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Measurement of viscoelastic properties of treated

AND UNTREATED CANCER CELLS USING PASSIVE

MICRORHEOLOGY

by

Devesh Bekah

HBSc, University of Toronto, 2008

A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Science

in the Program of

Biomedical Physics

Toronto, Ontario, Canada, 2010

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Measurement of viscoelastic properties of treated and untreated cancer cells using passive microrheology Master of Science 2010 Devesh Bekah Biomedical Physics Ryerson University

Experiments have shown that there is an increase in ultrasound backscatter from cells during cell death. Since cell scattering depends on the mechanical property variations, one step towards a better understanding of that phenomenon involves measuring the cells' viscoelastic properties. Two promising techniques used for such studies are particle tracking microrheology (1P) and two-point microrheology (2P). The main aim of this work is to develop and test the ability of both to measure changes in viscous and elastic moduli of breast cancer cells during chemotherapeutic treatments. First, the viscosities of glycerol-water mixtures measured using microrheology were found to be within 5% of rheometer values. The viscous and elastic moduli of 4% and 6% poly(ethylene oxide) solutions were successfully measured at 30°C and 37°C. For MCF-7 cells, a 10-fold increase in the elastic modulus was observed using 1P, without a corresponding increase in the viscous modulus. Thus, it was shown that MCF-7 cells undergo an increase in stiffness during apoptosis.

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Dedication

I would like to dedicate this thesis to my family: My father and mother, Doodave and Woormeela Bekah, my uncle and aunt, Loondeo and Anuradha Bekah, my brother, Sacheen Bekah and my cousins, Nandishi and Dhilan Bekah for their unconditional love and constant support throughout the course of this thesis. "Being a graduate student is like becoming all of the Seven Dwarves. In the beginning you're Dopey and Bashful. In the middle, you are usually sick (Sneezy), tired (Sleepy), and irritable (Grumpy). But at the end, they call you Doc, and then you're Happy." - Ronald Azuma

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Chapter 1

Introduction

1.1 Ultrasound Backscatter

The life cycle of a cell is finite and there are several ways in which they die and are disposed of, such as apoptosis and necrosis. In apoptosis, a series of controlled biochemical events result in the celllar remnants being disposed of without much damage to the organism. On the other hand, necrosis is not regulated, and can often lead to inflamation in the necrotic region, among other side effects. Some of the morphological changes of the cell that occur during apoptosis are nuclear condensation and DNA degradation. Also, the cell shrinks, and then fragments into apoptotic bodies that can be better cleared by the system. Nowadays, there are several chemotherapeutic drugs such as cisplatin and paclitaxel that have been be used to induce apoptosis in cancer cells. Cisplatin works by binding to DNA and causing cross-linking. This causes apoptosis. Paclitaxel, on the other hand, is a mitotic inhibitor.

It has been shown that high frequency ultrasound (20-60 MHz) has the ability to detect changes in dying cells. For example, the ultrasound backscatter was observed to increase by 6-13 dB as cells started undergoing apopsosis [10, 11, 23, 24]. Figure 1.1 shows the ultrasound

backscatter from a pellet of acute myeloid leukemia (AML) cells treated with cisplatin. Images taken at different timepoints show an increase in the ultrasound backscatter, with the highest intensity being at the 24 hour timepoint.



Figure 1.1: Ultrasound backscatter from an AML cell pellet treated with cisplatin at different timepoints. The colorbar at the bottom right of the image indicates the backscater intensity, which increases with time. The black line in the 48 hour image represents the scale of 1 mm. Adapted from Czarnota et al. [10].

If this change in the backscatter intensity could be fully characterized, it could potentially be used as a novel way of monitoring cancer treatments. However, this process is still not well understood. The cell is a complex structure, and it is not known exactly which part of the cell is mostly responsible for this change in ultrasound scattering. Theoretical models developed by Falou et al. [15] have aimed to develop finite element models of the backscattering from cells in order to understand the underlying physics. However, one of the inputs required for these models is the mechanical properties of both viable and non-viable cells. Therefore, developing a technique to measure properties of the whole cell, as well as different subcellular components, is crucial.

1.2 Shear-mode ultrasound Imaging

In conventional longitudinal-wave ultrasound imaging, very high frequencies are used (usually in MHz region) since higher frequencies yield better resolution. However, at those frequencies, the waves are also highly attenuating in tissue. The shear waves generated by ultrasound pulses have a much lower frequency (kHz range). Such shear waves also produce a much better soft tissue contrast when compared to conventional longitudinal waves. Whereas the bulk modulus of soft tissue spans only a small range, the range for the shear modulus is much bigger [34]. The main idea behind this technique is that in solids, both longitudinal and shear waves can travel. So, an ultrasound beam can be focused at different depths and locations within the tissue and the shear-wave generated by the radiation force of the beam can be detected [34]. The speed of the shear wave depends on the mechanical properties of the material. Hence, a mechanical map of the material at tissue level can be obtained. Recently, ultrasound imaging using shear-waves has been proposed as an alternative method of obtaining the changes in mechanical properties that occur in tissue due to pathology [1, 34]. For example, Aglyamov et al. [1] performed both numerical simulations and experimental measurements on bovine muscle tissue samples to show that shear waves can be used to estimate the viscous and elastic properties. In the shear-mode imaging technique, only information at the tissue level can be obtained. There is a dirth of published data on how those properties vary at the cellular level. Thus, developing a technique that can measure such properties at the cellular and even sub-cellular level, in the low frequency range, is important. For example, shear mode imaging could potentially be used as a treatment monitoring modality. If the stiffness of a tumor changes as a result of treatment, for example, images taken before and after treatment will be different. Thus, a novel imaging modality could be developed using shear-mode ultrasound imaging.

1.3 Cell Structure and Mechanics

The cell is one of the basic components of complex organisms. There are two main types of animal cells: eukaryotic (found in organisms such as mammals and prokaryotic (found in lower organisms such as bacteria). Eukaryotic cells have complex and dynamic structures, and their properties vary over different length and time scales. For example, the mechanical properties are altered in response to different kinds of chemical and physical stimuli [14]. A schematic of a typical eulkaryotic cell is shown in figure 1.2. The cell structure is highly heterogeneous. Different locations within the cell also have different properties due to the presence of a variety of sub-cellular structures Therefore, techniques with high spatial and temporal resolution are preferred when studying cellular mechanics.



Figure 1.2: A schematic of a eukaryotic cell showing some of the subcelular components. The cell is very heterogeneous over many lengthscales. Adapted from [14]

The cell's mechanical properties are primarily determined by its actin filament cytoskeleton [44]. Thus, one model that has been used to study cell mechanics is by reconstituting polymer networks (e.g of actin filaments) since there is more control of the properties of these networks [18, 45]. From experiments done in vivo, on cancer cells [22, 39, 28] it has been determined that cells are viscoelastic (for a comprehensive review, see Kaszra et al. [21]). In addition, there have been several mathematical models developed of the cell. Since the cell is very heterogeneous, modelling each and every component is extremely challenging. Several methods have been used. These include modelling the cell as a continuum, using a tensegrity model of the cytoskeleton or modelling the actin filaments as a foam [14]. In the continuum model, the viscoelastic behavior can be obtained using different arrangements of two elements connected in various ways: a linear spring for the elastic component, and a dashpot for a viscous component. Such models can provide a simple but good first approach in the study of cellular biomechanics. Bausch et al. [3], for example, measured the viscoelastic response of NIH 3T3 fibroblast cells using magnetic bead microrheometry and analyzed the viscoelastic response curves using models consisting of springs and dashpots. The tensegrity model uses actin microfilaments as the component providing tension and microtubules provide the compression component [14, 20, 37]. According to this theory, the interaction between these two components can be used to predict viscoelastic behavior. Finally, when modelling the actin filaments as an open-celled foam (random, solid matrix with open pores) as described by Gibson and Ashby [17], a unit cell model can be used for the cytoskeleton and the effective shear modulus can be estimated by calculatind the mechanical properties of individual fibers [14]. The cell's properties also change due to physiological processes; a cell in a diseased state will behave differently from a non-diseased state [28]. Thus, it is important to develop a better undestanding of the mechanical behavior of cells under various conditions.

