Nanorheology of regenerated silk fibroin solution

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Abstract. We have investigated the rheological properties of regenerated silk fibroin (RSF), a viscoelastic material at micro and nano length scales, by video microscopy. We describe here the principles and technique of video microscopy as a tool in such investigations. In this work, polystyrene beads were dispersed in the matrix of RSF polymer and the positions of the embedded beads diffusing were tracked using video microscopy. An optical tweezer was used to transport and locate the bead at any desired site within the micro-volume of the sample, to facilitate the subsequent free-bead video analysis. The position information of the beads was used to obtain the time dependant mean squared displacement (MSD) of the beads in the medium and hence to calculate the dynamic moduli of the medium. We present here the results of rheological measurements of the silk polymer network in solution over a frequency range, whose upper limit is the frame capture rate of our camera at full resolution. The technique is complementary to other microrheological techniques to characterize the material, but additionally enables one to characterize local inhomogeneities in the medium, features that get averaged out in bulk characterization procedures.

Keywords. Particle tracking video microscopy; rheology; optical tweezer; soft materials.

1. Introduction

Bulk rheology has proven to be important in studying the mechanical properties of viscoelastic materials such as strain rate dependant viscosity and frequency dependant viscoelastic moduli, for characterizing the materials. The extension of rheological studies at micrometer length scales in microrheology, and the techniques in microrheological studies, have the advantage of requiring smaller amounts of the sample, a consideration when only smaller sample volumes are available, or where the sample is expensive (Waigh 2005). They are also useful in situations where high throughput screening on a regular basis may be required (Breedveld and Pine 2003).

In microrheology, particles embedded in a complex fluid are used as probes to generate a strain field in the medium. By using spherical tracer particles of a known size (micro to nanometres), the motion of particles can be interpreted quantitatively in terms of the local viscoelastic properties of the surrounding medium. While often interpreted in terms of the macroscopic bulk modulus, the real power of microrheology lies in the fact that individual tracer beads probe microenvironments and the motion of the individual tracer particles reflects the local mechanical response of the surrounding material. Therefore, microrheology yields information about structure and inhomogeneities at micro and nano length scales in soft matter normally not accessible using conventional mechanical rheometers (Gardel *et al* 2005; Waigh 2005; Raghu *et al* 2007).

There exist several microrheological tools and techniques to characterize a viscoelastic sample. These are optical tweezer, magnetic tweezer, AFM, video multi particle tracking, diffusing wave spectroscopy, laser deflection particle tracking and dynamic light scattering, to mention a few. The first three are active techniques in which an external force/stress is applied to produce strain field in the medium. The remaining techniques are passive, wherein strain fields are produced by free beads diffusing due to the thermal energy of the medium in which they are embedded. Hence microrheological parameters measured with passive techniques will be in a linear viscoelastic regime at room temperature and no sample deformations occur (Breedveld and Pine 2003; Waigh 2005). In active methods the magnitude of the applied force decides the linear or nonlinear regime; if the force (or stress) applied is in a nonlinear regime then it can deform the local structural environment of the sample being probed. More information regarding different active microrheological techniques can be found in literature (Gardel *et al* 2005; Waigh 2005). These techniques are complementary to each other to probe different frequency regimes of microrheology.

In this paper, we describe the principles and technique of video microscopy as a micro and nanorheological tool. Initially, this technique was used to track single particles (Sheetz *et al* 1989; Crocker and Grier 1996), but later, was extended to multiple particle tracking (Apgar *et al* 2000; Breedveld and Pine 2003). This improvement has led researchers to extend the use of video microscopy as a rheological tool in the study of viscoelastic materials, as in, studies of the heterogeneities in solutions of actin filaments as a function of concentration using histograms

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(Apgar *et al* 2000), rheology of F-actin solutions that provides new insights into the length scaling behaviour of the rheology (Liu *et al* 2006), gelation processes in block copolypeptide solutions (Breedveld and Pine 2003), to name a few. As an application, we study a viscoelastic material such as regenerated silk fibroin (RSF) polymer solution by video microscopy. The unique mechanical properties of RSF make it especially interesting for many different applications. Apart from its wide use in the textile industry (Chen *et al* 2001), it also finds uses in making environmentally sensitive hydrogels, as a chemical valve material, as a food additive, in the cosmetic industry, and as enzyme immobilizer in biosensors (Chen *et al* 2001).

