PART I

Basic Concept and Preparation Culture Substrates for Cell Mechanical Studies

CHAPTER 1

Basic Rheology for Biologists

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Abstract

Many cellular processes lead to changes in elastic and viscous properties of cells. Rheology is the science that deals with deformation and flow of materials. Fundamental rheologic concepts are explained, and some of the main techniques are discussed. Nonperturbing oscillatory techniques are especially useful for monitoring structure formation including gelation, whereas other techniques such as steady shear flow and creep are useful for determining flow properties. Sample preparation is often a major obstacle, and advantages of different deformation geometries are discussed. Simple biological samples such as purified biopolymers can be investigated with a range of rheologic techniques, and factors affecting gelation of, for example, blood or cytoskeletal proteins can be studied in detail. More complex biological systems such as intact tissues can often only be studied with more qualitative techniques and results. With proper choice of experimental setup, rheologic techniques can give valuable information about cellular systems and dynamics on a timescale that is closely related to biological functions.

I. Introduction and Rationale

Rheology is the study of how materials deform when forces are applied to them. The word is derived from Heraclitus' expression "$\pi\alpha\nu\tau\alpha\ \rho\varepsilon\iota$" translated as "everything flows" which was adapted to create the term rheology in 1929 when the American Society of Rheology was founded (Barnes *et al.*, 2001). Although a separate discipline of rheology is fairly new and often not familiar to biologists, key experimental results and concepts on which it is founded are among the most widely known discoveries in mechanics. The concept of viscoelasticity draws from theories describing ideal materials: that of Robert Hooke's description of ideal elastic behavior in "*True Theory of Elasticity*" (1678) and of Isaac Newton's definition of ideal liquid behavior in "*Principia*" (1687) (Barnes *et al.*, 2001). Hooke described elasticity as a state where the extension of a material produced is proportional to the load. A Hookean solid in a deformed state will remain deformed as long as the applied stress persists. In contrast, Newton's theory describing ideal liquids states that a flow will persist as long as a stress is applied and the stress will be proportional to the rate of flow.

The property that rheological studies are designed to quantify is conceptually simple, namely a value that predicts how a material will deform when a force of

a certain magnitude is applied to it in a defined geometry for a given amount of time. Some of the complexity of the rheology field is due to the fact that quantitative measures of deformation are often not simple for samples with anything more than the most basic shapes, and the extent of deformation can depend strongly on how long and how quickly the force is applied. The molecular structure of a material can also change during the deformation, leaving the sample in a different state than it was before deformation, with different properties the next time it is deformed. Accounting for the different scenarios by which forces are applied and deformations are measured requires a certain amount of terminology, some of which has a more precise meaning as a rheological term than it does in common usage, and some of the jargon of rheology is likely to be unfamiliar to biologists. A basic set of terminology is listed in the glossary.

Two different ideal ways in which a material can deform are essentially related to the differences between liquids and solids. When a force is applied to an ideal solid, the material immediately deforms to a certain extent, and then stays put in that deformed state until the force is removed, when it returns to the shape it was before the deformation. This kind of perfectly recoverable deformation is called elastic, as the energy or work that was done to produce the deformation (the force integrated over the displacement) is stored within the deformed material and is entirely recovered when the force is removed. For such a simple elastic material in one dimension, most commonly exemplified by an ideal spring, a single number, the elastic constant—the ratio of force to displacement—suffices to predict how far the spring will stretch the next time a force of any given magnitude is imposed on it for any amount of time.

At the other extreme are ideal liquids. In this case, when a force is applied, the liquid will deform without limit for as long as the force is applied, and the liquid will remain in the deformed state when the force is removed. Although the extent of deformation is not limited, the rate at which the liquid will deform and flow is precisely determined by the magnitude of the force. The ratio of force to rate of deformation defines a viscosity that predicts how fast a liquid will flow whenever a force of any given magnitude is applied, and this rate will remain constant until the force is removed.

Real materials are neither ideal solids nor ideal liquids nor even ideal mixtures of the two. There are always effects due to molecular rearrangements and other factors that complicate deformation, transforming elastic and viscous constants to functions of time, and extent of deformation. Real materials, and especially biological materials, exhibit both elastic and viscous responses and are therefore called viscoelastic. They are also often highly anisotropic, showing different viscoelastic properties when deformed in one direction than when deformed in other directions. The goal of rheological experiments is to quantify the viscoelasticity of a material over as wide a range of time and deformation scales as possible, and ultimately to relate these viscoelastic properties to the molecular structure of the material.

Measuring the rheology of biological materials, cells, and subcellular organelles is currently motivated in part by recent demonstrations that cells respond to

physical stimuli as strongly as they do to chemical agonists, and that the morphology and developmental program of cells and multicellular aggregates also depend very strongly on the viscoelasticity of the extracellular matrix on which or within which cells grow. To gain perspective on how cells respond to physical forces, it is essential to determine the mechanical properties of the cell itself as well as those of its environment and extracellular matrix. These allow one to evaluate how much deformation different magnitudes of force can cause, and to identify subcellular structures that will be significantly deformed that might thereby serve as sensors, transducers, or effectors of the forces. In addition, cells change their rheological properties in response to genetic changes or acute responses to signals, and differences in cell stiffness, a characteristic that can be explained in part by studying the material properties of intracellular constituents, show promise as a criterion to differentiate normal from transformed cells (Chapter 17 by Lincoln *et al.*, this volume). In the context of mechanosensing, defining the rheology of a cell and its individual components is as important as determining levels of intracellular messengers before and after stimulation (Bershadsky *et al.*, 2005).

II. Rheological Concepts

Two fundamentally different modes of deformation can result when a force acts on a system. The volume of the system can decrease or the shape of the system can change. The former deformation is measured by the compressibility of the system, and relates to the Poisson ratio, but it will not be discussed further in this chapter, since most biological tissues are hydrated and water is nearly incompressible under the forces generally exerted in biology (Poisson ratio equals 0.5). However, local volume changes as would occur when water flows across a cell membrane or within the interstices of a polymer matrix may play important roles in the microrheological methods described in other chapters of this volume. For most biological systems the macroscopic volume stays constant during deformation, but there will be a change in shape. For example, in a simple shear deformation, as illustrated in Fig. 1A, a force is applied along a surface plane. The system will deform but the volume stays constant. In the case of simple elongation or extensional deformation, the force acts in a direction normal to a plane (Fig. 1B), and increases in length couple to decreases in cross section. Simple shear or elongation yields the same information about the material properties as long as the volume does not change. For some materials, it is more convenient to test materials in other deformation geometries such as bending or twisting.