1.3.1 Mechanical Properties Measurement

There have been several techniques developed with the aim of assessing the mechanical behavior of cells under a variety of conditions such as in response to chemical or physical stimuli, metastasis or migration. For example, there have been several attempts made to measure the response of cells to an applied force. Some of the techniques developed include atomic force microscopy (AFM), optical tweezers, magnetic beads and micropipette aspiration [14]. In AFM, a probe attached to a cantilever is used. When that probe touches the surface of a cell, it is deflected. In this way, very high resolution images of live cells can be obtained (probe deflections of up to 1 nm can be measured). The mechanical properties of cells can also be obtained using optical tweezers. For this method, a dielectric bead that has been injected into the cell is trapped in a potential well using two laser beams. From the motion of those beads, properties such as elasticity can be obtained. Magnetic bead rheometry is similar to the optical trap technique. However, instead of using lasers to trap the paramagnetic beads, a magnetic field is applied and this results in the motion of the beads. Finally, there is the method of micropipette aspiration. In this technique, a very thin glass pipette is used to generate a negative perssure after it has been brought in contact with the cell. By analyzing the deformation of the cell caused by the micropipette, one can extract the mechanical properties. For a more thorough description of the techniques mentioned above, see Ethier et al. [14]. Even though there are a host of techniques available to researchers, they all have limitations. For example, in AFM, only one frequency can be probed at a time. When the beads are moved by a magnetic field or using optical means, an active force is exerted on the cell, and this might have an effect on the cell mechanics.

One of the more recently developed techniques used to measure the viscoelstic properties of a wide variety of soft materials is microrheology. There are two main categories in which



Figure 1.3: Classification of microrheology techniques into an active method and a passive method. In the active method, an external force is applied to manipulate the probes. For the passive methods, the inherent thermal energy of the cell causes the probes to move. Adapted from [40].

microrheology can be classified: active and passive (see figure 1.3), both of which track the motion of particles present within the system to extract the viscoelastic properties. In active microrheology, an external force is used to manipulate articles embedded in the material of interest. For example, one active method depicted in figure 1.3 is using optical tweezers. In this method, laser beams are used to move particles and the response is measured. This technique can be used to measure the rheological response over a broad range of frequencies (theoretically can go up to MHz). Also, the response using a single probe particle at a time can be measured. This allows for very localized measurements [40]. However, by actively manipulating the system one risks causing damage. Also, the active methods usually require comparatively more sophisticated equipment. On the other hand, passive microrheology relies only on the Brownian motion of particles caused by material's inherent thermal energy. This method has the advantage of being minimally invasive, usually only requiring a way of getting the probes into the material. Also, passive microrheology requires less sophisticated and cheaper equipment. In passive microrheology, no external forces are exerted on the system

as the motion of particles is due to the inherent thermal energy of the system. In cells, any active force can modify the cell's properties due to cellular reorganization as a response to this force. Hence, passive microrheology possesses an advantage over active methods. Passive methods have been used by several groups in the study of cell properties. Denis Wirtz [44] has written a review of the various cell lines analyzed with particle tracking microrheology, a passive technique. In addition, there have been spatial maps of Swiss 3T3 fibroblast cells developed by Tseng et al. [39] showing that this technique can be used to measure the properties in different locations within the cell. They found that there is a lot more heterogeneity in the cytoskeleton, compared to reconstituted actin filament networks. Moreover, they observed that the cell behaved more like an elastic solid at high frequencies (63 rad/s), but was more like a viscous fluid at low frequencies (1 rad/s). There has also been work done on cancer cells. Li et al. [28] compared the viscoelastic properties of malignant (MCF-7) and benign (MCF-10A) breast cancer cells and showed that the mean squared displacements (MSD) in the malignant cell lines were consistently higher.

Another technique related to particle tracking microrheology, but developed more recently, is two-point microrheology. This technique, developed by Crocker et al. [8], was shown to more acurately measure the viscoelastic properties of complex materials. Instead of extracting the viscoelastic properties using data collected from single particle motion, this method aims to extract the same properties from the cross-correlated motion of particle-pairs. John Crocker's group demonstrated that the viscous and elastic moduli obtained using two-point microrheology were in better agreement with rheometer measurements for 0.25% guar solutions compared to one-point particle tracking microrheology [8]. Moreover, in TC7 cells, it was shown that the two-point MSD is consistently higher than the one-point MSD [7]. One-point and two-point particle tracking microrheology are two methods that could possibly be used in the analysis of a cell's mechanical behavior in response to treatment.

1.4 Motivation and Hypothesis

In this thesis, I aim to continue the work started by Ahmed El-Kaffas in our laboratory[13] and use both of the abovementioned passive microrheology techniques to measure the mechanical properties of treated and untreated cancer cells. *I hypothesize that both one-point particle tracking microrheology and two-point microrheology can be used to measure the mechanical properties (viscous and elastic moduli) of inhomogeneous viscoelastic materials, breast-cancer cells (MCF-7) and the changes that occur during cell death*. Preliminary work has shown that one point particle tracking microrheology can accurately measure the viscous moduli of glycerol/water mixtures as well as detect changes in the viscoelastic nature of guar/water mixtures [13]. In addition, Ahmed El-Kaffas showed that the viscous and elastic moduli of PC3 cells increase as a function of time after treatment using one-point microrheology, with the increase being more apparent in the elastic modulus [13]. However, the two-point microrheology method, which is more accurate in the measurement of the mechanical properties of specimens with elasticity, was not successfully developed.

The first step was to verify the accuracy and precision of both techniques for a purely viscous and a viscoelastic solution. This was done using different concentations of each solution to measure the range over which the techniques can be used. Moreover, since the cells had to be kept at 37°C, the measurements were also done at different temperatures. To do so, experiments were done with glycerol/water and poly(ethylene oxide)/water mixtures. Glycerol, being a Newtonian, purely viscous solution was ideal for the initial measurements. Poly(ethylene oxide) solutions are viscoelastic, inhomogeneous and non-Newtonian, which is why they were chosen to be used as a viscoelastic phantom. After the initial validation stages, both techniques were used to measure the MSD of fluorescent particles embedded within MCF-7 cells treated with a chemotherapeutic and to extract the viscoelastic properties at different frequencies. This thesis hence provided new insight (both qualitative and quantitative) into the changes in the mechanical properties that occur in breast cancer cells during cell death. The results from this work will also aid in obtaining more information on the mechanical properties of tumors at the cellular level, as well as help in developing more accurate models of the ultrasound backscatter from cancer cells undergoing cell death.

Chapter 2

Theory

2.1 Brownian Motion

The theory of Brownian motion forms the basis for both passive microrheology techniques described in this chapter. It is a mathematical model put forward by Albert Einstein, among others, to describe the random walk/diffusion of particles embedded in a fluid medium due to the bombardment of these particles by the molecules in solution. By characterizing this random walk, one can extract the mechanical properties of the medium (such as the viscous and elastic moduli). In this chapter, first, the theory of Brownian motion will be described and then the relationship between the material's viscosity and the particles' motion will be derived, following Einstein's formulation. These principles will then be extended to more complex, viscoelastic materials. Finally, there will be a discussion on the various ways in which particles can be embedded within cells.