2. Method

For a bead of radius, a , embedded in a given medium at a temperature, T and freely diffusing in a medium due to thermal energy, one can calculate its time dependent mean squared displacement (MSD = $\langle \Delta r^2(\tau) \rangle$) in that medium from its position information, $r(t)$, using the relation (Mason and Weitz 1995)

$$\langle \Delta r^2(\tau) \rangle = \langle |r(t + \tau) - r(t)|^2 \rangle, \quad (1)$$

where τ is the lag time and $\langle \rangle$ indicates the average being calculated for all starting times, t .

Once the MSD of particles in a given medium is determined, it is related to the mechanical properties of that medium through the generalized Stokes–Einstein relation (GSER). Mason and Weitz (1995) originally derived the GSER in the Laplace domain with Laplace frequency, s ; later GSER was modified for the Fourier domain with Fourier frequency, ω , to yield better accuracy for the complex shear modulus in the measured range. It has been shown elsewhere (Mason 2000; Gardel *et al* 2005) that the GSER recovers the frequency independent viscosity for a pure Newtonian liquid, in addition to its applicability for a viscoelastic material. Levin and Lubensky (2000) provided strong theoretical foundations to the GSER and established the conditions on the range of frequencies over which rheological measurements using GSER are valid. Due to the small probe size the upper limit of valid frequency extends to the MHz regime for microrheology (Levine and Lubensky 2000; Breedveld and Pine 2003). Several routines are followed to obtain the complex shear modulus, $G^*(\omega)$, the storage modulus, $G'(\omega)$ and the loss modulus, $G''(\omega)$ of the medium, in the Fourier domain, from the MSD values of beads in that medium (Waigh 2005). We follow an analytic continuation method originally developed by Mason *et al* (1997) to obtain different moduli of a viscoelastic medium. Dasgupta *et al* (2002) empirically modified these equations to improve the accuracy of moduli determined from the data where MSD data displays greater curvature. These expressions are

$$G^*(\omega) = \frac{k_B T}{\pi a \langle \Delta r^2(1/\omega) \rangle \Gamma[1 + \alpha(\omega)](1 + \beta(\omega)/2)}, \quad (2)$$

$$G'(\omega) = G^*(\omega) \{1/[1 + \beta'(\omega)]\}$$

$$\cos \left[\frac{\pi \alpha'(\omega)}{2} - \beta'(\omega) \alpha'(\omega) \left(\frac{\pi}{2} - 1 \right) \right],$$

and

$$G''(\omega) = G^*(\omega) \{1/[1 + \beta'(\omega)]\}$$

$$\sin \left[\frac{\pi \alpha'(\omega)}{2} - \beta'(\omega) [1 - \alpha'(\omega)] \left(\frac{\pi}{2} - 1 \right) \right]. \quad (3)$$

Here k_B is the Boltzmann constant and Γ the gamma function. $\alpha(\omega)$ and $\beta(\omega)$ are the first and second order logarithmic derivatives of the MSD, and $\alpha'(\omega)$ and $\beta'(\omega)$ are the first and second order logarithmic derivatives of $G^*(\omega)$ and are obtained by fitting the data locally to a second order polynomial. This method has good accuracy with < 5% error for the dominant modulus of the two in the whole frequency range (Dasgupta *et al* 2002).

3. Experimental

3.1 Sample preparation

Proper choice of probe particles is essential to characterize the unknown material by microrheology. The mere presence of the probe particles can lead to changes in the local environment surrounding the particles either through adsorption or depletion. To deal with such problems, the samples must be prepared with different probes of varying size and surface chemistry (Breedveld and Pine 2003). If the system under investigation is highly charge sensitive then both positive and negative particles affect the rheological properties. For such systems sterically stabilized silica particles can be used for microrheology.

For the present work, we have used unmodified polystyrene beads (Cat. no. 07310-15, $0.989 \pm 0.02 \mu\text{m}$, Polysciences Inc., USA) in aqueous solution as probes to measure the relation between stress and deformation in viscoelastic materials. They were added to the samples in low concentrations (1 μL in 1 mL of sample) to avoid inter-bead hydrodynamic interactions as well as to obtain mono dispersed suspensions of beads in the sample. The sample was agitated by shaking gently to achieve uniform mixing of beads in solution for about five min and then allowed to stabilize for 10 min before conducting trials. About 70 μL of sample was used for each trial and the sample was poured carefully into a single cavity micro-

scope slide and covered with number 1 grade cover slip to avoid air bubbles in the sample. Molten wax was used to seal the slide, which was then inverted so that the cover slip was in contact with the objective with a film of index matching oil in between.