In order to determine the elasticity or viscosity of a biological or any other system, it is necessary to be able to quantify forces and deformations. For a given material, an applied force will result in different magnitudes of deformation depending on the size of the material. In order to determine material properties, which are independent of size and shape of the system, two key rheological concepts are used: stress and strain. Stress is defined as force per area and has the SI unit of Pa (N/m^2). In older literature

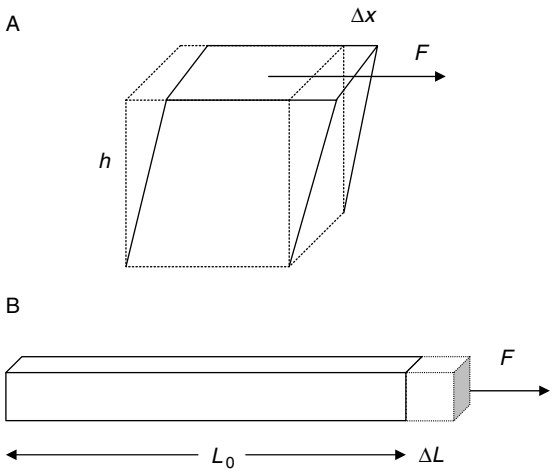

Fig. 1 Two common deformation geometries. In simple shear (A), a force F is applied along the surface of an undeformed box (fine lines) with height h. The top of the box is deformed Δx due to the force. In simple extension or elongation (B), the force is applied perpendicular to a surface. The undeformed length of a box, strip, or rod is L_0, and the extension due to the force is ΔL.

and in studies of vascular flow, stress is often reported in the cgs unit of dyne/cm^2, for which 1 Pa = 10 dyne/cm^2. The two forces shown in Fig. 1 result in a shear stress and in an elongational stress, respectively, and in both cases the stress is calculated as the force per area of the plane on which the force is acting. Strain is similarly defined as the relative deformation, which is the deformation divided by the height or length of the system and is a dimensionless quantity (length/length). The symbols σ and γ are often used for stress and strain, respectively, in a simple shear deformation. In simple shear $\gamma = \Delta x/h$, where Δx is the deformation and h is the height of the system (Fig. 1A). In simple elongation, the tensile strain $\varepsilon = \Delta L/L_0$, where L_0 is the unstrained length and ΔL is the extension resulting from the force, as shown in Fig. 1B. When the sample shape is more complicated than the simple forms in Fig. 1, calculation of strain is often difficult, requiring integration over complex volumes. Geometrical expressions called form factors allow calculation of dimensionless strains from measurable quantities such as the distance that a sample is stretched or the angle by which it is twisted in response to a given force. For some geometries, it becomes practically impossible to determine unambiguously the strain or the stress, and the rheological properties of a biological material are then reported in less informative empirical units such as force per displacement.

A. Elasticity

The mechanical responses of ideal elastic and viscous systems serve as important references when discussing more complicated biological systems, which often show both elastic and viscous characteristics. An ideal elastic system follows Hooke's law,

which states that the force is proportional to the deformation, or in terms of stress and strain

$$\sigma = G\gamma \tag{1}$$

where G is the elastic shear modulus and $\gamma = \Delta x/h$ as defined earlier. The value of G is a measure of the rigidity of the system. Typical values of many biological gels and the cytoskeleton are of the order 10^3 Pa. A rubber band has a shear modulus of the order 10^6 Pa, and harder materials such as glass, wood, and steel have characteristic shear moduli of the order 10^{10} Pa. While such order of magnitude estimates of moduli are commonplace for materials as well as cells, more accurate and reproducible determinations require attention to sample preparation and history or aging. Nonetheless, knowing G for an ideal elastic material enables a prediction of the elastic deformation in any type of deformation.

Some systems are more conveniently studied in simple elongation than in shear deformation. The stress applied in simple elongation will be proportional to the extension, and the proportionality constant is called the Young's modulus or E modulus. For isotropic incompressible materials E equals $3G$, which illustrates that the same information may be obtained from either elongation or shear measurements.

B. Viscosity

In contrast to ideal elastic materials, for the ideal Newtonian liquid stress σ is independent of strain γ, but is proportional to the rate of strain, which has units of $\sec^{-1}$ and is related to the liquid flow rate or more precisely the time derivative of strain $\mathrm{d}\gamma/\mathrm{d}t$. The proportionality constant relating stress to shear rate is viscosity, η.

$$\sigma = \eta \frac{\mathrm{d}\gamma}{\mathrm{d}t} \tag{2}$$

Newtonian liquids are fully described by the value of the viscosity, which often depends on temperature and slightly on pressure but is independent of strain and strain rate (Fig. 2). The SI unit of viscosity is Pa sec, and characteristic values are 0.001 and 1 Pa sec for water and glycerol, respectively. In older literature, Poise (P) is often used as the cgs unit of viscosity. 10 P equals 1 Pa sec. Non-Newtonian liquids are liquids that cannot be characterized by a constant viscosity. Many biological systems show a constant viscosity at low shear rates but a decreasing viscosity above a characteristic shear rate (Fig. 2). Flow curves are plots of viscosity against shear rate and allow determinations of the zero shear rate viscosity, the critical shear rate (rate at which viscosity starts to decrease), and how rapidly the viscosity decreases at high rates.

When a constant stress is applied to an ideal elastic material, a constant strain is obtained [Eq. (1)], and a constant strain corresponds to zero shear rate (or zero flow). Ideal elastic materials are therefore characterized by an infinite viscosity [Eq. (2)]. As a result, viscosity measurements of elastic and many viscoelastic

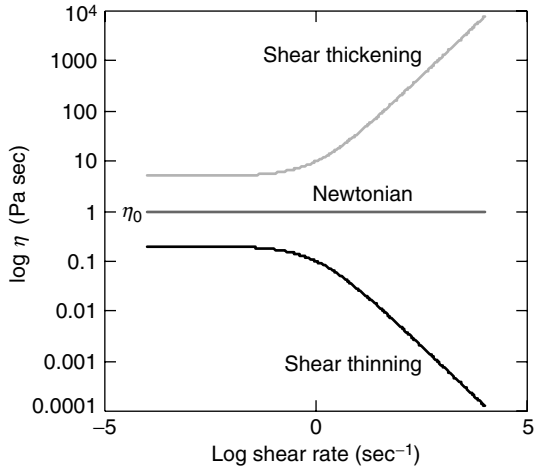

Fig. 2 Characteristic types of flow curves, showing the dependence of viscosity on shear rate. Newtonian liquids have a constant viscosity, whereas shear thinning and thickening solutions have a constant zero shear rate viscosity, η_0, only below a characteristic shear rate.

systems under steady shear are of limited use. Furthermore, in many cases, deformation caused by a steady shear rate will destroy fragile structures in the sample and provide little information about the unperturbed properties of the system. Empirical information about the amount of stress that a sample can withstand before it is damaged and flows, often called a yield stress, however, is generally useful for studies of bone fracture, blood vessel rupture, and other failure events.