2.1.1 Underlying Principles of Brownian Motion

The name "Brownian" motion arises from fact that botanist Robert Brown who is traditionally regarded as being one of the first to observe such a phenomenon. Using an inverted microscope, he observed the motion of clarika pollen grains in water. However, he did not adhere to the contemporary view that this irregular motion was due to the pollen grains being "alive". To prove his point, he stored the pollen in alcohol for 11 months. He then showed that the random motion was still observed. He observed similar behavior with other particles such as powdered metals, rocks, and carbon particles [4]. Even though he was unable to explain the theory behind this random motion, he is still acknowledged as the person who discovered "Brownian" motion.

It was not until the kinetic theory of gases was formulated that further development was made in the field of Brownian motion. In particular, the theory that the motion of particles could be due to the collision between the particles and molecules in the fluid was not proven. Ignace Carbonelle, Joseph Delsaulx and William Ramsey speculated, for example, that if small particles are bombarded from all sides, an imbalance would be caused and the particles would move in a certain direction. They noted that such motion was unpredicable, and hence a mathematical formulation would be impossible. However, there are several characteristics of Brownian motion that were noted [19]:

- The particles can move both in straight lines and rotate. Both of these types of motion are irregular.
- The Brownian motion of the particles are independent of one another, provided there are no collisions between these particles.
- If the particles are smaller in the medium, the motion is more vigorous.
- The motion does not depend on the chemical properties of the particles

- In less dense fluids, the motion is more vigorous.
- At higher temperatures, the particles move more vigorously.
- If effects such as evaporation are removed, the particles keep on moving.

One of the first people who attempted to describe Brownian motion mathematically was Thorvald N. Thiele in 1880 [26] in a paper on the principle of least squares. Louis Bachelier also proposed a theory in his PhD thesis in 1900. He based his theory on the stochastic behaviour of stock markets [2]. However, it was Marian Smoluchowski [36] and Albert Einstein [12] who independently formulated a solution to the Brownian motion problem. Smoluchowski published a one dimensional model that described the Brownian motion of a particle. There were, however, several assumptions made in this model. The mass, M, of the test particles were assumed to be much greater than the mass, m, of the bombarding molecules. He also assumed that the particle motion was only in one dimension, and that the probability for the test particle to be bombarded from any side was equal. Moreover, it was also assumed that after every collision, the test particle's velocity changed by an equal amount. These assumptions over-simplified the problem and as a result, his theory could only describe Brownan motion in a qualitative manner. For example, in a more realistic Brownian motion problem, it cannot be assumed that there are an equal number of collisions from each side of the test particle as it is moving. Also, the change in velocity of the particle after each collision won't be the same; it will follow a distribution.

Albert Einstein derived a more complete mathematical description of this phenomenon. He started by assuming that colloidal particles could be considered as large molecules [19] and that atoms did exist. Based on these assumptions, he tried to predict the behavior of other particles surrounded by atoms. By concentrating on the average behavior of a large number of
particles, he essentially proved that Brownian motion was a stochastic process characterized by a diffusion constant dependent on parameters such as Avogadro's number N, the absolute temperature, the test particle's size and the solution's viscosity. Indeed, his theory was not dissimilar to Bachelier's theory on the price fluctuation in the stock stock market [2]. This was all theoretical work, though. The experimental confirmation of Albert Einstein's theory, and hence proof that atoms did exist was brought by Jean Perrin at the Sorbonne in Paris [19, 33], work for which he received the Nobel prize in 1926.

2.1.2 Mathematical Formulation of Brownian Motion and the Stokes-Einstein Equation

As mentioned previously, Einstein's investigations on the osmotic pressure exerted by colloidal particles led him to an equation relating the diffusion coefficient to the fluid's viscosity, the test particle's size, the absolute temperature, and Avogadro's number [19]. The main assumtion he made was that the colloidal particles were much bigger than the particles in the solvent. Then, he went on to find the relationship between the diffusion of the particles and their displacement under the random walk. The following derivation has been adapted from Truskey et al. [38] and follows the same approach used by Albert Einstein in a one dimensional case. Consider the situation depicted in figure 2.1 in which N colloidal particles are suspended in a solvent in a region of length, L. Assume that the concentration of the particles is C(x).

If the solution is in thermodynamic equilibrium, C(x) is usually uniform, with an exception being when the particles are being acted upon by an external force, $F_x(x)$. In that case, the diffusion flux is balanced by a negative flux, $-v_xC(x)$, caused by convection. This can be



Figure 2.1: Particles (red circles) suspended in a solvent (blue circles). This is a closed system, where no particles are allowed to escape. The length of the container is L.

expressed using the following equation:

$$v_x \mathbf{C}(x) + \mathbf{D} \frac{d\mathbf{C}(x)}{dx} = 0$$
(2.1)

where v_x is the average velocity of all colloid particles due to thermal motion of the solvent particles and D is the diffusion coefficient. Now, if the Reynold's number is low (laminar flow regime), the velocity, v_x , is directly proportional to F_x . Thus,

$$F_x = \beta v_x \tag{2.2}$$

where β is a constant known as the frictional drag coefficient.

If equation 2.2 is substituted into equation 2.1, we obtain the following:

$$\frac{F_x}{\beta}C + D\frac{dC}{dx} = 0$$
(2.3)

A concept that will be used for the next few steps is that of "virtual displacement", $\delta u(x)$. In essence, it is an arbitrary displacement that preserves the equilibrium state of the system and goes to zero at the boundaries. Since this is a closed system which is in equilibrium, the virtual change, δ , in the Gibbs free energy, G, has to be zero. In other words,

$$\delta \mathbf{G} = \delta \mathbf{H} - \mathbf{T} \delta \mathbf{S} = \mathbf{0} \tag{2.4}$$

where H is the enthalpy, T is the absolute temperature and S is the entropy.

Moreover, since the system is closed and there is no expansion, the change in enthalpy, δH , is equal to the change in the particles' internal energy, i.e,

$$\delta \mathbf{H} = \int_0^L (F_x \delta \mathbf{u}) C A \, dx \tag{2.5}$$

The virtual change in entropy, δS , can be calculated using the Bolzmann equation. Generally, if the volume of a particle system increases from V_1 to V_2 , the net change in entropy is given by:

$$\Delta \mathbf{S} = k_b \, n_o \, \ln(\frac{V_2}{V_1}) \tag{2.6}$$

where

- *k_b*: Boltzmann constant
- n_o : Number of particles in the system

In the 1D case depicted in figure 2.1, let the initial volume, $V_1 = A\Delta x$. After the virtual displacement, the final volume, V_2 becomes

$$V_2 = A(\delta \mathbf{u}|_{x+\Delta x} - \delta \mathbf{u}|_x + \Delta x) \approx A(1 + \frac{\partial \delta \mathbf{u}}{\partial x}) \Delta x = V_1(1 + \frac{\partial \delta \mathbf{u}}{\partial x})$$
(2.7)

If we assume that $\frac{\partial \delta \mathbf{u}}{\partial x} \ll 1$, we can substitute equation 2.7 into equation 2.6 to obtain:

$$\Delta \mathbf{S} = k_b n_o \ln(1 + \frac{\partial \delta \mathbf{u}}{\partial x}) \approx k_b n_o \frac{\partial \delta \mathbf{u}}{\partial x}$$
(2.8)

If the above equation is integrated, the virtual change in entropy can be obtained:

$$\delta \mathbf{S} = \int_0^L k_b \mathbf{C} \mathbf{A} \frac{\partial \delta \mathbf{u}}{\partial x} \, dx = k_b \mathbf{A} [\mathbf{C} \delta \mathbf{u}|_0^L - \int_0^L \delta \mathbf{u} \frac{\partial \mathbf{C}}{\partial x} \, dx] = -k_b \mathbf{A} \int_0^L \delta \mathbf{u} \frac{\partial \mathbf{C}}{\partial x} \, dx \tag{2.9}$$

Note that to obtain equation 2.9, one has to make use of the boundary condition that the virtual displacement goes to zero at x = 0 and x = L.

