Published in final edited form as:

Ann Biomed Eng. 2012 August ; 40(8): 1654–1665. doi:10.1007/s10439-012-0532-5.

Eccentric rheometry for viscoelastic characterization of small, soft, anisotropic, and irregularly shaped biopolymer gels and tissue biopsies

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Abstract

Quantification of the physical properties of tissue biopsies and cell-remodeled hydrogels is critical for understanding tissue development and pathophysiological tissue remodeling. However, due to the low modulus, small size, irregular shape, and anisotropy of samples from these materials, accurate viscoelastic characterization using standard rheometric methods is problematic. The goal of this work is to utilize image analysis to extend rotational rheometry to these samples. In this method, the sample is offset to increase the torque generated; a custom clear glass geometry, right angle prism, and camera are used to determine the exact shape and location of the sample relative to the axis of rotation for calculation of the sample shear modulus, G' . Values of G' for standard polydimethylsiloxane gels tested in centered and eccentric configurations were not statistically different (respectively 137 ± 37 kPa and 126 ± 8 kPa, $p = 0.58$), indicating accuracy of the method. Additionally, G' values from circular and irregularly shaped collagen gels yielded equivalent results (31 ± 1.8 Pa and 31 ± 5.1 Pa, $p = 0.29$). A blood clot and a lipid plaque sample recovered from human patients ($G' \sim 4$ kPa) were successfully tested with this method, demonstrating applicability to clinical diagnostics.

Keywords

rheometry; viscoelasticity; stiffness; anisotropy; collagen gel; tissue biopsy

INTRODUCTION

Quantification of the viscoelastic properties of soft tissues and fibrous protein gels can provide insight into the physiological process of tissue development, wound healing, and disease pathogenesis⁹. It is becoming increasingly clear that the stiffness of the extracellular matrix (ECM) regulates cell differentiation^{3, 11}, and may modulate cell responses between normal and pathological phenotypes^{9, 14}. Furthermore, changes in matrix viscous properties, as quantified by the loss modulus, may affect cell morphology, proliferation, and differentiation potential¹. There is also evidence that cell behaviors, such as reorientation to stretch, are strain rate dependent⁸. In addition to matrix regulation of cell activity, the cells

also actively alter the mechanical properties of their ECM environment. The stiffness and viscosity of tissues evolve during growth, adaptation to new loading patterns, and pathological remodeling (such as the remodeling associated with atherosclerosis). This dynamic reciprocity between the cells and ECM is often studied using biopolymer gels (collagen, fibrin, Matrigel™) which serve as simple tissue models^{5, 16}. The cell behavior varies considerably in these fibrous hydrogels depending upon culture conditions and duration; yet changes in the viscoelastic properties of the matrix during this remodeling are rarely reported due to the difficulty of mechanical characterization of these small, soft gels¹⁷. Biopolymer gels are also used as scaffolds for engineered tissues, such as dermal component of Apligraf® skin substitute (Organogenesis Inc, Canton, MA). Characterizing the evolving mechanics of these tissue surrogates is essential for monitoring quality control of the engineered tissues and understanding the remodeling associated with wound healing.

Soft tissue biopsies, discarded tissues from surgery, and cell-remodeled protein gels are extremely difficult to characterize mechanically. These samples are often small, soft, anisotropic, and irregularly shaped. Uniaxial methods of testing require gripping the samples, which due to small size and low stiffness, can be problematic. Trimming irregular samples to a “dogbone shape” (as in common practice for uniaxial testing) wastes valuable material. Additionally, uniaxial methods of testing allow for testing along only one axis, preventing the assessment of anisotropy. Custom methods have been developed for testing small gels, such as the laser-trap¹⁵, optical tweezers¹², and magnetic bead twisting rheometry¹³; however, these techniques are highly specialized with equipment that is not generally accessible.

Rotational (shear) rheometry is a widely used method (common in both academic and industry labs) for viscoelastic characterization of gels. Commercial rheometers are highly sensitive and have large dynamic torque ranges, with many spanning over seven orders of magnitude. Technologies implemented in these machines, such as powerful new algorithms and magnetic bearings (which reduce friction), expand the range of samples that can be tested to include soft samples, such as viscoelastic polymer systems.

Although rheometry is widely used, rheometry of tissue samples and biopsies is not common in the literature, in part due to the large circular sample size required to be compatible with parallel-plate geometries that are available commercially. For example, Nasser and colleagues¹⁷ noted the difficulty of finding homogenous portions of swine kidney cortex large enough to completely fill the area required by their test geometry of diameter (ϕ) 25 mm. A seemingly simple solution would be to use a smaller geometry such as an ϕ 8 mm geometry available commercially. However for small soft tissue samples, the low torque generated often falls below the recommended resolution limit of the machine necessary for robust estimation of the shear moduli. For example, at a given level of applied strain, an ϕ 8 mm sample will generate only 3% of the torque generated by a ϕ 25 mm sample of the same material due to the torque being proportional to the third power of that radius. For this reason, research groups, such as Holt *et al.*⁶, reported difficulty in testing ϕ 6 mm skin punches. In addition, restricting samples to a circular shape also requires trimming the sample, which wastes valuable testing material. Thus, although rheometers are ideal for monitoring the evolution of viscoelastic properties of biopolymer solutions and gels during polymerization, traditional rheometry is not well suited for the testing of small, soft, and irregularly shaped tissues.

The goal of this work is to extend the utility of standard rotational rheometers for accurate and sensitive viscoelastic characterization of small, soft tissue biopsies and pre-polymerized gel samples. As torque increases linearly with distance away from axis of rotation, our approach is to locate the sample at a radial distance away from the axis of rotation, thus

increasing the amount of torque generated by the sample. A custom clear glass geometry, right angle prism, and high resolution camera are used to accurately determine the shape, location, and size of sample for straight-forward complex shear modulus calculation. As shear testing can be done non-destructively, multiple tests on a single sample placed at different orientations relative to the axis of rotation can be completed, thereby facilitating the assessment of anisotropy. Furthermore, the small sample size relative to that of the geometry creates a more uniform strain field than that of traditional parallel-plate rheometry, a feature which may allow for assessment of the nonlinear properties of gel and tissue biopsies. The validation of the novel method, as well as possible extensions and applications to other research areas, are discussed.