We used pure viscous materials such as water and glycerol to calibrate our system. We then measured the properties of RSF solutions, which were prepared by standard methods described elsewhere (Mathur *et al* 1997; Chen *et al* 2001). Undialysed RSF samples were used in this work, as they retain the polymer structure in solution through bonding between chains by metallic ions (Ochi *et al* 2002). Three different concentrations, 1%, 0.50% and 0.15% (w/v) of RSF, were prepared and used for micro-rheological analysis. Tracer beads were mixed to these samples as explained above.

3.2 Video microscopy

In video microscopy, we track the bead position in a plane along two orthogonal directions for each field of view by the following procedure: Video microscopy makes use of processing time sequenced image frames of the embedded beads in a given sample to study rheological properties of that sample. In our setup (figure 1), the sample is visualized on a computer monitor through a CMOS camera (EDC-3000, Electrim Corporation, USA), after magnifying the

sample image through a 100 $\times$ oil immersion objective (1.25 NA, EA100 $\times$ Long Barrel, Olympus, Japan). The temporal resolution of the video microscopy is determined by the frame rate of the camera, which is either 15 fps (for 1280 $\times$ 1024 pixels) or 30 fps (for 640 $\times$ 480 pixels) for our system. The spatial resolution of video microscopy is determined by the optical magnification and pixel density of the CMOS camera (Crocker and Grier 1996; Breedveld and Pine 2003). This is 166 nm/pixel and 63 nm/pixel, respectively, for 30 fps and 15 fps frame rates. Movies of Brownian motion of the beads in a given medium are stored on to a computer hard disk (Intel Pentium IV, 1GB RAM, 80 GB HDD). Subsequently, image analysis algorithms are used to analyse the bead positions in each frame. These algorithms can identify the bead's position by fitting local intensity maxima to a Gaussian distribution with sub pixel accuracy. If numerous features are tracked, this approach requires a further step of linking the features correctly between the images. We have used feature finding algorithm, originally written by Crocker and Grier (1996), and later incorporated into interactive data language virtual machine (IDLVM), RSI Systems, Boulder, USA) software by Ryan Smith and Gabe Spaulding (for link see reference). This program can detect displacements of a bead with a precision of few tenths of a nanometre in sequenced image frames (Crocker and Grier 1996; Breedveld and Pine 2003). Nearly 1000 images are acquired for each measurement, after focusing the microscope well above (about 30 μ m) the coverslip wall, to avoid any possible interaction between beads and cover-slip. An optical tweezer built with an open microscope setup (Raghu and Sharath 2005), is used to transport and locate the beads 30 microns above from the surface of the cover slip to avoid possible interaction with bead and the cover slip (Fauchaux and Libchaber 1994; Dufresne *et al* 2000). Figures 2(a) and (b) show the positions of a trapped bead in water near the cover slip and at 30 micron above the cover slip, respectively.

Through multi particle tracking, 3–5 beads are tracked simultaneously for each field of view. This saves time while also allowing one to obtain good statistics of the data from only a few movies. The smallest displacement observable with our image processing setup is in principle, 4 nm. By monitoring the apparent displacements of the microspheres stuck on a glass coverslip, over 900 frames, we detected an average standard deviation of 33 nm, from an ideal value of zero, as expected for stationary stuck beads. This noise is a reflection of the artefacts inherent in the imaging system and sets the upper limit on the accuracy of the data in our setup. By using (1)–(3) described above, the rheological parameters of the sample are obtained using the position information of the bead. We have analysed all the movies off line and this took nearly 2–3 h to get bead positions from each field of view using the IDLVM code.

Figure 1. Schematic of our video microscope setup. (MO, microscope objective; CL, collimated laser (830 nm, 100 mW, Thorlabs); CC, CMOS camera; PC, personal computer (see text for more details and part numbers).

Figure 2. (a) A 3 micron trapped bead (inside circle) in water near cover slip and (b) same bead elevated to 30 microns above the cover slip using an optical tweezer. The diffraction rings around the free beads in (a) are evidence that they are slightly out of focus and closer to the cover slip. (b) When the trapped bead, which is always in focus, is shifted 30 micron above the coverslip the diffraction rings around other beads disappear.