Now that expressions for δG , δH and δS have been obtained, equations 2.4, 2.5 and 2.9 can be combined:

$$\int_{0}^{L} (F_{x}\mathbf{C} + k_{b}\mathbf{T}\frac{\partial\mathbf{C}}{\partial x})\,\delta\mathbf{u}dx = 0$$
(2.10)

Since the virtual displacement δu is arbitrary, the integral is only equal to zero if the integrand is zero. Therefore,

$$F_x C + k_b T \frac{\partial C}{\partial x} = 0 \tag{2.11}$$

Comparing equation 2.3 and equation 2.11, it can be concluded that the diffusion coefficient, D, is

$$D = \frac{k_b T}{\beta} \tag{2.12}$$

For spherical particles of radius, a, in a solvent of viscosity η , the frictional drag coefficient is

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$$\beta = 6\pi\eta a \tag{2.13}$$

Therefore, the Stokes-Einstein equation can be rewritten as

$$D = \frac{k_b T}{6\pi\eta a} \tag{2.14}$$

If it is assumed that the average displacement of a particle is $\langle \Delta r(\tau) \rangle$ given within a certain lag time or time interval, τ , the diffusion coefficient in n dimensions, D_n , is obtained from the following equation:

$$\langle \Delta r(\tau) \rangle = \sqrt{2nD\tau} \tag{2.15}$$

Therefore, if the motion of particles embedded within a solution is monitored, and the tracks analyzed, the viscosity, η , of that solution can be obtained:

$$\eta = \frac{k_b T \tau}{3\pi a \langle \Delta r^2(\tau) \rangle} \tag{2.16}$$

2.2 Viscosity, Elasticity and Viscoelasticity

The viscosity of a material is a measure of that material's resistance to flow as a fluid in response to either a shear stress (force per unit area applied parallel to a material's surface) or tensile stress (force per unit area applied perpendicular to the material's surface). In everyday terms, viscosity can be regarded as a measure of a fluid's "thickness". For example, water is "thin", implying it has a low vicosity. Honey, on the other hand, is "thick", meaning it has a high viscosity. The elasticity of a material is a measure of the propensity of a solid to return back to its original shape after deformation due to an applied stress. In a purely viscous fluid such as glycerol, energy is always dissipated due to viscous flow, and the material never regains its shape. On the other hand, for a purely elastic material, energy is stored in the material and when the deformation-causing stress is removed, the material regains its original shape. One example is a spring. The energy is stored when it is stretched, and it reverts back to its original shape when the external force is removed (assuming the elastic limit is not exceeded, and the object does not undergo permanent plastic deformation). A viscoelastic material is one that exhibits both viscous and elastic behaviour. The strain for a purely viscous material is linear with time. For a purely elastic material, it is instantaneous. However, for a viscoelastic material, there is a time-dependence on the strain (which might be nonlinear) [31].



Figure 2.2: Stress-strain curve for a viscoelastic substance. The red area represents the energy dissipated during a loading and unloading cycle.

There are several properties associated with viscoelastic materials:

1. The stress-strain curve exhibits **hysteresis** (see figure 2.2). So, when a load is applied and then removed, there is energy lost (for example, as heat).

- 2. When a constant strain is applied, the material tries to relieve the stress. This is known as **stress relaxation**.
- When a constant stress is applied, the material tends to deform slowly (increasing strain).
 This is called the creep.

The creep and stress relaxation are two very important terms needed to understand viscoelastic substances. The creep, J(t), is given by the ratio of the instantaneous strain in response to the applied stress whereas the stress relaxation, G(t), is given by the ratio of the instantaneous stress to the applied strain.

$$J(t) = \frac{\epsilon(t)}{\sigma} \tag{2.17}$$

$$G(t) = \frac{\sigma(t)}{\epsilon} \tag{2.18}$$

There were several attempts made by scientists such as Maxwell, Voigt and Kelvin to develop models for how viscoelastic substances respond to forces. Their model were mainly based on the use of springs to represent the elastic component and dashpots to represent the viscous component of the material. For example, the Maxwell model used a dashpot and spring connected in series and the Kelvin-Voigt model connected them in parallel [31]. However, these models grew more and more complicated with time. Ludwig Boltzmann came up with a model based on the relaxation function of the material. He showed that the complex shear modulus, $G(\omega)$, can be used as a measure of a substance's viscoelasticity. The complex shear modulus can be broken down into its real and imaginary components. The real component, $G'(\omega)$, is known as the elastic (storage) modulus and the imaginary component, $G''(\omega)$, is called the viscous (loss) modulus.

$$G(\omega) = G'(\omega) + iG''(\omega) \tag{2.19}$$

It should be noted that both the viscous and elastic moduli have a frequency dependence, as shown in equation 2.19. Thus a material might behave more like an elastic solid at high frequencies, but behaves like a viscous liquid at low frequencies. One example used by Wirtz et al. [44] is *S illyPutty*[®]. When left on its own for long timescales (i.e, sampled at very low frequency), it will flatten out, thus behaving more like a liquid. However, Silly Putty can also bounce and regain its shape when thrown against a wall, behaving more like an elastic solid during the short timescale impact (high frequency).

A rheometer is used to measure the viscous and elastic properties of different materials as a function, for example, of frequency and temperature. It typically works by applying a sinusoidally varying strain, $\epsilon(t)$, of a given amplitude, ϵ_0 , and frequency, ω , and then measuring the resulting stress in the material. This stress, $\sigma(t)$, can be broken down into a sine and a cosine component.

$$\sigma(t) = \sigma' \sin(\omega t) + \sigma'' \cos(\omega t) = \epsilon_0 (G'(\omega) \sin(\omega t) + G''(\omega) \cos(\omega t))$$
(2.20)

From equation 2.20 above, one can extract the elastic modulus from the sine (in-phase) component with $G'(\omega) = \frac{\sigma'}{\epsilon_0}$ and the viscous modulus from the cosine (out of phase) component with $G''(\omega) = \frac{\sigma''}{\epsilon_0}$ [44].

2.3 Microrheology

Rheometers have been used to probe the properties of various viscoelastic materials and cells are known to have a behavior that is dependent on the rate at which the deformation is applied. However, it is not possible to probe the full behaviour of cells using a conventional, macroscopic rheometer. To compensate for the inadequacy of using a rheometer to probe a cell's viscoelastic behavior over a wide range of frequencies, the technique of microrheology was developed. Microrheology refers to a family of techniques developed to study the mechanical properties of soft materials (such as polymer solutions and cells) at very small length-scales (sub-micron). For rheometer measurements, typically a few milliliters of sample are required. As a result, the study of rare samples cannot be done. In microrheology, only a few microliters are required. In addition, a rheometer can only measure the average properties of materials whereas for microrheology, the local viscoelastic response can be measured. This can be particularly advantageous for inhomogeneous materials such as cells. There are two passive microrheology techniques that will be used in this thesis, namely one-point particle tracking microrheology and two-point microrheology.

2.3.1 One Point Particle Tracking Microrheology (1P)

In one point particle tracking or 1P microrheology, the thermally-induced Brownian motion of thousands of micron size particles is tracked using video microscopy. The motion of such particles can be determined to sub-pixel accuracy using specialized software written by Crocker et al. [6]. Then, by calculating the mean-squared displacement (MSD) and using this in the Stokes-Einstein equation, the viscoelastic parameters can be obtained.