C. Oscillatory Measurements

For reasons discussed above, rheological information for viscoelastic systems is often obtained by applying small amplitude oscillatory strains or stresses to the sample rather than steady flows. If an oscillatory strain deformation with an amplitude γ_0 and an angular frequency ω is applied, the stress will also oscillate in time t but will be phase shifted by δ with respect to the strain

$$\gamma(t) = \gamma_0 \sin \omega t \tag{3a}$$

$$\sigma(t) = \sigma_0 \sin (\omega t + \delta) \tag{3b}$$

where σ_0 is the stress amplitude and the angular frequency ω (in rad/sec) equals $2\pi\nu$, where ν is the frequency in Hz (Fig. 3). The phase shift, δ, is always between 0° and 90°. For an ideal elastic system the phase shift is 0° [Eqs. (1) and (3)], and for an ideal Newtonian liquid it is 90° [Eqs. (2) and (3)]. Materials with phase shifts between 0° and 90° are viscoelastic and the stress in Eq. (3b) is expressed as a sum of elastic and viscous contributions.

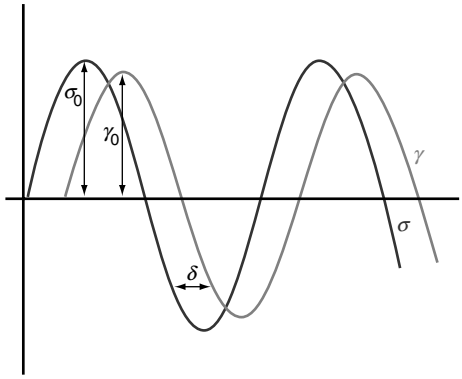

Fig. 3 Stress and strain against time in an oscillatory deformation. Stress and strain amplitudes are marked with σ_0 and γ_0, respectively. The stress and strain signals are phase shifted by an angle δ.

$$\sigma(t) = \gamma_0(G' \sin \omega t + G'' \cos \omega t) \tag{4}$$

In this equation, G' is referred to as the elastic storage shear modulus, and G'' is the loss shear modulus because it is related to the viscous properties and associated energy loss in the sample. For an ideal Newtonian liquid $G'' = \omega\eta$ and $G' = 0$. For an ideal elastic system $G' = G$ and $G'' = 0$. For viscoelastic materials G' and G'' depend on angular frequency, and oscillatory measurements as a function of time at a fixed frequency can be used to monitor structure formation in biological systems.

According to Eqs. (1) and (4), stress is proportional to the strain or strain amplitude. This is valid for all materials at small strains or amplitudes and is called the linear elastic or viscoelastic range. At larger strains or strain amplitudes, stress and strain will not be proportional and the material will be in the nonlinear range. Most materials will exhibit strain softening with a smaller G at large strains. However, some systems exhibit strain stiffening where G increases above a critical strain.

Steady shear and oscillatory measurements are probably the two most important types of rheological measurements. However, many biological systems experience a sustained force such as gravity or blood pressure. It is therefore useful to monitor how such systems deform under a constant load or stress. This type of measurement is called a creep experiment, and in such an experiment the strain $\gamma(t)$ is monitored as a function of time for a fixed stress σ_0 (Fig. 4). The compliance J is then obtained as

$$J(t) = \frac{\gamma(t)}{\sigma_0} \tag{5}$$

After a certain time period under a constant stress, the stress can be removed and the system's ability to recover toward the original undeformed state can be investigated in a creep recovery experiment.

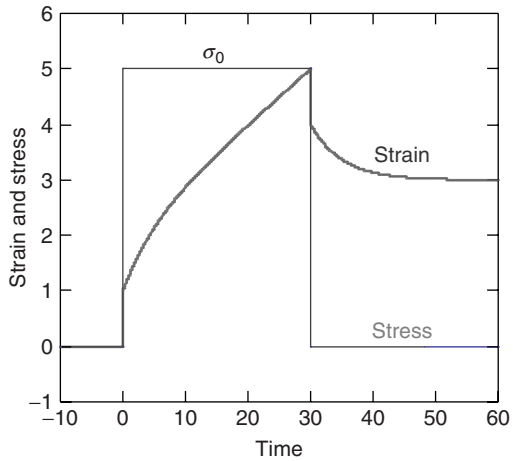

Fig. 4 An example of a creep recovery experiment. A constant stress, σ_0, is applied to the system at time 0. The stress (thin line) is removed again at time 30 and the rest of the experiment is the recovery measurement. The strain (thick line) shows an initial elastic response at time 0, followed by a more gradual increase in strain. When the stress is removed, a partial rapid recovery is seen, followed by a more gradual recovery of strain.

Creep experiments are generally performed on a controlled stress (CS)-type instrument, which applies a defined stress and measures the resulting change in strain. A closely related type of experiment, stress relaxation, can be performed on controlled rate (CR)-type instruments. In a stress–relaxation experiment, the sample is rapidly deformed to a fixed strain, γ_0, and the stress is monitored as a function of time (Fig. 5). The stress–relaxation modulus, $G(t)$, is then calculated as

$$G(t) = \frac{\sigma(t)}{\gamma_0} \tag{6}$$

The relaxation modulus contains information about how rapidly structures can reorganize to relieve the stress in the system.

III. Rheological Instrumentation

A rheometer is any instrument that enables determination of rheological properties. Very simple rheometers such as capillaries have been used for centuries to determine flow of liquids, and in the art of cooking people have used simple kitchen tools or teeth and fingers as a means to probe rheological properties of food products. Modern rheometers allow a more precise quantification of material properties in well-defined geometries, and they can be divided into two main types. In a CR-type instrument, a motor, often controlled by a computer, deforms

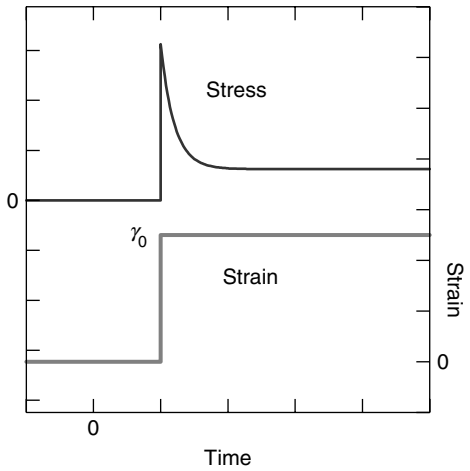

Fig. 5 Principle of a stress–relaxation experiment. A sample is quickly deformed to a constant strain γ_0 (lower curve) and the stress is measured as a function of time (top curve). The stress signal is characteristic for a viscoelastic solid with some stress relaxation and a finite equilibrium stress at long times.

the sample in a controlled manner and a force transducer monitors the force or stress resisting the deformation of the sample. The strain or strain rate is controlled and the forces or stresses are measured. In a CS-type instrument, a stress is applied to the sample and the resulting deformation is monitored. The flow of a liquid through a simple capillary viscometer is an example of a CS-type instrument where gravity determines the stress and the flow rate is determined from the flow time of a fixed volume of the flowing liquid.