Figure 3. (a) MSD (squares) of water (filled) and glycerol (open). Dotted line indicates the operational limit of our setup and (b) frequency dependence of shear moduli (circles) and viscosity (hexagonals) of water (filled) and glycerol (open).

4. Results

4.1 Calibration of setup

To calibrate our set up, we have used $0.989 \mu\text{m}$ polystyrene beads in pure viscous media such as double distilled water and pure glycerol. Ensemble averaged, two-dimensional MSD of 14–16 beads were obtained from the bead position data measured at different parts of each sample, in both solutions using (1). Figure 3(a) shows the measured MSD as a function of lag time of water and glycerol. The slope of the curves of figure 3(a) confirms that the media is pure viscous (Apgar *et al* 2000; Breedveld and Pine 2003). Note that we have omitted the data of MSD below $0.006 \mu\text{m}^2$, the operational limit of our setup while calculating the different moduli. This limit is bearing in mind the reasons explained in the earlier section. This is indicated in figure 3b, through a dotted line, and the data below the line should be ignored. Omission of this data limits the upper frequency value of the measured rheological parameters for glycerol. These MSD values are used to calculate the complex shear moduli by (2) for the two fluids. The storage, $G'(\omega)$ and loss moduli, $G''(\omega)$, are obtained by (3) by polynomial fitting the $G^*(\omega)$ data to a Gaussian with a width of 0.07. The $G'(\omega)$ and $G''(\omega)$ are clipped at 0.03 times $G^*(\omega)$ as they are almost certainly meaningless below that point unless the data is extremely clean (Crocker and Grier 1996; Dasgupta *et al* 2002). The storage moduli are zero, for this reason, at all frequencies as is expected for pure viscous materials. The loss moduli coincide with complex shear moduli (not shown in the plot for clarity) for both the fluids, at all frequencies. The loss modulus grows linearly with frequency obeying the relation (Mason 2000),

Figure 4. (a) MSD (squares) of beads in 0.15%, 0.50% and 1% RSF and (b) storage (stars) and loss moduli (triangles) of 1%, 0.50% and 0.15% (w/v) RSF concentrations.

$$G''(\omega) = \eta\omega. \quad (4)$$

The viscosity ' η ' of the medium can be calculated by (4). The frequency dependence of viscosities of water and glycerol are shown in figure 3(b) along with their complex shear moduli. The viscosities are constant at all measured frequencies and found to cross the complex shear modulus at 1 Hz for both the fluids as expected from (4). Mean viscosity of water and glycerol are 1.08 ± 0.04 mPas and 0.94 ± 0.06 Pas, respectively and these are in agreement with the standard values at 22°C. These measurements serve to calibrate our setup.

The lower and upper limits of shear modulus which could be measured with our set up are $\sim 5 \times 10^{-4}$ Pa and 1 Pa, respectively as seen in figure 3(b). Since our camera has a fixed frame rate of 15 Hz at full resolution, further increase in the upper limit of the shear modulus value measured is not possible with the current spatial resolution of our setup. Although it is possible to increase the frame rate of our camera to 30 fps, this results in a trade off in the spatial resolution of the imaging system due to smaller pixel density and also due to a lesser number of photons seen by the camera for shorter exposures (Neuman and Block 2004). In principle, with the use of a camera

of high spatial and temporal resolution, this measurement limit can be extended further.

4.2 Regenerated silk fibroin solutions

We have used this calibrated setup to measure the rheological properties of viscoelastic material such as RSF at different concentrations. We have added 0.989 microns beads in low concentrations to RSF samples and tracked the bead positions in these materials for nearly 1000 images with 15 frames/s. All these measurements were carried out at 22°C. Ensemble averaged MSD of 3 RSF concentrations are determined using (2) and is shown in figure 4a. The curves in this plot show different slopes in different regions varying between 0 and 1. Initially the curves show small increment with lag time and later show large increment approaching the slope of 1 at higher lag times. By using (2) and (3), we have obtained storage and loss moduli of three RSF concentrations. Figure 4b shows the storage and loss moduli of RSF for three concentrations. Note that the code (Crocker and Grier 1996) used to obtain storage and loss moduli by the analytical continuation method (Dasgupta *et al* 2002) clip these curves at

Cross over Frequencies of 3 RSF Samples

Figure 5. (a) Storage and loss moduli of three RSF concentrations (same data as in figure 4b) after enlargement and (b) cross over frequency increases slightly with concentration of RSF with a slope of 0.16.