In theory, the frequency range measureable by 1P microrheology exceeds that of a rheometer. It has been shown that this upper limit is about 100 kHz for 1P as a result of inertial effects [16, 27]. In practice, however, this upper limit depends on the equipment available. When using a CCD camera coupled with a microscope, for example, frequencies of only up to 100 Hz can be achieved, depending on the desired spatial and temporal resolution. This limit depends on the maximum amount of time each particle remains in the field of view, which varies depending on the different microenvironments. Another advantage of 1P is that it can be used to probe spatially localized behavior by only tracking specific particles. This is especially help-ful in inhomogeneous materials, if both the average behavior and the local behavior needs to be determined. For example, Tseng et al. [39] used particle tracking microrheology to create a mechanical map of Swiss 3T3 fibroblast cells by analyzing tracks from different locations within the cell separately.

The MSD depends on the lag time, which is the time interval betwen two measurements of a particle's location. In purely viscous fluids, the MSD scales linearly with the lag time. In these cases, equation 2.16 can be used. However, in viscoelastic fluids, the relationship between the MSD and lag time is not as simple. Mason et al. [29] proved that for such complex fluids, there is a weak power law dependence:

$$\langle \Delta r^2(\tau) \rangle \propto \tau^{lpha}$$
 (2.21)

For a purely elastic material, $\alpha = 0$. Thus the MSD does not scale with lag time. On the other hand, in purely viscous, Newtonian fluids, $\alpha = 1$. Sometimes, the slope on a graph of log(MSD) vs $log(\tau)$ could be >1. This is the super-diffusive regime. For example, if there are active forces acting on the probes in 1P measurements, the slope might be bigger than 1. At higher lag times, a substance might exhibit more elastic behavior. This will be indicated by a decrease in the slope of the graph. Such behavior has been observed in fibroblast cells. Hence, these power law relationships can provide valuable insight into the mechanical behavior of cells at various timescales. As mentioned previously, the Stokes-Einstein equation in equation 2.16 cannot be used for complex fluids. A more generalized form has been developed to account for the non-linear dependence of the MSD on lag time and the presence of an elastic term in

addition to a viscous term. This equation used to obtain the complex shear modulus, $G^*(\omega)$, is called the Generalized Stokes-Einstein equation:

$$G^*(\omega) = \frac{k_b T}{\pi a i \omega \mathcal{F}_u \langle \Delta r^2(t) \rangle}$$
(2.22)

where $\mathcal{F}_u \langle \Delta r^2(t) \rangle$ is the unilateral fourier transform. The unilateral fourier transform has a range of zero to infinity. This range cannot be obtained using particle tracking microrheology since this technique has lower and upper limits as explained previously. There are several numerical methods that have been used to estimate the fourier transform from the finite range obtained in experiments. The one used in this thesis was developed by Thomas Mason [30]. In this method, the complex shear modulus is estimated by measuring the log-derivative of the MSD around each time-point. This estimate is then used to fit a line around the time point, producing a smoothed fit of the MSD. The slope of this line of best fit is $\alpha(\tau)$.

$$\alpha(\tau) = \frac{d \ln \langle \Delta r^2(\tau) \rangle}{d \ln \tau}$$
(2.23)

From the slope, the magnitude of the complex shear modulus can be calculated.

$$|G^*(\omega)| = \frac{k_b T}{\pi a \Gamma(1 + \alpha(\omega)) \langle \Delta r^2(\frac{1}{\omega}) \rangle}$$
(2.24)

where Γ is the gamma function. After computing the complex shear modulus at each timepoint and MSD, the viscous and elastic moduli can be obtained using:

$$G'(\omega) = |G^*(\omega)| \cos(\frac{\pi\alpha(\omega)}{2})$$
(2.25)

$$G''(\omega) = |G^*(\omega)| \cos(\frac{\pi \alpha(\omega)}{2})$$
(2.26)

2.3.2 Two-point Particle Tracking Microrheology (2P)

One point particle tracking microrheology has been shown to work very well for homogeneous systems such as glycerol. However, its ability to determine the mechanical properties of more complex fluids that are heterogeneous has been questioned. This is particularly the case when the probes are much smaller than the length scale of heterogeneity. To visualize the problem with 1P, consider the situation depicted in figure 2.3. This might for example be a polymer solution. One of the probes might be present in an area with a lot of polymer strands, while another probe might be in a region with no polymer chains. The MSD of those two probes are going to differ by a significant amount and will scale differently with lag time. As a result, the viscoelastic moduli calculated from those two probes will be different.



Figure 2.3: Heterogeneous system with spherical particles (blue) in two different regions. The mechanical properties of each pore is expected to be different due to differing concentrations of polymer strands (black).

To account for the shortcomings of 1P microrheology, the technique known as two point particle tracking or 2P microrheology was developed by Crocker et al. [8] and is based on the principle that all soft materials can undergo a form of internal Brownian motion. Instead of tracking individual particles, the aim of this method it to monitor the "cross-correlated Brownian motion" of all possible pairs of particles in the material. Thus, the long-range fluctuations of the material are measured instead of getting more localized meaurements. This is done by calculating the cross-correlation of the displacement vectors of each pair of particles along a certain direction (see figure 2.4). The technique is based on the principle that the strain field caused by the motion of one particle will affect the motion of another particle and that this effect will vary depending on the distance, d, between each of them. As a result, the crosscorrelation of particle pairs can be used to obtain a measure of the whole material's strain field [40]. In a homogeneous solution, the two-point cross correlation function, D_{rr} is proportional to each particle's MSD and decays as $\frac{a}{d}$, where a is the particle radius. However, this is not the case in heterogeneous system, where the $\frac{a}{d}$ dependance only occurs over a small range. Therefore, when doing data analysis for 2P, one has to choose a range over which the proportionality relation stated above holds when computing the two-point equivalent of the MSD.

The computational power required for 2P data analysis is greater than for 1P since crosscorrelation between all possible particle pairs over all lag times has to be calculated. In addition, the number of tracers required is larger. In our lab, it has been estimated that about 3000 distinct tracks are required to obtain statistically significant values for the MSD, which is in agreement with the arguments presented by Crocker et al. [8] in his discussion on the statistical requirements for computing the two-point MSD.

The first step in calculating the 2P MSD is to obtain the vector displacements of all the particles at all the specified lag times, τ , for all time points, t:

$$\Delta r^i_{\alpha}(t,\tau) = r^i_{\alpha}(t+\tau) - r^i_{\alpha}(t) \tag{2.27}$$

where $r_{\alpha}^{i}(t)$ is the displacement for particle i at time t using a coordinate , α , from a given coordinate system. In this thesis, the polar coordinate system is used. So, when computing the



Figure 2.4: Initial (blue) and final (red) position of two tracers a distance, d, apart. Displacement vectors $\Delta r^i(t, \tau)$ and $\Delta r^j(t, \tau)$ of particles i and j, respectively calculated along the line connecting the centres of both particles. The 2P MSD can be calculated by cross-correlating the two vectors.