METHODS

Approach

For traditional rheometry using parallel-plates, the sample is centered and fills the total volume created by the geometry of radius R_g and the gap height, h , as depicted in Figure 1a. Our approach is to offset the sample radially to increase the measured torque to values above the noise levels of commercial rheometers and to image the surface area of the sample to obtain the shape, size, and position of the sample, as shown in Figure 1b. The torque, T , applied by a rotational rheometer is counteracted by the shear stress, τ , at each point on the surface of the sample of area A , with the following relationship:

$$T = \int_A \rho \tau(\rho) dA \quad (1)$$

where ρ is the radius from the axis of rotation to an incremental area dA . Therefore, portions of the sample which are located further from the axis of rotation yield a higher torque at a given shear stress, and contribute more to the total torque of the sample; material near the rotation axis contributes relatively little to the total torque measured.

Assuming a linear viscoelastic material, the shear stress is proportionally related to the shear strain as depicted in Figure 1a:

$$\tau(\rho) = G^* \gamma(\rho) \quad (2)$$

For a parallel-plate geometry shear strain is related to the radius, ρ , gap height, h , and rotation angle, θ , by the following equation:

$$\gamma(\rho) = \frac{\rho \theta}{h} \quad (3)$$

As can be seen from Eqn. 3, the strain is zero at the axis of rotation and maximum at the outer edge of the sample. Sample strain values reported herein refer to the maximum strain (at the outer edge of the sample). The complex modulus, G^* , of the sample is related to torque and rotation angle, θ , by the equation,

$$T = G^* \frac{\theta}{h} \int_A \rho^2 dA \quad (4)$$

The integral is equivalent to the polar moment of inertia, J , of the sample's projected surface area, which simplifies Eqn. 4 to the following form:

$$T = G^* \frac{\theta}{h} J \quad (5)$$

For a circular sample of radius, r , undergoing a rotation about its center, the polar moment of inertia is:

$$J_0 = \pi \frac{r^4}{2} \quad (6)$$

By the parallel axis theorem, for a sample offset a distance d from the axis of rotation (as depicted in Figure 1b) the polar moment of inertia is:

$$J = J_0 + d^2 A \quad (7)$$

where J_0 is the polar moment of inertia of the circular sample about its centroid, and A is the projected area of the sample. For the case of a circular sample of radius, r , with its center at distance d from the axis of rotation, the torque-angle relationship is:

$$T(d) = G^* \frac{\theta}{h} \left(\frac{\pi}{2} r^4 + d^2 (\pi r^2) \right) \quad (8)$$

The full integral derivations of Eqn. 8 are provided in the Appendix A. An example of the torque increase (for a given angular displacement) obtained when an $\phi 8$ mm sample is offset from 0 to 28 mm is shown in Figure 1c.

Implementation

To implement the offset method, we manufactured a transparent circular geometry from a $\phi 64$ mm glass slide which allows direct visualization of the sample, and attached the slide to an aluminum rod that interfaces with a standard rotational rheometer (AR-G2, TA instruments, New Castle, DE). The glass slide ($\phi 64$ mm) was etched using dilute hydrofluoric acid (Armour Etch, Armour Products, Hawthorne, New Jersey) for 24 hours to ensure adequate friction between geometry and sample to reduce slippage during testing. The rheometer attachment was created out of aluminum with a low mass moment of inertia to minimize the impact on allowable testing frequency. A custom aluminum jig was created which allowed for the etched glass slide to be glued to the center of the aluminum rheometer attachment. The glass was affixed to the aluminum attachment with epoxy glue.

To calculate sample inertia, J_s , the position, size, and shape of the sample must be accurately determined. A combination of an orthogonal prism and high resolution camera were used to capture the image of the projected surface of the sample and the edge of the geometry as shown in Figure 2b. After rheological measurements, the prism was placed just above (without touching) the clear glass geometry using a custom jig. A ring flash (Macro Ring Lite MR-14EX, Canon) was used to provide even lighting. To ensure that the camera and prism remained orthogonal to one another during image acquisition, an alignment jig was created. A custom MATLAB (Mathworks, Natick, MA) program was created for analysis of the image of the sample and geometry. The image was thresholded and converted to binary with the sample becoming white (value of one) and all else black (value of zero) as shown in Figure 3. Sample inertia, J_s is calculated as the sum of each pixel multiplied by the square of distance from the axis of rotation to that pixel.

To calculate G_s^* , the modulus value output from the rheometer analysis software, G_{rheo}^* is multiplied by a correction factor, b . The correction factor b is the ratio of J_s and J_{rheo} , the polar moment of inertia that the sample would have if it filled the entire area under the geometry. It is obtained by rearranging the terms in Eqn. 5:

$$\frac{T}{\theta} h = G_{rheo}^* J_{rheo} = G_s^* J_s \quad (9)$$

Further rearrangement yields:

$$G_s^* = \frac{J_{rheo}}{J_s} G_{rheo}^* = b G_{rheo}^* \quad (10)$$

J_{rheo} is calculated using Eqn. 6 with $r = R_g$ where R_g is calculated by fitting five user-selected points on the edge of the geometry in image described above (prethresholding) to the equation for a circle. All parameters (geometry radius, sample dimensions, etc.) are computed in pixels. No conversion to engineering units is needed since the correction factor is a unitless ratio.

