided by a third-party biotherapeutic company. The viscosities measured on the NeoVISC™ were compared against those obtained on the RheoSense m-VROC (Figure 4B). The results from the two microfluidic-based rheometer systems are in good agreement.

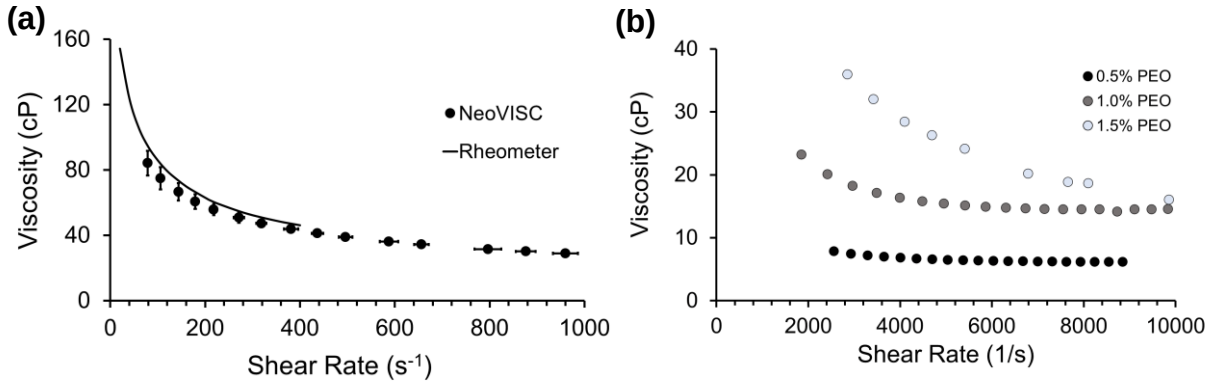



**Figure 3. Viscosity of PEO as a function of shear rate.** (a) Comparison of NeoVISC™ viscosity of 1% PEO as a function of shear rate to cone-and-plate rheometer. (b) Viscosity of increasing PEO concentrations as a function of shear rate.

## *Protein Denaturation*

To mimic potential prolonged exposure to high temperatures during the manufacturing process, we placed four BSA solutions of 10, 50, 100 and 200 mg/mL concentrations in a 60 °C oven for 30 minutes. The viscosity of the native protein was significantly lower compared to the denatured protein solution (Figure 5A). The difference in viscosity was largest for the 200 mg/mL BSA solution but still apparent at 10 mg/mL. This is to be expected, as protein tends to aggregate at high concentrations, and any unfolding or denaturing of BSA would lead to the exposure of hydrophobic domains in the protein, which in turn would result in increased protein-protein