2P MSD in the radial direction, $\alpha = d$, which is the distance between particle pairs as shown in figure 2.4. Then, the ensemble-averaged cross correlation of all possible pairs of particle displacements, can be calculated using the tensor product between particles i and j that are a distance, d, apart for a lag time of τ . This is then averaged over all possible particle pairs $(i, j)_{i \neq j}$ and times, t, as shown in the equation below:

$$D_{rr}(d,\tau) = \langle \Delta r_r^j(t,\tau) \delta[d - R^{ij}(t)] \rangle_{i \neq j,t}$$
(2.28)

where

- D_{rr} : Cross-correlation function in radial direction
- R^{ij} : Separation between tracers i and j

• δ : Dirac Delta function

For the range over which D_{rr} is proportional to $\frac{1}{d}$, the 2P MSD can be calculated as follows:

$$\langle \Delta r^2(\tau) \rangle = \frac{2d}{a} D_{rr}(d,\tau)$$
 (2.29)

As mentioned previously, 2P microrheology has been shown to measure the bulk viscoelastic properties more accurately than 1P for inhomogeneous systems such as cells. Moreover, it has been shown that this method is not as sensitive to the interactions between tracer particles and the materials. However, it also has some disadvantages. One of them is related to the number of particles required to obtain statistically significant results. Whereas for 1P only about 100 particles are needed (for homogeneous materials), for 2P up to 3000 different tracks are required. Crocker et al. [7] have also reported similar results. In addition, 2P is very sensitive to noise caused by vibrations and sample drift because particle motion due to these are highly correlated. This is particularly relevant to cells where active processes within the cell can cause super-diffusive motion of the probes. Hence, bead selection is a very important step in the data analysis.

2.3.3 Methods for Embedding Beads into Cells

For both 1P and 2P, fluorescent particles embedded within the sample have to be tracked. It is important that those particles undergo only Brownian motion and are not affected by other processes within the cell. Some have tracked endogenous particles such as lipid granules or mitochondria present in the cell [25, 46]. An advantage of this technique is that no foreign materials are introduced into the cell. However, the particles are more susceptible to motion caused by intracellular processes.

Valentine et al. [41] showed that carboxyl-modified particles injected directly into the cell

using a Xenoworks Micromanipulator and microinjection system (Sutter Instrument, California: http://www.sutter.com) do not interact significantly with materials in the cytoplasm (e.g. proteins) and their motion is mostly Brownian. One drawback of this technique, though, is that the cells can only be outside of the incubator for a maximum of 30 min to reduce stress due to changes in the environment and minimize contamination. As a result, only a limited number of cells can be injected, which means that there are not enough beads to properly perform 2P analysis.

It has also been shown that polystyrene microspheres can enter the cell via endocytosis [22, 42]. For this technique, no injection needles are used, so the cell is not damaged. Moreover, there are about 10 times more beads per cell compared to the microinjection technique in our experience. Hence more tracks are obtained. However, some of those particles can be trapped in vesicles that are bound to the cell membrane, which will affect their motion [42]. In addition, some of those particles can be actively transported within the cell. Exogenous particles such as fluorescent beads can also disrupt the cell structure as they can, for example, bind to the cytosleketon [42] whereas endogenous particles do not.

In the end, a compromise was required between having enough particles for tracking, reducing cell-microsphere interaction and not damaging the cell. With all of the above taken into consideration, it was decided that for any subsequent experiments, bead uptake by endocytosis would be used. the only difference was that care was taken not to track particles close to the cell membrane or particles that exhibited directed motion (an indication that these were being actively transported).

2.3. MICRORHEOLOGY

Chapter 3

Materials and Methods

3.1 Overview of Experiments

This chapter is divided into four sections, namely, sample preparation, experimental setup, experimental procedures and data analysis. Validation of both 1P and 2P microrheology was done using 2 solutions: glycerol (purely viscous phantom) and poly(ethylene oxide) (viscoelastic, non-Newtonian phantom). Glycerol was used to verify whether the current setup could be used to detect changes in viscous modulus with change in concentration and temperature as well as asses the precision and accuracy of the technique. The viscous and elastic moduli of poly(ethylene oxide) were measured at two different concentrations and temperatures. The analysis was done using both 1P and 2P to demonstate the following:

- 1. The ability to detect changes in viscous and elastic moduli with changing concentration using 1P.
- 2. The limitations of 1P microrheology property measurements for viscoelastic phantoms.
- 3. The accuracy and precision of 2P microrheology when measuring both viscous and elas-

tic modulus.

MCF-7 (Michigan Cancer Foundation-7) breast cancer cells were treated with paclitaxel and used to study the changes in mechanical properties that occur during cell death over a time period of 48 hours. Both 1P and 2P were used as data analysis methods. The last section of this chapter details the data analysis methods used to obtain the mechanical properties by tracking the motion of particles in the material of interest.

3.2 Sample Preparation

3.2.1 Glycerol

The stock glycerol solution (Medisca, Plattsburgh, NY: www.medisca.com) was diluted with distilled water to five different weight-by-weight (w/w) concentrations: 30%, 50%, 69%, 70% and 90%. To each of these solutions, 500 μ l of 0.20 μ m diameter fluorescent beads (catalogue no. G200, Thermo Scientific, Fremont, CA: http://www.thermoscientific.com) was added and the solution was allowed to mix overnight using an IKA C-MAG HS7 magnetic stirrer (Rose Scientific ltd., Edmonton, AB: http://www.rosesci.com).

3.2.2 Poly(ethylene oxide) (PEO)

Poly(ethylene oxide) of molecular weight 900 kD was purchased in powder form from Acros Organics (New Jersey, USA). Two different w/w solutions (4 % and 6 %) were made by dissolving the powder in distilled water as outlined by Williams et al [43]. In addition, both solutions were centrifuged at 2000 rpm for 10 min to remove any air bubbles and other residues (such as undisolved powder). Then, 500 μ l of the 0.20 μ m diameter fluorescent beads were added and the solutions were again gently stirred overnight.

3.2.3 MCF-7 Cells

Two weeks prior to any experiment, fresh MCF-7 cells were thawed and transferred to a culture flask containing Dulbecco's Modified Eagle Medium (DMEM), 10 % fetal bovine serum (FBS), and 0.10 % insulin. For cell microrheology, carboxyl-modified fluorescent polystyrene microspheres of 0.10 μ m diameter (Invitrogen Inc., Burlington, ON: www.invitrogen.com) were used. A volume of 2 ml from the 2 % stock solution was first dialyzed in Dulbecco's Phosphate Buffered Saline (DPBS, Cat. No. 14040-133 from Invitrogen) as outlined by Kole et al. [22] and the resulting solution was diluted in DPBS to a final volume of 40 ml. To allow for endocytosis of the polystyrene beads, 50 μ l of the above bead solution was added to the MCF-7 culture flask. After 48 hours, the cells were trypsinized and transferred to Delta-T culture dishes (Bioptech Inc., Butler, PA: www.bioptechs.com).

3.3 Experimental Setup

The experimental setup used for all particle tracking microrheology experiments is shown in figure 3.1. The microscope used was an Olympus IX-71 inverted microscope (Olympus Inc., Markham, ON: www.olympus.com). Three different objectives were available (10X, 40X, and 100X), the latter being used with oil immersion. In light microscopy, the smallest object that can be resolved is on the order of 0.40 μ m. Therefore, to allow for the use of smaller polystyrene beads, a fluorescence illumination system (EXFO Inc., Quebec, QC: www.exfo.com) was connected to the apparatus.