Most modern rheometers are rotational type instruments. The sample to be measured is confined in a narrow gap between a stationary and a moving part of a measuring cell. Different measuring cells are used depending on the sample properties. The most common measuring cells are cone and plate (CP, Fig. 6A), parallel plate (PP, Fig. 6B), and concentric cylinders (couette). The couette geometry is often the choice for liquid samples. The PP cells are typically used for films or disk-shaped materials, but have the disadvantage that the sample confined between the plates is not deformed to the same degree, because the strain depends on the distance from the center of rotation. The maximum strain is obtained at the perimeter of the measuring plate, whereas the strain is zero at the rotational axis. The surface properties of the measuring cell are also important, since the sample has to stick to and follow the tool surface during measurements. Slippage between sample and cell surface, for example, as a result of syneresis, can be a problem for some biological systems.

Two signals from a rotational rheometer, the angular position and the torque, are used to compute strains and stresses. A position sensor registers the angular position of the moving cell part. The angle of rotation, ϕ, is proportional to the strain and the proportionality constant depends on gap size and geometry.

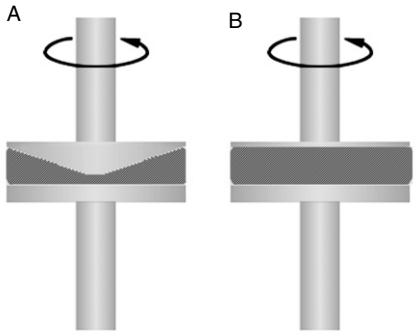

Fig. 6 Two common measurement geometries in rotational rheometers. The sample is confined between a cone and plate (A) or two parallel plates (B).

The stress is proportional to the torque (force times lever arm length) and the proportionality constant depends on the sample geometry and especially on the surface area of the measuring tool. Tools with a large area are used for soft materials and low-viscosity liquids, whereas smaller tools are used for harder materials. A summary of proportionality constants for various geometries can be found in (Ferry, 1980).

In oscillatory tests, the computer monitors both the position and torque signals as a function of time. The time-dependent strains and stresses are calculated and fitted to sine waves [Fig. 3; Eq. (3)]. The stress and strain amplitudes, as well as the phase shift, are obtained from the fits and from these G' and G'' are calculated. Modern rotational rheometers typically cover a frequency window from 0.001 to 100 Hz. The upper limit is often determined by mechanical resonances in the tool and detector. G' and G'' are only defined in the linear range where both strains and stresses are simple sinusoidal curves [Eq. (4)]. In order to ensure this, materials should always be tested as a function of stress or strain amplitude. In the linear range, G' and G'' are independent of these amplitudes.

IV. Experimental Design

Biological systems can be investigated by a number of different rheological techniques. The choice of technique depends on the type of information desired and the type of system. Some common techniques will be described with respect to typical uses and limitations. The techniques can be divided into steady and oscillatory measurements.

A. Stress–Strain Relation

Elastic systems can be studied in both simple shear and simple elongation by use of either CS or CR instruments. A stress–strain curve is a plot of steady stress against strain. The slope of this plot at low strains is the shear modulus (or the

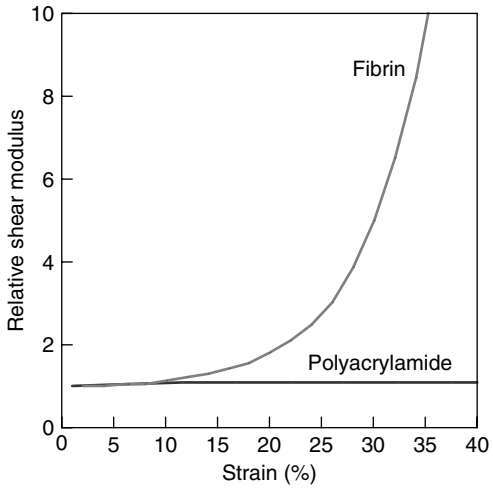

Fig. 7 Strain amplitude dependence of polyacrylamide and fibrin gels. The measured shear modulus divided by the modulus at small strain amplitudes is plotted against strain amplitude. G' of the polyacrylamide gel is seen to remain constant over the measured strain range, whereas fibrin gels are strain hardening.

Young's modulus in simple elongation). The part of the stress–strain curve, where stress is proportional to strain, is the linear range. At larger strains, stresses will not be proportional to strain and the measurement will be performed in the nonlinear range. Some systems are strain softening and some are strain stiffening as illustrated by data for a fibrin gel in Fig. 7.

Stress–strain measurements are most readily performed on systems that mechanically equilibrate in time rather than flow. Measurements can be done in both the linear and the nonlinear range. However, care should be taken that measurements be performed well below the yield stress, where structural integrity starts to be affected by the stress.

B. Stress Relaxation

Stress–relaxation measurements can be performed in both simple shear and simple elongation, and they are of special interest for viscoelastic systems. In a stress–relaxation experiment, the sample is rapidly deformed and the stress is monitored as a function of time, keeping the sample in the deformed state. The stress–relaxation curve is a plot of the shear modulus, $G(t)$, as a function of time. A typical example is shown in Fig. 8. For a viscoelastic material, $G(t)$ will decrease with time and the decrease will occur on a timescale which is determined by the relaxation time of the sample. At long (infinite) times, the relaxation modulus will

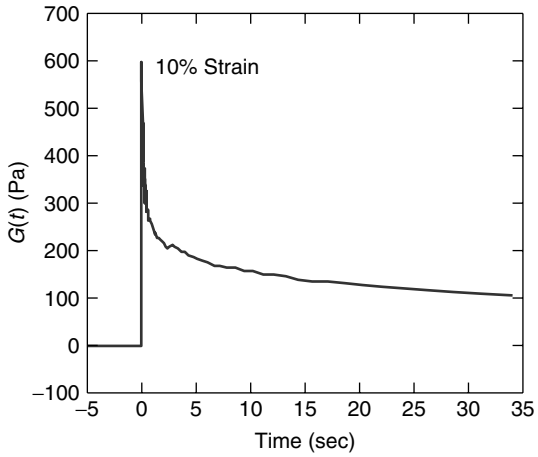

Fig. 8 Stress relaxation of adult rat brain exposed to 10% strain between parallel plates with a gap of ~3 mm. From a shear modulus of nearly 600 Pa at application of strain, the tissue will relax to a modulus of 125 Pa.

either relax to zero (characteristic of all liquids) or reach a constant value which will be the equilibrium modulus of the system characteristic of a viscoelastic solid.

Stress–relaxation experiments are especially useful for studies of systems with long relaxation times. Experiments should be performed in the linear range where the relaxation modulus is independent of the strain magnitude. Stress–relaxation measurements should be performed on CR instruments.