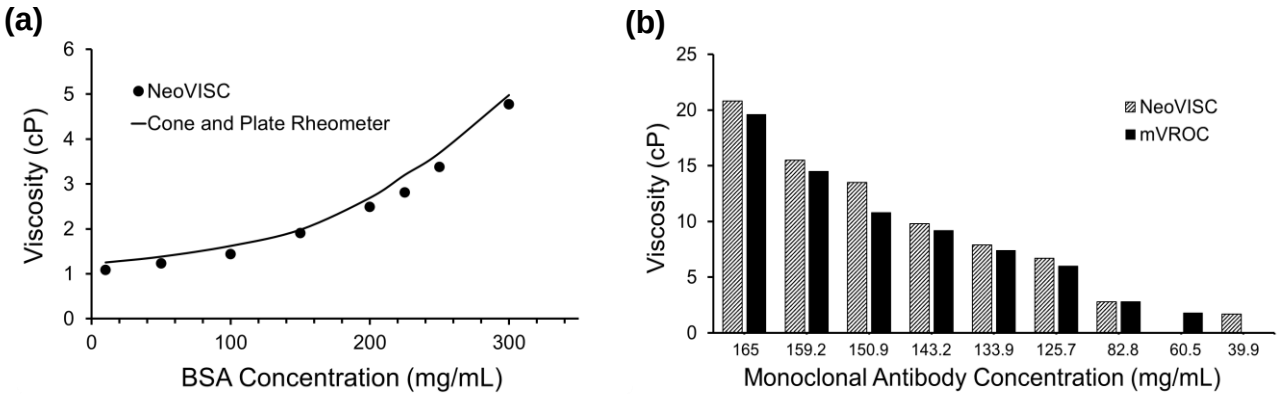



**Figure 4. Viscosity of protein solutions compared to current macroscale and microfluidic viscometers.** (a) Viscosity of BSA solutions as a function of concentration compared to a cone-and-plate rheometer. (b) Viscosity of blind-coded monoclonal antibody solutions as a function of concentration compared to RheoSense m-VROC.

interactions. Higher concentrations increase the probability that initial protein-protein seed aggregates will grow and increase the bulk viscosity. We also present viscosity as a function of volume fraction to compare to well-characterized colloidal rheology models and data obtained by Sharma et. al. using the RheoSense m-VROC[13] (Figure 5B). Here, we show the Russel et. al. rheology model $\frac{\eta(\phi)}{\eta_0} = 1 + 2.5\phi + s\phi^2 + O(\phi^3)$ which predicts viscosity of charged colloids based on concentration[15]. In the quadratic term, *s* is a function of the effective diameter of the colloid, which is dependent on the colloid and its specific interactions in a particular solvent. Batchelor found that *s* = 6.2 in the case of solely hard sphere repulsive interactions[13,15]. However, the native BSA data follows closely with the model where s = 10, as is the case for the Sharma et. al. RheoSense values.