Videos were recorded using a Retiga EXi (QImaging Inc., Surrey, BC: www.qimaging.com) CCD camera connected to a PC. The software chosen to record videos was Streampix 3.0 (Norpix Inc., Montreal, QC: www.norpix.com). This software allows for the modification of various



Figure 3.1: The experimental setup consists of an IX-71 inverted microscope to which a fluorescent light source and a CCD camera are connected. The Delta-T dish temperature controller and the objective heater system are used to keep the cells at 37°C.

parameters that can affect the framerate and contrast. For example, reducing image exposure time can increase the frame-rate, but will reduce contrast between the beads and their background. Moreover, longer exposure time will lead to dynamic errors because the particle moves during the finite frame acquisition time [35]. One of the requirements for particle tracking is that each particle must cover at least 4 pixels (otherwise the position of each particle cannot be determine accurately). Since binning the video pixels increases contrast and frame-rate, but decreases the number of pixels per bead, a compromise was required between having a good contrast and a high framerate. It was decided that a framerate between 26 frames-per-second (fps) was acceptable for the cell experiements (where $0.10 \,\mu m$ diameter beads were used). To achieve that, a 400x400 pixel frame size was used with an exposure time of 10 ms. The pixel size at 100X magnification and no binning has been measured previously by Ahmed El-Kaffas to be 0.0645 μ m [13]. Thus, for 2x2 binning, the pixel size is 0.1290 μ m, which results in about 4 pixels per microsphere under fluorescence. A Delta-T dish temperature control system (Bioptechs Inc., Butler, PA: www.bioptechs.com) was also used to keep the cells at 37.4°C during live cell imaging. Since the objective can act as a heat sink, especially under 100X magnification where oil immersion is used, an Objective Heater from Bioptechs (Bioptechs Inc., Butler, PA: www.bioptechs.com) was also purchased and used to keep the objective at the same constant temperature. For the glycerol and PEO experiments, the framerate was optimized for the sample used. For example, for all glycerol experiments, a 36 fps framerate was used.

3.4 Experimental Procedures

3.4.1 Glycerol

Four PC20 chambers (Grace Bio-Labs Inc., Bend, OR: www.gracebio.com), each containing 30 μ l of glycerol of different concentrations (30%, 50%, 69%, 70% and 90%) and sealed with a 0.17 mm thick coverslip (Fisher Scientific, Ottawa, ON: www.fishersci.ca) were prepared. Each sample was placed, with the coverslip facing down under the microscope and allowed to settle for one hour to reduce sample drift. In addition, the objective heater system was used to heat the sample to the appropriate temperature, but the Delta-T dish tempreature controller was not used. Due to the small sample size, the temperature stablized after 1 hour. First, the temperature was fixed to 30°C using the objective heater. Under 100X magnification, ten videos, each 5000 frames long and at different locations in the sample chamber, were captured at 10 min intervals. This was to allow the solution to settle after moving to a different location. Then, using the 70% glycerol solution, videos (5000 frames at 36 fps) were captured at eight different temperatures between 30 and 42°C. This experiment was repeated three times.

The viscous modulus at 30°C and the temperature dependance of the viscosity for the 69% glycerol solution were also measured using a Physica MCR-301 rheometer (Anton-Paar, USA: www.anton-paar.com). For these experiments, the appropriate strain amplitude was determined by performing a strain test. It was set to 0.5%. Nian-Sheng Cheng has formulated equation 3.1 below relating the viscosity of a glycerol-water mixture, μ , to its concentration and temperature [5]. The experimental results for 1P were compared to the theoretical predictions to validate the experimental procedure for a purely viscous solution. In addition, the viscosity was measured using the rheometer for all five solutions. The experimentally determined viscosity of the 69% and 70% solutions were compared to the theoretical predictions to determine the sensitivity of

the microrheology method. The formula used for the theoretical calculations was:

$$\mu = \mu_w^\alpha \mu_g^{1-\alpha} \tag{3.1}$$

where

- μ_w : Viscosity of pure water,
- μ_g : Viscosity of pure glycerol,
- α : Weighing factor dependant on temperature and concentration (0-1).

3.4.2 Poly(ethylene oxide)

Similar volumes of PEO solution, one for each concentration (4% and 6%), were sealed in PC20 chambers using microscope coverslips. Again, for each experiment, the temperature was set to 30°C and the solutions were allowed to settle for 1 hour. For each concentration, twenty videos were captured, at 10 min intervals using the same settings as for glycerol. The rheometer was also used to measure the bulk viscous and elastic moduli of each solution for comparison. Finally, in order to test the accuracy with which 2P microrheology could measure the viscous and elastic moduli at another temperature (that of cells), ten videos were captured of the 4% solution at 37°C.

3.4.3 MCF-7 Cells

The cells plated in the Delta-T dishes mentioned in section 3.2.3 were FBS and insulin starved 24 hour prior to any experiments. Paclitaxel, a chemotherapeutic agent, was introduced at a concentration of 20 ng/ml to two of those culture dishes and two other dishes were left untreated. After placing one dish in the Delta-T dish temperature controller and allowing the

sample to settle for 10 min, thirty videos (1000 frames each, different locations) were captured at 0, 12, 24 and 48 hours after exposure to paclitaxel using the 100X magnification objective. Care was taken to set the focal plane about halfway between the surface of the cells and the bottom of the culture dish to avoid boundary effects and microspheres attached to the cell membrane. This procedure was then repeated for each of the culture dishes (including the controls). Usually, for each run of the experiment, sixty "treatment" videos and sixty "control" were obtained for each timepoint. It should be noted that even though the temperature was maintained at 37°C during video capture, the culture dishes were only allowed to be outside of the incubator for a maximum of 30 min to avoid cell contamination. Also, the contrast between the fluorescent particles and background is smaller for cell imaging compared to glycerol or PEO. There are also fewer beads in each field of view for the cells since the cell plating density was about 20% and each cell contained 10 beads on average. Therefore, to minimize loss of contrast due to photobleaching and to allow for recording from a greater number of fields-of view, the cell videos were kept shorter.

Finally, in collaboration with Ahmed El-Kaffas, the above experiment was repeated with 0.19 μ m microinjected polystyrene beads to determine whether the results would change if a different embedding method was used (For a detailed explanation of the bead injection procedure see [13]). Five culture dishes had to be used since the number of beads available per dish was much smaller (about 10 per dish). Again, the cells were serum and insulin starved 24 hours prior to treatment, and videos were captured at 0, 12, 24 and 48 hours. However, due to the limited amount of beads present, only 1P data analysis was feasible.

3.5 Data Analysis

After capturing the videos using Streampix, each of them was exported to a different TIFF stack for further analysis using Matlab 2009b. The code used for particle tracking was originally written by Crocker et al. [6] in IDL. This was then transcribed to Matlab by Daniel Blair and Eric Dufresne (http://physics.georgetown.edu/matlab/). The first step in the tracking process is to filter each frame of the video so as to remove noise, correct for image imperfections and hence improve signal-to-noise ratio between the particles and the background (as shown in figure 3.2(a) and (b)). A bandpass filter is used to remove both low spatial frequency and high spacial frequency noise. The lower limit for the bandpass filter is always set to one pixel whereas the upper limit is set to a few times the radius of the particle.

Next, as shown in figure 3.2(b), the locations of potential particles are determined by finding bright spots in the image that meet certain criteria such as minumum brightness threshold (to avoid tracking particles that are out of focus or just not bright enough) and radius of gyration (to avoid tracking microspheres that are stuck together or too close to each other). For example, if 0.20 μ m diameter particles are being used, the maximum radius of gyration (average size of each bright spot, assuming that each bright spot's location is its brightness-weighted centroid) can be set to 12 pixels, which corresponds to 1 μ m. Since the microspheres appear to be twice ther actual size under fluorescence, the 1 μ m radius of gyration is equal to twice the apparent diameter. In addition, by using all the information about each bright spot, such as the brightness distribution, the centre of each particle could be determined to sub-pixel accuracy. All of the above information (brightness, size, shape) can be used to distinguish unsuitable particles from suitable ones. The suitable particles tend to be circular, bright, and of a specific size. In contrast, the unsuitable particles and noise tend to be elliptical, less bright, and/or irregularly sized. The usual strategy for feature-finding was to change the input parameters and look at what kind of particles were being accepted or rejected by the algorithm. For cell experiments, more care was taken and each particle track was carefully analyzed. Particles found near cell boundaries were rejected since they were more likely to be bound to the cell membrane. Oversized particles were also rejected since those were most probably several beads that had



(c)

Figure 3.2: (a) Unfiltered image of 4% PEO solution (b) Filtered image with beads meeting specified criteria in red circles (c) Filtered image after tracking for 5000 frames, with a minimum track length of 100 frames

formed aggregates. Also, tracks that showed particles moving in a specific direction instead of undergoing Brownian motion were discarded. This was done by comparing the distribution of particle displacements in the x and y planes and check whether it was normal.