C. Creep and Creep Recovery

In a creep and creep recovery measurement, a constant stress is applied to the system for a period of time and then removed. An example of creep recovery of a blood plasma clot is shown in Fig. 9. The strain is monitored as a function of time. Results are often plotted as compliance, J, as a function of time, as shown in Eq. (5). Creep experiments are of interest primarily for viscoelastic materials. The elastic properties are seen in the rapid deformation and recovery when the stress is applied or removed. The slow subsequent deformation is characteristic of the viscoelastic time-dependent processes in materials. If this deformation (strain) increases linearly with time, one can obtain the viscosity at a low shear based on the inverse relationship between viscosity and the slope. The viscous or plastic deformation can also be calculated from the nonrecoverable compliance at long times.

Creep tests are also of interest for viscoelastic systems with long relaxation times because they measure flow at very low shear rate based. Care should be taken to ensure that the experiments are performed in the linear range where the compliance

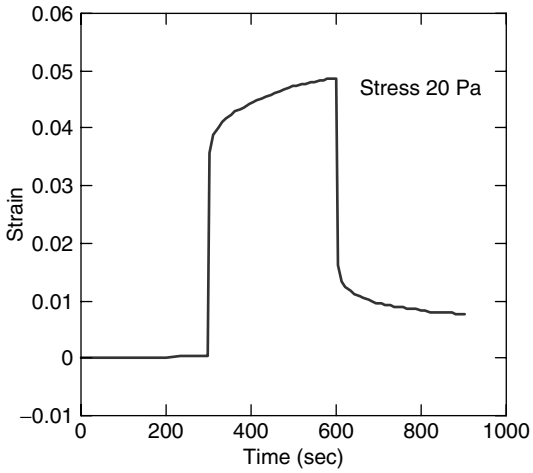

Fig. 9 Creep recovery experiment on a plasma clot. A stress of 20 Pa is applied to the clot between 300 and 600 sec. The Figure shows a rapid elastic deformation to a strain of 0.035, which corresponds to a shear modulus of 20 Pa/0.035 = 600 Pa. The clot nearly recovers completely at the end of the recovery part.

is independent of the stress magnitude. Creep measurements should be performed on CS-type instruments.

D. Frequency Sweep

Oscillatory measurements at low strain or stress amplitudes allow a determination of G' and G'' as a function of frequency. Such measurements are important because they can give information about both structure and dynamics. For viscoelastic liquids, G'' will dominate at low frequencies. At higher frequencies, relaxation of structures cannot take place within the oscillation cycle, resulting in an increase in G'. An example of a frequency sweep is given in Fig. 10, which shows the frequency dependencies of a hyaluronic acid solution. The Figure illustrates G'' dominating at low frequencies, where G'' is related to the zero shear rate viscosity and angular frequency through $G'' = \omega\eta$. At higher frequencies, G' dominates and a plateau is almost seen as expected for an ideal elastic material. The crossover angular frequency is approximately the inverse of the relaxation time, which for long biopolymers is strongly dependent on molecular mass and concentration.

Oscillatory tests can be performed on both CR and CS instruments. Care should be taken to perform measurements in the linear range where G' and G'' are independent of stress or strain amplitudes, and both stress and strain can be fit by sinusoidal functions as shown in Fig. 11A. Otherwise, as shown in Fig. 11B, the stress response to a sinusoidal strain is no longer purely sinusoidal, and G' and G'' are not well defined.

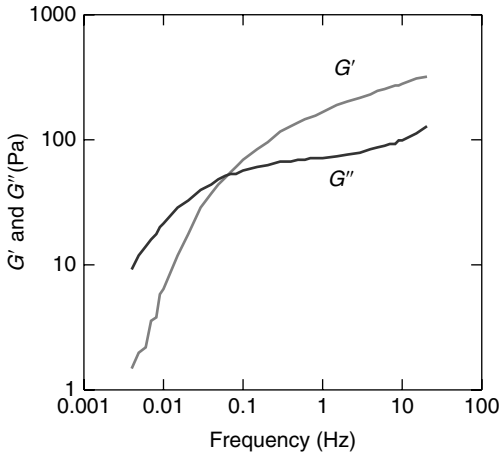

Fig. 10 Frequency sweep of a high molecular mass hyaluronic solution. G' and G'' are plotted against frequency. G'' dominates at frequencies below 0.1 Hz, whereas the elastic properties and G' dominate at high frequencies. The crossover frequency is determined by the longest relaxation time in the system.

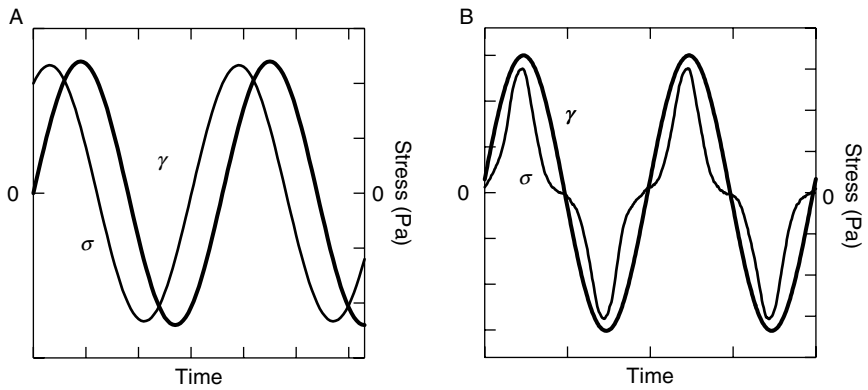

Fig. 11 Stress and strain in an oscillatory experiment on a hypothetical linear viscoelastic system (A) and on a strain-hardening system (B, plasma clot). The strain signal in B is a sine wave but the stress signal is not a sine wave. G' and G'' are therefore not well defined, but the very small phase shift demonstrates that the elastic properties dominate.

E. Time Sweep

A steady rate of shear should not be used to follow structural changes since structures are likely to rupture during steady shearing. Oscillatory measurements are an ideal method to overcome this problem for monitoring structural changes over time. In a time sweep, the system is subjected to small strain amplitude deformations at a single frequency. Time sweeps are useful to study, for example,

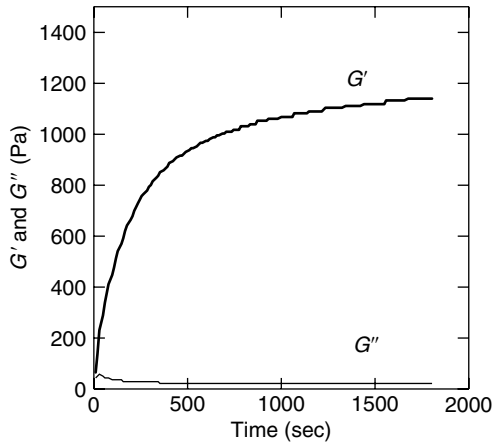

Fig. 12 Shear modulus measurement over 30 min of an 8 mg/ml fibrin gel exposed to 2% oscillatory shear strain at a frequency of 10 rad/sec. The slope of G' provides polymerization kinetics and the takeoff point indicates gelation time.

gelation or kinetics of enzymatic reactions. An example is given in Fig. 12, which shows a time sweep on a gelling system, a fibrin gel. The gelation time is sometimes defined as the time where G' and G'' are equal just before G' begins to rise rapidly.