G_{rheo}^* is utilized in our calculations rather than raw torque and rotation angle data to take advantage of the powerful algorithms utilized by the TA-instrument software which filter noise and correct for the mass moment of inertia during oscillation. This step simplifies the analysis for easy implementation and is valid if the strain applied is within the linear region of the sample's stress-strain curve. However, standard commercial rheometry software does not incorporate higher order terms when fitting the T - θ data (e.g., utilizing Fourier analysis), thus nonlinear material properties may be obscured (see Discussion). A stress controlled rheometer was used for all experiments; test protocols were all stress sweeps of increasing nominal stress (defined below) every three to five cycles at a constant frequency of 6.28 rad/s (1 Hz). The test was set to terminate at 15% strain which is beyond the strain at which the samples slipped (generally in the range of 5-10% strain). The stress-controlled oscillatory stress sweep protocol went from a very low stress (where noisy data was obtained as determined by sine wave quality) to a level above which the sample slipped. Sample slippage was observed when there was a sudden decrease in G' and a corresponding increase in strain. The G' and G'' values were stable over a wide range of strain values from at least 0.3 to 3% applied maximum strain, indicating linear viscoelastic behavior and lack of slipping within this range. The values reported in this paper correspond to ~1% strain for all samples.

A small offset sample in eccentric rheometry experiences much higher stress levels than output by the rheometer since the calculation in the commercial software assumes that the sample fills the entire space created by the parallel-plate geometry and peltier plate. The actual (maximum) shear stress applied to the outer portion of the sample, τ_s , is larger than nominal stress (τ_{nom}) set in the protocol of the rheometer control software as described below.

The (maximum) shear stress, τ_{max} , applied to a sample is related to the torque by Eqn. 11:

$$T = \int \left(\frac{\rho}{R_s} \tau_{max} \right) \rho dA = \frac{\tau_{max}}{R_s} \int \rho^2 dA = \tau_{max} \frac{J_s}{R_s}, \quad (11)$$

where R_s is the distance to the outer edge of the sample (not to be confused with the sample radius, r , as in the case of a circular sample); J/R is often referred to as the "shear stress factor." As the calculation of τ_{max} is valid for any sample, regardless of offset location or

size, τ_s can be obtained from the nominal stress, τ_{nom} , calculated by the rheometer software (which assumes the sample fills the gap under the entire geometry) with the following equation:

$$\tau_s = \frac{\tau_{nom} J_{rheo}}{R_g} \frac{R_s}{J_s} = \tau_{nom} b \frac{R_s}{R_g}, \quad (12)$$

where R_s and R_g are the distances to the outer edge of the sample and the radius of the geometry, respectively. For a sample offset to the edge of the geometry, the ratio of R_s to R_g is equal to 1. Protocols herein are reported in terms of the nominal stress value.

Similar to Eqn. 11, sample torque, T_s , at a given strain can be calculated by:

$$T_s = G^* \gamma \frac{J_s}{R_s} \quad (13)$$

where J_s is the polar moment of inertia of the offset sample, γ is the strain, and R_s distance from the axis of rotation to the outer radius of the sample.

Thus the equivalent torque of a centered sample at a given strain can be computed from the torque of an offset sample by taking the ratio of sample torque at center to sample torque at edge and rearranging terms to yield:

$$T_c = T_s \left(\frac{J_c}{J_s} \right) \left(\frac{R_s}{R_c} \right) \quad (14)$$

Where J_c is the polar moment of inertia of the centered sample, and R_c is the radius of a centered sample.

Equivalent centered sample torques were calculated for 1% strain to quantify the benefit of the offset method. For rectangular samples and irregularly shaped samples, equivalent centered sample torque was estimated using the R_{max} in Eqn. 14 (R_{max} being defined as the distance from the axis of rotation to the farthest point on the edge of the sample).

Gap height was set to be sample specific. The normal force created by the geometry was noted and ~1 mN for all samples tested.

Validation

Accuracy—To validate the accuracy of the method, the shear moduli of small elastomeric discs were tested both with the traditional centered configuration and with the offset method. To test small samples in the centered configuration, a $\phi 12.7$ mm geometry was custom manufactured. Polydimethylsiloxane (PDMS, Sylgard Silicone Elastomer Kit, Dow Corning) was chosen as the test material as it can be made sufficiently stiff such that small, centered samples generate enough torque to provide an adequate signal-to-noise ratio. PDMS was prepared in a 20:1 ratio of silicone base to curing agent. The mixture was poured into a petri dish until the depth was approximately 2 mm. Vacuum was used to remove any bubbles that may have formed in the gel. The petri dish was then placed in an oven at 60°C and the solution was allowed to polymerize for 24 hours. Four $\phi 12.7$ mm samples were cut from the PDMS. Each centered sample was tested with a nominal stress sweep from 100 Pa to 2500 Pa using a $\phi 12.7$ mm geometry; offset samples were tested with a nominal stress sweep from 10 Pa to 200 Pa using a $\phi 64$ mm geometry. Samples were tested at 25°C at 1 Hz. G' and G'' values at ~1% strain are reported.

Effect of location and shape on accuracy—To test if the calculated shear moduli are independent of radial offset distance and to demonstrate the applicability of this method to non-standard specimens, $\phi 15$ mm circular and irregularly shaped collagen gel samples were created. Collagen gels of 2.4 mg/ml were prepared in the following manner: 0.8 ml of 3 mg/ml acid-extracted rat tail (RTT) collagen was neutralized with 40 μ L of 0.1M Sodium Hydroxide (NaOH) and 200 μ L of 5x Dulbecco's Modified Eagle Medium (DMEM). The solution was mixed via a syringe and pipetted into a well of a 24-well plate and allowed to polymerize at room temperature for 1 hour. The gels were removed from the wells and placed on the rheometer under the clear $\phi 64$ mm geometry. The samples were tested at three locations (center, middle, and edge of the geometry) to assess the effect of location on G' and G'' . To create irregular shapes, the circular samples were halved into two semi circles, which were arranged form an oblong shape. The samples were tested using an oscillatory stress-controlled sweep with nominal stresses ranging from 0.01 Pa to 100 Pa, three points per decade, gap height of 2000 μ m for each sample. Samples were tested at 25°C at 1 Hz.