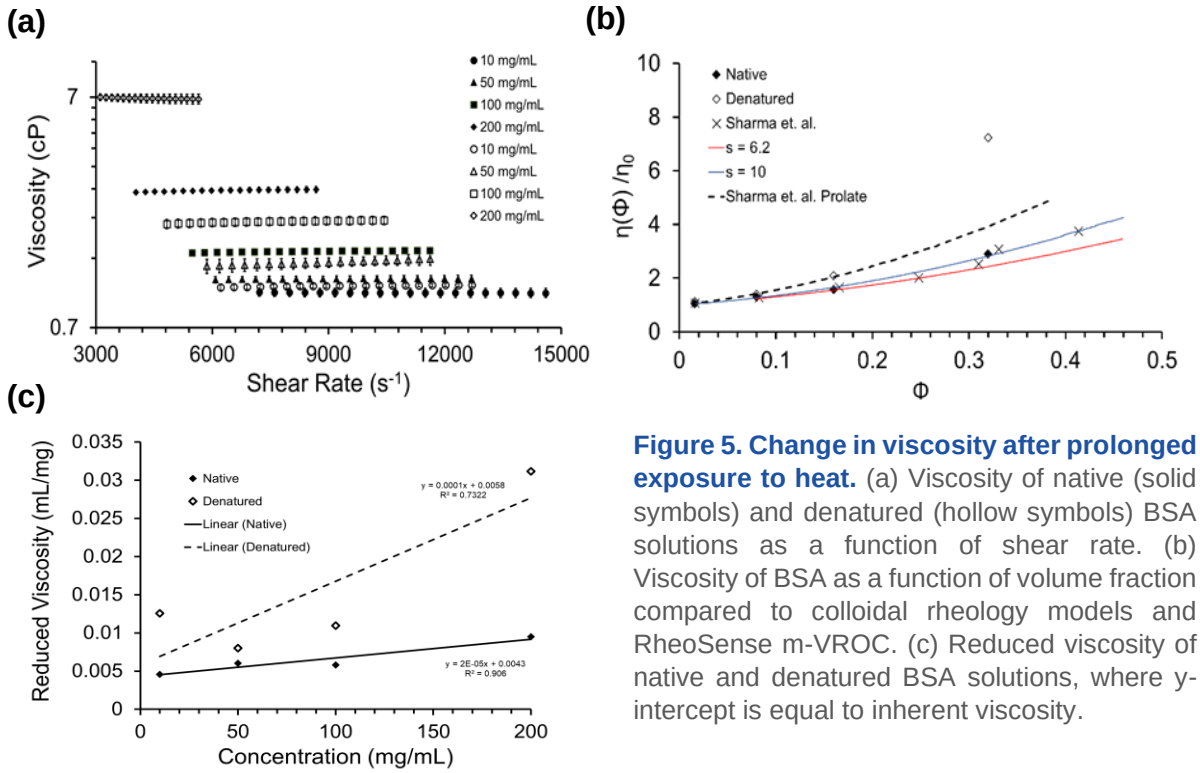



**Figure 5. Change in viscosity after prolonged exposure to heat.** (a) Viscosity of native (solid symbols) and denatured (hollow symbols) BSA solutions as a function of shear rate. (b) Viscosity of BSA as a function of volume fraction compared to colloidal rheology models and RheoSense m-VROC. (c) Reduced viscosity of native and denatured BSA solutions, where y-intercept is equal to inherent viscosity.

The intrinsic viscosities of native and denatured BSA were found from Equation 5 by plotting reduced viscosity as a function of concentration and obtaining the y-intercept (Figure 5C). Denatured BSA showed roughly a 35% higher intrinsic viscosity than the protein in its native state (4.3 mL/mg for native and 5.8 mL/mg for denatured). Applying Equation 6 and Equation 7, we observed that the hydrodynamic radius of denatured BSA was also significantly higher (~15%) than that of the unaltered protein (4.09 nm compared to 3.57 nm for native). The experimental value obtained for the radius of native BSA matches closely with reported values in literature (3.48 nm)[16].

## DISCUSSION

The NeoVISC™ microfluidic rheometer is shown to produce data for a range of fluid types that closely agrees with results from conventional techniques, while significantly increasing throughput and decreasing volume requirements. This is especially relevant for biotherapeutic applications, where viscosity must be tested at multiple concentrations, and available sample is limited. We show how the NeoVISC™ can simultaneously measure viscosity of a range of protein concentrations and generate shear rate sweeps that give the user a complete understanding of their formulation's rheological properties. While viscosity is a critical parameter for manufacturability and delivery of biotherapeutics, it is often neglected in early-stage formulation screening due to time and/or volume constraints. The NeoVISC™ solves the issues observed in conventional rheometer systems and gives biopharmaceutical manufacturers the power to test more formulations earlier to make sure the most promising candidates are moved forward.

We also demonstrate how the NeoVISC™ can detect increases in protein viscosity and hydrodynamic radius after heat denaturation, indicating chain elongation and protein-protein interaction, which can be detrimental to therapeutic efficacy and safety. While proteins can often be exposed to high temperatures during processing, there are a number of additional manufacturing steps that can have adverse effects on protein quality, including high shear pumping, freeze-thaw-cycles during storage, purification and concentration processes, and long-term storage. These stresses can result in protein unfolding and aggregation that can be identifiable by an increase in intrinsic viscosity. We show how viscosity values measured with the NeoVISC™ offer a low-volume, low-effort technique to screen for adverse protein interactions throughout biotherapeutic manufacturing.

## CONCLUSION

The NeoVISC™ is capable of measuring viscosity rapidly and reliably over a wide range of viscosities, temperatures, shear rates, and fluid types. Our breakthrough system minimizes sample volume and maximizes throughput with ten simultaneous measurements, solving the problems of conventional methods and allowing for viscosity to be performed in applications where it was previously impractical or impossible. Here, we show the potential of our system to measure viscosity of biotherapeutics to assess protein processability and injectability in early screening and subsequently measure protein quality throughout manufacturing and storage. The NeoVISC™ provides invaluable information about formulations at each step of the biotherapeutic development process.

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