Time sweeps should be performed at small strain amplitudes and preferentially on CR instruments, or on a CS instrument run in a controlled strain mode (Section VI). For experiments over a long time period, care should be taken to avoid evaporation or drying of the sample.

F. Strain or Stress Amplitude Sweeps

Oscillatory measurements at a fixed frequency with increasing strain or stress amplitudes are called strain sweeps. The linear range, where G' and G'' are constant, should always be observed at low amplitudes, but at larger strains G' and G'' will often depend on the strain amplitude. In many gel cases G' exceeds G'' at low strains, but G'' will dominate when the gel structure is broken down at higher stress amplitudes. The stress amplitude where nonlinear effects are observed has been used to determine the yield stress. In addition, if a strain sweep is repeated on the same sample in the rheometer, it is often possible to determine if the large strain has had an irreversible effect on structures (Kerst *et al.*, 1990). Some gel systems primarily consisting of long fairly stiff biopolymer structures show pronounced strain hardening (Storm *et al.*, 2005), that is G' increases with increasing strain amplitudes (Fig. 7).

The values of G' and G'' in the nonlinear range are not well defined, since the strain signal on a CS instrument and the stress on a CR instrument will not be true sine waves (Fig. 11B). The error made in estimating elastic moduli by fitting the

data to sine functions, however, can be small, as demonstrated by direct comparison of oscillatory and steady shear measurements for a highly nonlinear material (Storm *et al.*, 2005).

G. Rate-Dependent Viscosity

Newtonian liquids are characterized by a viscosity that is independent of shear rate. Flow curves are plots of viscosity against shear rate and are of interest for non-Newtonian liquids. Any viscoelastic liquid should have a constant "zero shear rate viscosity," η_0, at sufficiently low shear rates. The example in Fig. 13A shows a typical flow curve for a biopolymer solution at fairly high concentrations. The η_0 value is very dependent on molecular mass and concentration. Above a critical shear rate, the viscosity decreases linearly with shear rate on the log–log plot. Such flow curves are very common for biopolymer solutions and the sample is said to behave as a power law fluid, since the viscosity is proportional to some power of the shear rate. The exponent should always be between 0 (Newtonian liquid) and a value not less than -1 (limit of indeterminate flow). The critical shear rate is related to the inverse of the longest relaxation time of the system.

Flow curves can also be used to determine yield stresses of elastic materials. If the shear rate is measured with increasing steady stresses, the elastic properties will dominate at small stresses, with a vanishing shear (flow) rate (Fig. 13B). At stresses above the yield stress, structures will be broken and the system will flow. The example in Fig. 13B is typical for many systems and is referred to as a

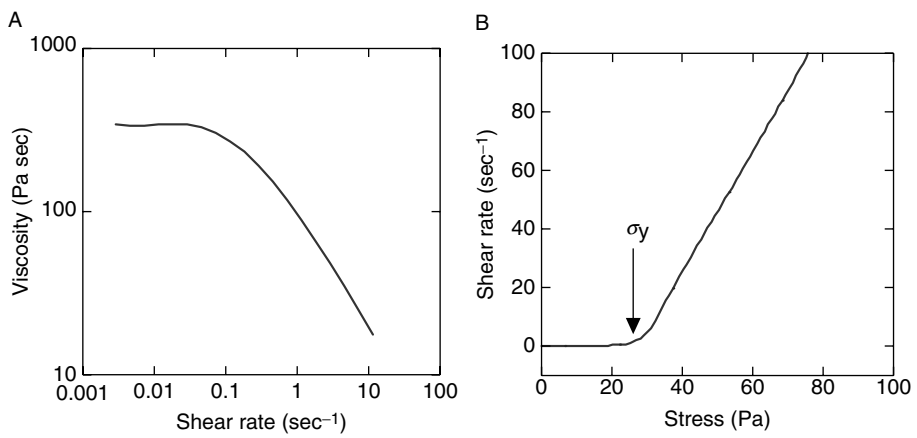

Fig. 13 Steady shear flow curves of hyaluronic solution (A) and a flocculated micellar system (B). The hyaluronic solution is a shear-thinning solution with a zero shear viscosity of about 300 Pa sec and power law behavior above shear rates of 0.3 sec^{-1}. The micellar system behaves like a Bingham liquid with an elastic response (i.e., shear rate ≈ 0) at stresses smaller than the yield stress σ_y. The linear relationship between shear rate and stress above the yield stress is characteristic for a Bingham liquid.

"Bingham liquid," with a Bingham viscosity which is the slope of the curve at higher stresses and a yield stress corresponding to the intercept of this curve at vanishing shear rates.

Flow curves should represent steady state values of the viscosity against shear rate. This means that viscosity should be independent of measurement time. It is important at each shear rate to allow sufficient time for the stress to reach a constant value before the stress is measured. Otherwise the calculated viscosity will not only depend on shear rate but also on time and history of the sample. Systems for which the viscosity depends on history are called thixotropic.

H. Flow Oscillation

During a steady shear flow, structures are often reversibly destroyed or aligned in the flow field. In some cases, it can be of interest to determine how rapidly structures are reformed. This can be investigated by a flow oscillation test, in which the system is first sheared at a given shear until steady shear flow is obtained, after which steady shearing is stopped and small amplitude oscillations as a function are immediately started. The resulting time dependence of especially G' contains information about the kinetics of recovery.

V. Sample Preparation

Shear, compression, and elongational deformations of soft biological materials require well-defined interactions of the material with the plates or holders of the rheometer, and these requirements often limit the types of measurements that can be made. Elongation strains require that the sample be held in place at two ends, usually by a clamp, while the sample is stretched. This manipulation generally works well for stiff elastic materials such as skin, tendon, or bone, but is usually impractical for fragile tissues like brain or liver, and impossible for viscoelastic liquids or gels that flow out of the holder. Compression studies are easier in the sense that clamping is unnecessary, but evaluation of the stress–strain curve requires accurate information about whether the surfaces resting on stationary plates are adherent or can slide as the sample is compressed, and whether liquid is squeezed out of the hydrated material during testing.

Most rheological measurements of biological gels, liquids, and soft tissues are done under shear deformation because shear strains preserve sample volume, and the materials can be more easily placed between the plates of the rheometer. Figure 6A illustrates a typical CP geometry in which liquids or gelling systems are held.