Sensitivity—With decreasing sample size, the torque generated by a sample at a given rotation angle decreases, the variation in measured G' increases and, eventually, the signal-to-noise becomes too low to obtain meaningful G^* values. To determine the sensitivity of the offset method, soft acellular collagen gel punches of decreasing diameter were tested (collagen polymerization process described in previous section). Collagen gel punches of sizes $\phi 8$ mm, $\phi 7$ mm, $\phi 5$ mm, and $\phi 4$ mm were tested with an oscillatory shear stress sweep with nominal stress ranging from 0.01 Pa to 100 Pa, five points per decade, and a gap height of 500 μ m for all samples. Each sample underwent four consecutive tests, with $n = 4$ in each category. Samples were tested at 25°C. Collagen gels were used for this analysis as they are much softer than PDMS, resulting in lower torque values for a given sample size, and thus represent a more difficult test case. Samples were tested at 25°C at 1 Hz.

Applicability to cellular remodeled gels—To show the versatility of this method, gels with free boundaries and anchored (fixed) boundaries were created. Human lung fibroblasts (passage 15) at a density of 1×10^6 cells/ml were seeded into a 1.6 mg/ml RTT collagen gel (from a 2 mg/ml stock concentration). The solution was pipetted into a 12-well plate. After 1 hour of polymerization, a needle was used to detach the collagen from the sides of the well ($n = 5$). The anchored boundary gels were left undisturbed ($n = 6$). The gels with both the free boundary and the fixed boundary were allowed to incubate for 36 hours at 37°C prior to testing with an oscillatory shear stress sweep with nominal stresses ranging from 0.01 Pa to 100 Pa, five points per decade, and a gap height of 800 μ m for all samples. Samples were tested at 25°C at 1 Hz.

Anisotropy—An anisotropic cellular remodeled gel was tested in two orthogonal orientations relative to the axis of rotation to demonstrate the ability to detect subtle sample anisotropy using eccentric rheometry. The procedure for creation of the cell populated gel is as follows: Normal Human Fibroblasts (passage 15) were seeded at a density of 1×10^6 cells/ml into a 1.6 mg/ml RTT collagen gel (from a 2 mg/ml stock concentration) having free (collagen gel removed following polymerization) and fixed (gel allowed to polymerize against nylon) boundary conditions. The cells were cultured for 72 hours before the gel was removed from the well and tested at the edge of the glass geometry, both parallel and perpendicular to the direction of uniaxial constraint, with an oscillatory shear stress sweep with nominal stresses ranging from 0.01 Pa to 100 Pa, five points per decade, and a gap height of 300 μ m for both orientations of test. Samples were tested at 25°C at 1 Hz.

Extension to pathological tissue biopsies—Blood clot models (generously provided by the Gounis lab, University of Massachusetts Medical School) were tested with this

method to demonstrate applicability to other research areas. Additionally, to demonstrate the ability to physically characterize tissue biopsies, a lipid plaque and a blood clot were obtained from University of Massachusetts Medical School. All human specimens were collected with approval from Institutional Review Board at the University of Massachusetts Medical School. The lipid plaque was removed from a 64 y.o. male with a history of cardiovascular disease (CVD) presenting with hypertension, hyperlipidemia, insulin-dependent diabetes mellitus, peripheral vascular disease, chronic kidney disease, and obesity. The blood clot was removed from a 65 y.o. woman with CVD presenting with hypertension, hyperlipidemia, and cerebrovascular disease. Both specimens were placed in embedding medium, frozen, and stored in -80°C to prevent ice crystals from forming and damaging the specimens. Samples were thawed to 25°C while being soaked in phosphate buffered saline (PBS). The arteries were splayed open using a scalpel while being restrained with forceps. The scalpel was used to excise the clot and it was placed on the rheometer near the edge of the glass geometry. The geometry was lowered until the normal force was approximately 0.1 N. The lipid plaque was extracted and prepared for testing in a similar manner. Each sample was tested with an oscillatory shear stress sweep with nominal stress range from 0.01 Pa to 1000 Pa at 1 Hz with three points per decade. Samples were tested at 37°C .

Statistics

Two tailed equal variance student t-test were conducted in Microsoft Excel and Sigma Plot for statistical comparison between the samples used in the accuracy, sensitivity, effect of location and shape, and cellular remodeled gels experiments. Certain experiments were done solely to demonstrate applicability. Due to low numbers, statistics were not done on the sample used in the anisotropy experiment, as well as the lipid plaque and blood clot obtained from human patients.

RESULTS

All samples exhibited stable G' and G'' values in the range of 0.3% to 3% strain. For consistency, all values reported herein correspond to approximately 1% maximum shear strain (at the outer edge of the sample).

Accuracy

Eccentric rheometry and traditional centered rheometry yielded G' values for PDMS samples that were not significantly different G' (126 ± 8.0 kPa and 137 ± 37 kPa, respectively; $p = 0.58$). Further, the variability of G' values obtained with the offset method is lower than those obtained with centered configuration indicating a better signal-to-noise ratio (SNR) with the offset configuration (Figure 4a). Collagen gel samples placed at different radial offsets (20 mm and 24.5 mm) did not produce statistically different values for G' (31.0 ± 3.5 Pa and 31.0 ± 5.1 Pa, respectively; $p = 0.29$) (Figure 4b). Further, irregularly shaped samples having the same area as circular samples returned similar values for G' as the circular samples (31.7 ± 1.3 Pa and 31.0 ± 5.1 respectively; $p = 0.74$). Additionally, image analysis was found to add less than 5% error (see error analysis in Appendix B).