Figure 6. Trajectory of a bead in 1% RSF, used to estimate the mesh size of a RSF polymer network.

lower frequencies where higher curvature is displayed. This limits the low frequency values of these parameters. The moduli measured for the three RSF samples are well within this measurable limit of our setup. The magnitudes of storage and loss moduli are found to increase with increase in concentration at all frequencies. At small lag times the loss moduli is greater compared to the storage moduli, for the three concentrations. This is a signature of liquid like behaviour of the sample. The curves are similar to that observed in rheometry measurements (Chen *et al* 2001) in the low frequencies, but show a small decrease

in the slope of loss modulus (open triangles in figure 4) at higher frequencies. Upper frequency measurement restrictions preclude us from comparing our data with that acquired using a rheometer to frequencies above 2.25 rad/s. Elastic moduli become dominant at higher frequencies (above 0.5 rad/s) suggesting a solid-like behaviour of the sample. This crossover was observed for all the three measured RSF concentrations. The RSF solution plots are magnified and replotted (figure 5) in linear scale to obtain the crossover frequency values for the three concentrations. This cross over frequency is found to increase with increase in concentration of RSF sample with a small slope of 0.16. To make an estimate of the mesh size in 1% RSF we have plotted in figure 6, trajectory of a bead showing restricted motion in that sample and it was found to be nearly 200 nm wide. Average mesh size of 4 such beads in 1% RSF was calculated and found to be nearly 250 nm. This is less than the bead size (1 micron) used in the analysis and the use of the expressions derived from the assumptions of continuum viscoelasticity is reasonably justified.

Next, we examine the distribution in MSD of a set of beads in 1% RSF solution by calculating the percentage coefficient of variation (% CV) of MSD at different lag times. This is plotted in figure 7. Percentage CV of MSD at a given lag time is the percentage ratio of standard deviation of MSD of beads to the square root of mean of MSD at that time. At small lag times (figure 7) %CV of MSD in 1% RSF remains almost constant and represents uniform distributions of MSD. After a lag time of 1 s, the % CV starts to increase initially slowly but later steeply

Figure 7. Percentage coefficient of variation (%CV) of MSD in 1% RSF (filled triangle) and that of water (open triangle) as a function of lag time (Inset: bar plot of MSD distribution in 1% RSF at 50 s lag time shows two distinct families of beads, one with higher MSD and another, with lower MSD).

with lag time with a slope of nearly 1. This increment in % CV of MSD at higher lag times indicates a large distribution of MSD, signifying heterogeneity in 1% RSF. For comparison, % CV of MSD in water, a Newtonian fluid, is also shown in the same plot, wherein the curve remains almost constant, except a small rise at higher lag times partly due to dispersion in MSD values and partly due to the inherent noise in the system. The inset in figure 7 shows the bar plot of MSD values of 18 beads in 1% RSF at a lag time of 50 s. Though the numbering of particles shown in the plot is arbitrary, one can clearly distinguish a family of beads with $\text{MSD} < 1 \mu\text{m}^2$ and another, with $\text{MSD} > 1 \mu\text{m}^2$ at a lag time of 50 s. This serves to display the structural heterogeneity in the RSF solution. Apgar *et al* (2000) identified the presence of inhomogeneity in *F* actin network as defects due to the presence of small and large pores within the sample. We expect a similar reason, i.e. the presence of pores of different sizes, for the structural inhomogeneity displayed in RSF.

5. Conclusion

The results demonstrate that video microscopy is a powerful technique to characterize the rheology at micro and nano levels and with micro volumes of the test sample. With the improved camera speeds (say 100–1000 Hz) and with higher spatial resolution, we can, in principle, make measurements at even reduced length scales with the same setup. Through video microscopic technique, we have characterized the RSF polymer solution for a small

frequency range limited by the camera frame rate and imaging system. We have identified the presence of structural heterogeneity in 1% RSF solution. Further studies, in progress, are necessary to quantify the heterogeneity of RSF solution and to study RSF rheology in various physical and chemical conditions.

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