A. Solids

Solid tissues are generally excised and cut into disk-shaped samples with dimensions typically 5–25 mm in diameter and 0.1- to 5-mm thick. They are placed on a flat lower rheometer plate. A parallel top plate is lowered onto the sample until

contact is made, as judged by a positive normal force measured by a force transducer. Generally, hydrated specimens bind well enough to the metal (usually stainless steel or titanium) rheometer plates so that slipping does not occur during the shear deformation required for the rheological measurements. Use of a serrated top and bottom metal plate can further prevent the problem of slipping. If required, adhesives can be used to glue the sample to the plate, as long as the adhesive causes no chemical changes in the sample and is much stiffer than the sample, such that it remains stationary as the sample deforms. Since the tissue is expected to be predominantly elastic, slippage can be detected by an irrecoverable deformation when the sample is subjected to creep and creep recovery, or as an anomalously high mechanical loss, characterized by a large phase angle between stress and strain in an oscillatory measurement. Slipping can also be eliminated by decreasing the gap between plates to compress the sample slightly. Typical compressions of 5% have been used in several studies of tissue rheology. There is a complication in compression, however, in that studies of brain and liver rheology show that the shear modulus is a strong function of compression (or squeezing), and therefore a series of shear modulus measurements should be made over a range of compressions and the modulus extrapolated to zero compression to reflect the shear elasticity in the uncompressed state (Fig. 14).

Since PPs are generally used for solid samples, shear moduli are only well defined for deformation in the linear range, since the shear strain in this geometry increases linearly with the distance from the center of the plate. Often, however, the physiologically relevant rheology occurs in the nonlinear range, and indeed the nonlinear elasticity, often in the form of strain stiffening, is an essential feature of tissues such

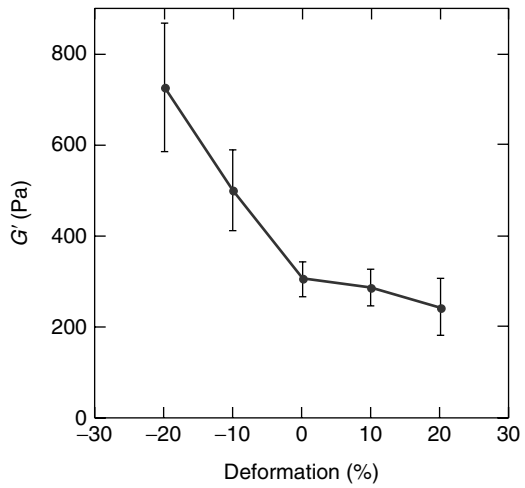

Fig. 14 Effects of compression or elongational deformation on shear modulus of intact rat brain tissue under 2% oscillatory shear strain. Compression of brain tissue (negative deformation) increases the shear modulus, and at 20% compression the shear modulus G' has increased by nearly a factor of 3.

as the arterial wall (Shadwick, 1999). The PP geometry will exaggerate strain stiffening since most of the stress is carried at the edge of the sample, and therefore a geometric correction needs to be made to relate these data to those made under uniform strains.

B. Liquids and Gelling Systems

Liquids and gels cannot be clamped and are therefore held in place between rheometer plates by surface tension and adhesion to the plate surface. This requirement limits the height of the sample to ~1 mm, depending on the tension at the sample interfaces. One important advantage of liquid systems is that they can be placed between a flat bottom plate and a truncated cone-shaped upper plate lowered to a prescribed separation so that the shear strain is uniform throughout the sample. This geometry is especially important for studying nonlinear materials such as strain-stiffening biopolymer gels.

The viscous properties of biological fluids such as blood and blood plasma have been extensively studied. Typical experiments measure viscosity as a function of shear rate. Shear-thinning properties of many fluids are important for their ability to transit through confined spaces in the vasculature, lymphatic system, and elsewhere. The range of biologically relevant flow rates is very large, from near zero in occluded blood vessels to over 1000 sec^{-1} in arteries.

Gelling systems require special consideration in sample preparation. Ideally, a gel is formed *in situ* between the rheometer plates, by initiation of the polymerization or cross-linking reaction right after the sample is placed in the rheometer so that the gel forms in an unstressed state before the rheological measurements begin. A common assay for gelling systems is a time-dependent oscillatory measurement of G' and G''. In a CR-type rheometer, a small amplitude strain is imposed during polymerization and the elastic and viscous components of the shear stress are measured. For a purely viscous solution placed before the gel forms, G' is near zero and G'' is relatively small. As the polymers form and become cross-linked, G' abruptly becomes finite, and then both G' and G'' will increase as more material is incorporated into the gel network. The time at which G' become nonzero is often denoted the gelation time or gel point. This method in principle is used clinically to define the clotting time of blood samples and is useful for monitoring the kinetics of polymerization reactions in many other systems.

VI. Special Considerations for Biological Samples

A. Biological Polymers

Biological samples are often more difficult to measure rheologically and their rheological characteristics are often more difficult to relate to specific molecular structures compared to synthetic polymer systems. In addition, biological samples

are often fragile and nonlinear materials that can survive only a limited range of strains. Perhaps more importantly, they are also usually far from equilibrium, and the biologically relevant properties often depend on specific chemical states that can change during the course of an experiment. For example, one of the most commonly studied biopolymer gels—cross-linked F-actin—contains polymers that are continuously hydrolyzing ATP and exchanging subunits from the filament ends. The cross-links that bind filaments together have finite off rates that are often not known, and the geometry of the networks they form is usually kinetically determined, with slow rearrangements occurring as the rheological experiment proceeds. This slow time-dependent change in the chemical or morphological state of the material, even held at rest, makes studies such as frequency-dependent measurements difficult to interpret unless they are performed rapidly enough so that the chemical changes are negligible during the period of the measurement.

B. Intact Tissue

Intact tissues also undergo changes in rheology after isolation and storage. Biochemical changes during such reactions as ATP hydrolysis and proteolysis can be inhibited or allowed to reach a steady state, but other changes may be more subtle and potentially interesting. For example, most native tissues are in a state of tension due to the activity of motor proteins. Such internal stress within a biological material can greatly alter its rheology due to the nonlinear elasticity of biopolymer networks and to the specific nonrandom geometry of tissues. As a tissue ages and these internal tensions relax, they can alter tissue elasticity even without a gross change in tissue architecture.

The main complication in relating tissue rheology to molecular structure is perhaps the heterogeneity of biological materials. Tissues have complex architectures that are frequently connected to their *in vivo* function. They are often multilayered and composed of numerous cell types, each type with its own mechanical properties, contractility, strength, and orientation within the tissue. The situation might seem incomprehensibly complicated, but materials in common use today can also be complex and include carbon fiber composites (used in planes, cars, bikes) in which fibers are pretensed as they are layered into cured epoxy laminates.