Sensitivity

Smaller samples generated lower torques for a given angular displacement due to having less area. The method was sensitive enough to accurately measure the shear moduli of very small ($\phi 4$ mm, 500 μm thick), very soft collagen gel samples when tested with an offset of 25 mm. As expected, with smaller sample size the variability increased (COV of 9%, 10%, and 16% for the $\phi 8$ mm, $\phi 7$ mm, $\phi 4$ mm collagen gel samples, respectively); although, the

average measurements were not statistically different between samples of diameters ranging from 4 mm to 8 mm, as shown in Figure 4c.

Cell-remodeled gels

Cell-remodeled gels cultured with free and anchored boundary conditions yielded G' values of 131 ± 24 Pa and 99 ± 15 Pa respectively, although the differences were not statistically significant ($p = 0.07$). Results displayed in Table 1.

Anisotropy

An anisotropy ratio of 1.25 was calculated from the G' values determined for the cell-populated collagen gel sample that was cultured uniaxially constrained then tested both parallel and perpendicular to the direction of the fixed boundary (225 Pa and 180 Pa, respectively).

Pathological tissue biopsies

Blood clot models yielded G' of 369 ± 128 Pa, whereas the human blood clot and lipid plaque yielded much higher G' values of 4036 Pa and 4037 Pa, respectively. See Table 1 for complete results of all applicability experiments.

Discussion

In this work, we demonstrate the development and validation of an image-based modification to standard parallel-plate rotational rheometry which allows for the measurement of the viscoelastic properties of small, soft, irregularly shaped, anisotropic samples. Accuracy of the method was verified by comparing results from small centered and offset samples. We demonstrated that the method is sensitive enough to accurately measure the shear moduli of very small ($\phi 4$ mm), very soft ($G' \sim 100$ Pa) samples when tested with an offset of 25 mm; this measurement could not be accomplished with the standard centered method due to low signal-to-noise ratio. The ability to accurately characterize irregularly shaped samples was also verified, and the moderate anisotropy was able to be detected by altering the orientation of the gel relative to the axes of the test geometry. It should be mentioned that for anisotropic samples, rotational rheometry will not shear all samples perpendicular/parallel to the predominant fiber direction unless sample dimensions are small relative to offset distance. Following this validation, we were able to successfully characterize the viscoelastic properties of a range of samples including cellular remodeled gels, model blood clots, a human vascular plaque, and a human blood clot. Our results demonstrate that this method has broad applicability for viscoelastic characterization of soft, irregular gels and can be readily implemented with a standard rheometer, digital camera, and prism.

For the samples tested in this study, the offset configuration yielded 10- to 150-fold increase in torque relative to the centered configuration (estimated by Eqn. 8) as shown in Table 1. This substantial increase in torque is necessary for accurate characterization of small, soft samples. For example, the sample torque from the $\phi 4$ mm collagen gel was estimated to be only 0.01 μNm in the centered configuration at $\sim 1\%$ strain, whereas it was 1.28 μNm in the offset configuration. Although the reported minimum oscillatory torque for the AR-G2 rheometer used in these experiments (which has an advanced magnetic bearing) is 0.003 μNm , a minimum torque of 0.1 μNm is usually required for accurate results (personal correspondence with TA engineers). In our experience, greater than 1 μNm of sample torque at 1% applied strain is needed for reproducible measurements; for example, the $\phi 4$ mm collagen gel (CG) sample could only be reliably measured in the fully offset configuration, not at smaller distances from the center, and the $\phi 15$ mm CG samples did not provide

reliable data when tested in the centered configuration (sample torque $\sim 0.32 \mu\text{Nm}$). These values are also proportionally less at lower applied strain levels, such as 0.3% strain where we were able to obtain similar shear moduli values. Further, for robust analysis, the entire cycle of torque-angle vs. time are analyzed by the rheometer software for the estimation of the complex shear modulus, thus accurate intermediate torque values (at lower than peak strain) are required. The sample torques reported in Table 1 are the calculated maximum torques generated by the samples at the peak of the oscillatory loading cycle.

Eccentric rheometry has previously been used for measuring the shear moduli of both muscle²⁰ and brain tissue⁷. Offsetting the samples was necessary due to insufficient torque resolution obtained for centered specimens. The anisotropy of the brain tissue was also assessed by testing offset samples at different orientations relative to the machine axes⁷. However, in these prior studies, image analysis was not used to determine sample shape; the slight change of shape due to lateral expansion that occurs when the normal force is applied to a soft sample leads to an overestimation of sample stiffness if it is not accurately taken into account by direct visualization. The use of image analysis also facilitates the testing of irregularly shaped samples, such as those of tissue biopsies and discarded clinical tissue samples where shape is not controllable. In addition, this feature allows for samples to be collected in their natural state, where shapes might provide evidence to degree of pathology. Additionally, material is not wasted due to trimming for a certain size as required for previously described methods. One of the challenges of testing irregularly shaped samples is the possible problem of edge effects, a concern in all mechanical testing. The properties of the sample are often different at the edge than in the bulk due to drying, over hydrating, skin formation during polymerization, and other surface effects. As the edge increases relative to the bulk, measurements could reflect this change. The irregularly shaped collagen gels tested in this method, first as a circle and as an oblong shape, yielded comparable results for G' and G'' in both configurations indicating that this method can be used with most irregularly shaped samples.

Here we demonstrate that the correction factor, b , utilized for converting between the rheometer-software-calculated modulus (G^*_{rheo}) and the sample modulus (G^*_{sample}) is simply the ratio of the theoretical polar moment of inertia of the sample if it filled the entire area under the geometry and the true polar moment of inertia determined directly by image analysis. The maximum stress applied to the sample is also related to the nominal stress by the same correction factor when the sample is placed at the edge (Eqn. 12). In prior studies utilizing eccentric rheometry, the derivation of the shear stress is complex and contains mathematical errors that lead to inaccurate results²⁰. Our derivation has been verified by full integration for the case of a circular sample (see Appendix A).