C. Instrument Selection for Measuring Gelation Kinetics

Gelation kinetics generally require a CR instrument, rather than a CS instrument. Limiting the strain to a small, defined value ensures that the fragile polymer network that appears just at the point where filaments first make a continuous network throughout the sample—the so-called percolation threshold—is not destroyed by the measurement. When the network is very weak, the strain may be so small that the rheometer transducer cannot accurately measure the stress, and the data will be noisy. In contrast, in a CS rheometer, the value of stress chosen, even if very small, will usually be enough to induce continuous flow in the

sample before it gels and becomes stiff enough to resist the stress. Therefore, the first measurements of a gelling system, before or near the gel point, can destroy the sample and cause flow alignment of filaments that have not yet formed a network, resulting in anomalously low values for G' at later time points. Many gels formed *in vitro* by purified cytoskeletal polymers such as actin and microtubules are very soft with G' on the order of 1 Pa and therefore very susceptible to damage in conventional CS rheometers.

VII. Conclusions

Forces and mechanical effects can direct cell function and tissue formation as specifically as chemical stimuli, and integrating physical studies into cell biology is increasingly used in bioengineering efforts and other biomedical studies. Since a cell or tissue's response to forces is defined by its viscoelastic parameters, quantitative measurements of cell and extracellular matrix rheology are necessary for a full explanation of how cells interact with their environment.

Rheology can be applied to a vast range of biological and medical problems (Gabelnick and Litt, 1973). The actin, intermediate filament, and microtubule networks of the cytoskeleton each have distinct rheological responses that affect multiple cell processes ranging from migration to division (Janmey, 1991). Soft tissues, as another example, maintain mechanical properties within a specific range that varies according to tissue function, development, or disease. Fibrotic liver is more than three times as stiff as healthy liver and this characteristic could possibly be used in surgery to assess which areas are extensively damaged and to be removed (Kusaka *et al.*, 2000). Stress–relaxation properties of tissues vary with their functions: the bladder exhibits a high degree of stress relaxation to minimize internal pressure despite large volume changes (Andersson *et al.*, 1989), while the aorta shows a lower degree of stress relaxation in response to volume change in order to maintain adequate blood pressure (Shadwick, 1999). Brain is a particularly soft mammalian tissue, with an elastic modulus $\sim$10 times lower than liver and nearly 50 times lower than muscle and may, therefore, be more susceptible to damage by shear (Donnelly and Medige, 1997; Liu and Bilston, 2002). Rheological oscillatory shear or stress–relaxation tests can be performed to attain short-term and long-term values of the stiffness of intact tissue or extracellular matrix, and the information used toward modeling relevant strains induced by cellular processes such as growth cone or lamellipodial advance. It is likely that these rheological properties of the tissue play an important role in maintaining specific cellular functions.

Macroscopic rheological methods as outlined in this chapter have been extensively used to provide empirical measurements of how biological materials deform when stressed, and some of these studies have suggested ways to relate the molecular structures within tissues to their rheology. However, macroscopic rheology has several important limitations that have motivated efforts to design and

implement microrheological methods as are discussed in several chapters of this volume. The major limitations of macrorheology are associated with the size of materials needed, the magnitude of forces involved, and the heterogeneity of biological materials. Issues of sample size and forces that may disrupt the material have been addressed in Section VI and can in many instances be alleviated by appropriate sample preparation.

Heterogeneity of biological tissues presents a fundamental problem for macrorheology that in most cases makes a molecular or structural interpretation of the rheological behavior impossible. For example, it is difficult to relate the rheology of a tissue such as liver or brain to the rheology of the specific cell types or extracellular matrices that differentiate one tissue from another. Any tissue is a composite of cells linked to each other either directly by cell–cell junctions or indirectly through the extracellular matrices, and each cell undergoes biochemical reactions during the course of a measurement that can take between a few seconds and many minutes to perform. Therefore, the viscoelastic properties of the whole composite cannot be easily linked to the viscoelasticity of any particular element such as a cytoskeletal network, the membrane, or the extracellular network; however, mixture theories based on relative volume fractions have been successful in understanding materials with inclusions. Specific molecular manipulations such as the loss of a single actin cross-linking protein can be detected as a significant change in a macroscopic sample of cells each bearing this defect, but predicting the rheology of a tissue from knowledge of the concentration of various filaments and cross-linkers is presently possible only for simple systems of purified proteins.

As cell and tissue mechanics become more of an integral part of basic cell biologic studies, a comprehensive understanding of micro- and macrorheology may help develop a unified model for how specific structural elements are used to form the soft but durable and adaptable materials that make up most organisms. The results of these studies also have potential for developing materials and methods for wound healing, cell differentiation, artificial organ development, and many other applications in biomedical research.

Glossary

Anisotropic: variable properties with respect to direction
Compliance (J): the relative extent to which a body yields to deflection by force
Gel point: time at which shear modulus of a system becomes greater than zero
Elasticity: the property of a material to deform to a defined extent in response to a force and then return to its original state when the force is removed
Form factor: geometric expression that quantifies deformation through calculation of a dimensionless strain from an observable quantity such as distance moved or angle of rotation
Inertia: tendency of a body to resist acceleration
Isotropic: directionally invariant
Linear elasticity: Young's or shear modulus constant over range of strains

Loss modulus (G''): measure of energy lost during a strain cycle; often expressed as the imaginary part of the complex modulus: $G'' = (\sigma_0/\gamma_0)\sin(\delta)$

Newtonian viscosity: viscosity independent of shear strain rate; linear relationship between shear stress and shear strain rate

Nonlinear elasticity: Young's or shear modulus that changes with strain

Non-Newtonian viscosity: viscosity dependent on shear strain rate; nonlinear relationship between stress and strain rate (i.e., shear thickening or thinning)

Phase angle (δ): The angular shift between the sinusoidally varying stress and strain in an oscillatory measurement. The value of δ is 0° for a purely elastic solid and 90° for a purely viscous liquid

Shear Modulus (G): a constant describing a material's resistance to deformation in shear; $G = \sigma/\gamma$.

Shear strain (γ): unitless parameter quantifying the extent of deformation after application of shear stress. For a cube, shear strain is ratio of lateral displacement over sample height. For other shapes, the form factor relates measured displacement to unitless strain

Shear strain rate: rate of change of shear strain; $\mathrm{d}\gamma/\mathrm{d}t$

Shear stress (σ): force parallel to a material's axis per unit area; $\sigma F/\mathrm{A}$

Stiffness: resistance of a body to deflection by force

Storage modulus (G'): measure of energy stored during a strain cycle; under sinusoidal conditions, the part of shear stress in phase with shear strain divided by shear strain; often expressed as the real part of the complex modulus: $G' = (\sigma_0/\gamma_0)\cos(\delta)$

Elongational strain (ε): fractional change in length or elongation; $\varepsilon = \delta/L$.

Stress (σ): force per unit area; $\sigma F/\mathrm{A}$

Viscosity (η): measure of resistance of a fluid to shear stress; $\eta = \sigma/\mathrm{d}\gamma/\mathrm{d}t$

Young's modulus (E): a constant describing a material's resistance to deformation in extension; $E = \sigma/\varepsilon$

Yield stress (σ_y): maximum stress applicable to a system before rupture occurs

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