Errors in the image analysis measurements used in the calculation of the correction factor b add to the instrument error; however, the increase in torque provided by offsetting the sample more than compensates for this error for soft samples (see Appendix B for error analysis). For example, the coefficient of variance was lower in the offset configuration (6%) compared to the centered configuration (27%) for the same size sample of PDMS. However, the offset, d , cannot be increased indiscriminately since an increase in R_g comes at the expense of a concomitant increase in mass moment of inertia of the geometry ($I_z = \frac{1}{2} \text{mass} \cdot R_g^2$ for a thin disk rotated about its center) which must be compensated by the rheometer motor and software and limits the frequency response of the device.

Values reported herein for G' of acellular collagen gels are comparable to values found in the literature^{16, 21}, which are dependent on polymerization conditions such as temperature and pH^{4, 12, 19}. The concentration is within the range commonly utilized (0.3 to 30 mg/ml)¹⁶; however, most previous studies utilize pepsin-extracted collagen which forms less

stiff gels at a given concentration than the acid-extracted (telopeptide-rich) collagen used in this study. Unlike most rheometric analyses of collagen gels, to simulate the sample conditions for our long-term culture experiments, the gels tested in this device were created outside the rheometer (pre-polymerized) rather than polymerized between the geometry and the peltier plate (base plate that is capable of temperature control). As these samples have a constant thickness, they must be tested using a parallel-plate geometry which does not have a uniform strain field as would be generated by a cone-and-plate geometry. However, a benefit of the eccentric method is that the strain field is more uniform than for a standard centered sample tested between the parallel plates. For example, for a $\phi 5$ mm sample with an offset of 25 mm from the axis of rotation, the strain varies by only 10% from the strain value at the center of the sample, compared to ranging from zero at the center to the maximum strain at the edge for a traditional centered sample. Despite the relative uniformity in strain of the eccentric method, nonlinearity of the samples cannot be accurately assessed using the G^* value supplied by standard rheometers due to the algorithm by which the data are analyzed (e.g., FFT analysis). For nonlinear analysis, the raw torque and angle data must be analyzed using an analytical framework which analyzes higher order Fourier coefficients such as Large Amplitude Oscillatory Shear (LAOS) analysis¹⁰. Further, without polymerization of the gel directly onto functionalized plates, the maximum strain we were able to obtain for most samples before slipping was $\sim 5\%$. To achieve this strain level, which is within the standard range reported for collagen gels in the literature¹⁸, the glass slide was etched using dilute hydrofluoric acid. Previous researchers have reduced slipping of biological samples by using sandpaper and glue; however, this solution is not ideal as this modification adds an unknown thickness to the sample making the gap height used in strain calculations inaccurate.

Rheometry of tissue biopsies and pre-formed/cell-remodeled biopolymer gels is not common in the literature in part due to the difficulty of testing the small samples¹². Tissue biopsies and remodeled gels are fragile and can be destroyed with incorrect testing conditions even with this method. Care must be taken during testing to ensure that the samples are tested in the linear region and do not experience high strains especially when testing small or soft samples. Further, testing of tissue samples and pre-cast gels requires a parallel-plate configuration so that even compression of the sample occurs when the normal force is applied; such geometries require that the sample have uniform thickness which necessitates some trimming of the samples. However, the amount of material that needs to be removed is much less than with other testing scenarios where a circular or rectangular shape is required.

In this work we developed and validated a new method for obtaining the viscoelastic properties of challenging samples such as those that are small, soft, irregularly shaped, and anisotropic. This method is potentially useful for many research areas, from tissue engineering to mechanobiology, and even clinical diagnostics where it may prove both useful and insightful to combine mechanical testing with cellular analysis for better understanding of both physiological and pathological processes. Prior to the development of this method, characterizing the mechanical properties of tissue biopsies and cellular remodeled gels was challenging and rarely undertaken. This method facilitates such characterization and is straightforward, easily implemented, and accessible to many researchers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Tom Jones for aid in rheological measurements and machining; Adriana Hera, John Meklenburg, and John Favreau for assistance with the Matlab code; Matthew Gounis, Ph.D., and Juyu Chueh (University of Massachusetts Medical School) for model thrombus and human plaque samples. This research was supported in part by NIH grant 1R15HL087257-01A2 to K.L.B.

Appendix A: Integral Derivation for a Circular Sample

The following is a derivation for torque for an offset circular sample. It is represent by a small circle of radius r_s , inside a larger circle of radius R_g offset a distance, d , from the axis of rotation, so that it lies along the edge of the larger circle. In the following equations, ρ is the distance from the axis of rotation to an incremental area dA , G is the elastic modulus, T is the torque, h is the gap height between the peltier plate and bottom surface of the geometry. Integrating over domain D which is a circle of radius r_s centered at $x = R_g - r_s (= d)$ and $y = 0$

$$T = \iint_D \rho \tau(\rho) dA \quad (A1)$$

Integration in Cartesian coordinates gives:

$$\rho \tau(\rho) = \frac{G\theta}{h} \rho^2 = \frac{G\theta}{h} (x^2 + y^2). \quad (A2)$$

Eqn. 1 from text becomes

$$T = \frac{G\theta}{h} \iint_D (x^2 + y^2) dy dx. \quad (A3)$$

Symmetric about x-axis allows for doubling of the integration over top of disc:

$$T = 2 \frac{G\theta}{h} \int_{R_g - 2r_s}^{R_g} \int_0^{y(x)^+} (x^2 + y^2) dy dx. \quad (A4)$$

Integration of Eqn. A4 with respect to y gives:

$$T = 2 \frac{G\theta}{h} \int_{R_g - 2r_s}^{R_g} x^2 y + \frac{1}{3} y^3 \Big|_0^{y+(x)} dx. \quad (A5)$$

$$T = 2 \frac{G\theta}{h} \int_{R_g - 2r_s}^{R_g} \left(x^2 + \frac{1}{3} \left(r_s^2 - (x - (R_g - r_s))^2 \right) \right) \sqrt{r_s^2 - (x - (R_g - r_s))^2} dx. \quad (A6)$$

Let $V = \frac{x}{r_s} - \left(\frac{R_g}{r_s} - 1 \right)$ and $dV = \frac{1}{r_s} dx$

Substitution of terms in Eqn. A6 gives:

$$T = 2 \frac{G\theta}{h} r_s^4 \int_{-1}^1 \left[\left(V + \left(\frac{R_g}{r_s} - 1 \right) \right)^2 + \frac{1}{3} (1 - V^2) \right] \sqrt{1 - V^2} dV. \quad (A7)$$

Expanding and collecting terms gives:

$$T = 2 \frac{G\theta}{h} r_s^4 \int_{-1}^1 \left[\frac{2}{3} V^2 + 2 \left(\frac{R_g}{r_s} - 1 \right) V + \left(\frac{R_g}{r_s} - 1 \right)^2 + \frac{1}{3} \right] \sqrt{1 - V^2} dV. \quad (\text{A8})$$

With further expansion, Eqn. A8 becomes:

$$T = 2 \frac{G\theta}{h} r_s^4 \left[\frac{2}{3} \int_{-1}^1 V^2 \sqrt{1 - V^2} dV + 2 \left(\frac{R_g}{r_s} - 1 \right) \int_{-1}^1 V \sqrt{1 - V^2} dV + \left(\left(\frac{R_g}{r_s} - 1 \right)^2 + \frac{1}{3} \right) \int_{-1}^1 \sqrt{1 - V^2} dV \right] \quad (\text{A9})$$

Which, upon integration yields:

$$T = 2 \frac{G\theta}{h} r_s^4 \left[\frac{2}{3} \frac{\pi}{8} + 2 \left(\frac{R_g}{r_s} - 1 \right) 0 + \left(\left(\frac{R_g}{r_s} - 1 \right)^2 + \frac{1}{3} \right) \frac{\pi}{2} \right]. \quad (\text{A10})$$

Let $r_s = r$, $d = R_g - r_s$, then $\frac{R_g}{r_s} - 1 = \frac{R_g - r_s}{r_s} = \frac{d}{r}$

Rewriting Eqn. A10 with the following substitutions gives:

$$T = \frac{2G\theta}{h} r^4 \left[\frac{2}{3} \frac{\pi}{8} + \frac{d^2}{r^2} \frac{\pi}{2} + \frac{1}{3} \frac{\pi}{2} \right]. \quad (\text{A11})$$

Multiplying and combining constants in Eqn. A11 gives:

$$T = \frac{G\theta}{h} r^4 \left[\frac{\pi}{2} + \frac{d^2}{r^2} \pi \right]. \quad (\text{A12})$$

Simplification of Eqn. A12 gives:

$$T = \frac{G\theta}{h} \left[\frac{\pi}{2} r^4 + d^2 \pi r^2 \right]. \quad (\text{A13})$$

Eqn. A13 is equivalent to Eqn. 8 in text.

Appendix B: Error Analysis

To estimate the error associated with image analysis, the derivative of the equation for b ($= J_{rheo}/J_{sample}$) was used:

$$\delta b = \sqrt{\left(\frac{\partial b}{\partial J_{rheo}} \delta J_{rheo} \right)^2 + \left(\frac{\partial b}{\partial J_s} \delta J_s \right)^2}. \quad (\text{B1})$$

For a theoretical perfectly circular sample of radius r_s at an offset, d , with $J_s = \pi r_s^4 + d^2 \pi r_s^2$ (Eqn. 8), and J_{rheo} given by Eqn. 6 with $R = R_g$, the relative error is given by:

$$\frac{\delta b}{b} = \sqrt{\left(4 \frac{\delta R_g}{R_g}\right)^2 + \left(\frac{4(r_s^2 + d^2)}{(r_s^2 + 2d^2)} \frac{\delta r_s}{r_s}\right)^2 + \left(\frac{4d^2}{(r_s^2 + 2d^2)} \frac{\delta d}{d}\right)^2}. \quad (\text{B2})$$

Repeated manual measurements on actual images of circular samples yielded 1% error for $\delta R_g/R_g$, $\delta r_s/r_s$, and $\delta d/d$ (e.g., ± 1 pixel for a 100 pixel distance). Eqn. B2 shows that the error associated with the image analysis depends upon the size of the sample and the degree of offset; it also highlights the impact of having the polar moment of inertia dependent upon the square of the offset and 4th power of the radii. For example, for a sample of radius 7.5 mm and offset 24.5 mm (at the edge), with 1% error for R_g , r_s , and d , the total error is 4.90%, whereas if it were offset to only 12.5 mm, the total error is 4.92%. For the smallest sample tested (radius 2 mm with offset 30 mm (at the edge)), the error is 4.9%. However, for the first case ($r_s = 7.5$ mm and $d = 24.5$ mm) with $\delta r_s/r_s = 2\%$, the total error in the rises to 6.09%. These hypothetical cases indicate that the error is $\sim 5\%$ for representative circular samples, and that the sample dimensions and location have very little impact on the overall error, whereas the measurement error for each variable (i.e., $\delta r_s/r_s$) has a large impact on the total error.

In the actual implementation, J_{rheo} is calculated from R_g which is estimated from 5 points and fit to equation of a circle in MATLAB, and J_s is numerically calculated (r_s and d are not applicable for non-circular samples), thus the following equation is used:

$$\frac{\delta b}{b} = \sqrt{\left(4 \frac{\delta R_g}{R_g}\right)^2 + \left(\frac{\delta J_s}{J_s}\right)^2}. \quad (\text{B3})$$

When analyzed repeatedly for different images by two separate users, we found $\delta R_g/R_g = 1.6\%$. Although J_s is calculated in an automated fashion, variability associated with measurement of J_s could arise from camera focus and aperture, lighting, prism alignment, and the filtering and threshold used to determine sample the sample boundaries. With proper focus and light levels, even lighting using a ring flash, appropriate filtering and threshold setting, and automated perimeter selection, analysis of multiple independent images taken of the same sample after systematic readjustment over the widest applicable range of camera settings and prism placement yielded 0.99% error in J_s . The combined estimated error due to image analysis using $\delta R_g/R_g = 1.6\%$ and $\delta J_s/J_s = 0.99\%$ is 3.3% for the correction multiplier, b .

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Figure 1.

(a) Schematic depicting a geometry of radius R_g and gap height h . In traditional rotational Rheometry, the entire volume between the geometry and plate is filled with the sample. (b) For eccentric Rheometry, the amount of torque that the sample generates increases as the distance from the axis of rotation to the centroid of the sample increases. (c) Plot showing the theoretical increase in ratio of measured torques (T is the torque at offset d , T_0 is the torque the centered configuration) for a $\phi 8$ mm sample as it is moved from the center of a $\phi 64$ mm geometry to the edge. This offset results in ~ 100 -fold increase in torque relative to the centered configuration.

Figure 2.

(a) Schematic of the experimental setup in frontal view. A prism is placed on top of the clear glass geometry as shown in order to get exact location and shape of sample. (b) Schematic of experimental setup in side view shown with camera for clarity.

Figure 3.

Schematic depicting the process of image analysis. (a) The user selects points (shown with red crosses) on the edge of the circular geometry, from which the program calculates its radius, R_g . (b) Image of irregularly shaped sample projection through orthogonal prism when filtering is used to obtain sample profile. Each incremental area, dA , times its radius, r , from center of rotation is squared then summed to calculate the polar moment of inertia of the sample.

Figure 4.

(a) The stiffness of PDMS samples tested in the centered and offset configurations were not statistically different ($p > 0.05$) verifying the accuracy of the method. (b) The accuracy was further demonstrated by testing soft circular collagen gels ($\phi 15$ mm, 3mg/ml RTT) in two offset locations (pink open circles) and irregularly shaped gels of same area. Note that G' is independent of shape and position and that collagen gels of this size could not be tested in the centered configuration due to low torque generated. (c) Sensitivity of the method was shown by testing collagen gel samples (3 mg/ml RTT extracted collagen) of decreasing diameter from $\phi 8$ mm to $\phi 4$ mm. As sample size decreased, the variability of the G' measurements increased (G' shown with closed circles, torque with open) illustrating that, while still within the resolution capabilities of the rheometer, it is approaching the limits of the machine.

Table 1

Shear moduli, geometric factors, and torques for a wide range of samples tested using eccentric rheometry.

Sample Name	n	Sample Diameter (mm)	G' (Pa)	G'' (Pa)	Tan (δ)	Gap height (μm)	Correction factor, <i>b</i> (-)	Torque @ 1% strain (μNm)	Equivalent Centered Torque (μNm)
φ4 mm CG (3 mg/ml)	4	5.3 ± 0.6	129 ± 27.9	27.1 ± 7.8	0.20 ± 0.03	500	93.0 ± 23.8	1.28 ± 0.22	0.01 ± 0.03
φ15 mm CG (1 mg/ml)	4	16.1 ± 1.2	29.1 ± 4.3	3.6 ± 0.3	0.13 ± 0.01	2000	14.6 ± 1.69	3.20 ± 0.52	0.32 ± 0.06
PDMS (centered)	4	12.7	137,000 ± 37,700	13000 ± 1090	0.10 ± 0.03	1800	N/A	534 ± 120.4	N/A
PDMS (offset)	4	12.7 ± 0.1	126,000 ± 7,950	7240 ± 703	0.10 ± 0.01	1800	21.1 ± 0.9	3280 ± 40	569 ± 52.8
FPCG Free	5	14.6 ± 0.6	129 ± 21.6	20.5 ± 3.03	0.15 ± 0.01	800	15.8 ± 1.8	5.19 ± 0.90	1.21 ± 0.32
FPCG Fixed	6	18.7 ± 0.8	99.1 ± 12.2	17.3 ± 5.4	0.17 ± 0.02	800	15.2 ± 1.2	3.56 ± 3.16	1.13 ± 0.92
FPCG Anisotropic Parallel	1	22.7 × 6.2 (rectangular)	225	34.5	0.19	300	44.9	5.10	1.98
FPCG Anisotropic Perpendicular	1	22.7 × 6.2 (rectangular)	180	46.1	0.18	300	16.4	7.79	2.87
Model Thrombi	5	15.1 ± 1.5	370 ± 134	47.6 ± 23.4	0.10 ± 0.03	800	13.6 ± 3.0	8.98 ± 5.38	1.42 ± 1.21
Blood Clot	1	6.60 × 11.0	4037	764	0.18	1900	56.0	41.7	3.20
Lipid Plaque	1	8.50 × 4.30	4036	677	0.19	1900	40.4	32.4	2.88

Data presented as average ± standard deviation. CG = collagen gel made from acid extracted rat tail tendon collagen; PDMS = polydimethylsiloxane; FPCG = Fibroblast-populated collagen gel culture 18 hours; Anisotropic Parallel = tested parallel to the remodeling orientation; Anisotropic Perpendicular = tested perpendicular to the remodeling orientation. Equivalent centered torque is the estimated value of torque (Eqn. 14) at 1% strain for the sample if it were tested in the standard centered configuration coaxial with the axis of rotation. Human Blood clot and Lipid Plaque were tested at 37°C; all other samples were tested at 25°C. All samples were tested at a frequency of 1 Hz.