Particle tracks were formed by relating the locations of particles in each frame. First, the user had to specify a maximum displacement threshold. This threshold was chosen so that it is smaller than the maximum distance between particles (so as not to link two different particles into one track) but smaller than the expected maximum frame-to-frame displacement of a particle. This threshold, of course, depends on the material being used. For example, it was determined that for the 70% glycerol solution, a 0.20 μ m polystyrene microsphere had a maximum displacement of approximately 5 pixels. Thus, if the minimum particle separation is 10 pixels, the threshold above has to be between 5 and 10 pixels. Sometimes, in a very noisy video, spurious particles can get tracked by the algorithm despite all the precautions taken above. These tracks are, however, short and the maximum displacement is small. Therefore, by specifying a minimum track length in the routine, one can eliminate those tracks. The microspheres move in three dimensions, but the tracking is only done in two dimensions. Thus, particles might move in and out of the focal plane. To avoid tracking the same particle twice simply because it went out of the focal plane for a few frames, a "memory" parameter was used. For example, setting the "memory" to 5 would mean that if a particle is out of the focal plane for a maximum of five consecutive frames, then reappears at approximately the same location, it counted as a single particle. Otherwise, it counted as the beginning of a new track. An example of tracked particles is shown in figure 3.2(c).

Dr Maria Kilfoil from McGill University (Montreal, QC) provided the Matlab code that was used to compute the 1P and 2P viscoelastic moduli using the MSD (code is found here: http://www.physics.mcgill.ca/~kilfoil/downloads.html). This algorithm was originally written by Crocker et al. [8] in IDL and can be used to compute the complex shear modulus in addition to the viscous and elastic moduli (both 1P and 2P). Before calculating the MSD, any particle drift had to be removed. For example, if the microscope stage is not perfectly level or if it exhibits hysteresis, it can cause all the particles to drift in a certain direction. By applying this dedrifting routine, only the Brownian motion component was retained. A more detailed explanation of how the dedrifting routine works created by John Crocker and Eric Weeks can be found at [9]. The routine computes the average distance moved by each tracked particle from frame to frame in the x and y directions. The drift can then be reduced by substracting this average from the actual distance moved.

The MSD can then be calculated from the dedrifted tracks using the mean of all particle displacements from frame to frame for every possible lag time:

$$\langle \Delta r^2(\tau) \rangle = \langle (r(t+\tau) - r(t))^2 \rangle \tag{3.2}$$

where

- $\Delta r^2(\tau)$: MSD at lag time τ ,
- $r(t + \tau)$: position of one particle at time, $t + \tau$,
- r(t): position of one part at time, t.

For example, if the 5000 frames long video has a framerate of 50 fps, the minimum lag time is $\frac{1}{50}$ s and all frames are sampled to calculate the MSD. On the other hand the maximum lag time is $\frac{5000}{50} = 100s$ and to calculate the MSD, only the first and last frames are used. The 1P elastic and viscous moduli can then be directly computed using the code provided by Dr. Kilfoil using the MSD, bead radius and temperature as input parameters as required by the generalized Stokes-Einstein equation. The computation for the 2P moduli is more involved. There were four main parameters required by the algorithm. First, one had to specify a minimum and

maximum distance over which to compute the cross-correlation function. Usually, the minimum distance must be set to a value that is larger than the length-scale of heterogeneity. Lau et al. [25] found that a minimum correlation distance of 2 μ m is good. As mentioned previously, the two-point correlation approximation, D_{rr} , is only valid over the range where is inversely proportinal to $\frac{1}{d}$. The actual range depends on different factors such as the solution being used, the number of beads present. Thus, the maximum correlation distance has to be chosen bearing this in mind. From experience, it was found that a maximum correlation distance of 10 μ m was usually acceptable. Another input parameter needed to calculate the 2P correlation is the number of bins between the minimum and maximum correlation distances in which the data is stored. If a large number of bins is used, more points are obtained. However, the statistical power of each point is smaller. On the other hand, choosing fewer bins gives more statistical power. This also depended on the material being studied and the bead concentration. For our experiments, 25 bins was found through trial and error measurements to give accurate results. The fourth parameter needed is the maximum lag time over which the microspheres will be correlated. Since the particles drift in and out of the focal plane, at the higher lag times, there might be less particles being tracked. Hence, one has to set the maximum lag time to be less than the time during which a particle remains in focus. This value is usually set to one or two seconds, depending on the number of particles available at the higher lag times. If there are not enough particles available at the higher lag times, the uncertainty in the MSD will be greater, and this will lead to a bigger uncertainty in the viscoelastic moduli. The two-point correlation function can be then converted into a 2P MSD, from which the viscous and elastic moduli can be calculated (similar to 1P). It should be noted that for the 2P analysis, all field of views are processed simultaneously in contrast to 1P where each field of view is analyzed separately.

Chapter 4

Results and Discussion

4.1 Results

4.1.1 Glycerol

Figure 4.1 shows the comparison between the viscous moduli obtained using 1P and 2P for a 70% glycerol solution measured at 30°C. In both cases, the line is an average of the viscous modulus calculated using 10 different fields of view. For the rheometer data, only one run of the experiment was carried out. There is very good agreement between 1P, 2P and rheometer for that particular concentration and temperature. Furthermore, the slopes of the lines of best fit for 1P, 2P and rheometer were measured using the built-in matlab data fitter. The results and percentage difference between the theoretical predictions of Nian-Sheng Cheng [5] and the experimental results are shown in table 4.1. The theoretical prediction from equation 3.1 was 14.37 cP, which led to the maximum percentage difference being 5.15% (in the 2P case).



Figure 4.1: Viscous modulus vs frequency for a 70% glycerol solution at 30°C. The beads used were $0.20 \,\mu$ m in diameter. There is a very good agreement between 1P, 2P and rheometer values.

	1P	2P	Rheometer	Theoretical
Viscosities (cP)	14.87	15.10	14.79	14.37
Percentage Difference	3.55	5.15	3.00	X

Table 4.1: Percentage difference for the viscous modulus of a 70% glycerol solution at 30°C between experimental (calculated from slope of lines in figure 4.1) and theoretical prediction.

To verify the range over which the experimental results agreed with the theoretical predictions of Nian-Sheng Cheng [5], the viscosities calculated using both 1P and 2P were measured over a range of temperatures for a 69% glycerol solution, and at 30°C for the four other concentrations (30%, 50%, 70% and 90%). The results are graphed in figures 4.2 and 4.3. Also shown in figure 4.2 are the theoretical predictions for a 68% and a 70% solution. These were included to display the sensitivity of the viscosity to changes in concentration.

In addition, the viscosities at 30°C for all of the concentrations mentioned above were measured and the percentage deviation from the theoretical predictions was calculated. The results are shown in table 4.2.



Figure 4.2: Change in viscosity with changing temperature for a 69% glycerol solution determined by 1P microrheology and using a rheometer. For the microrheology experiments, 0.20 μ m beads were used. Also shown are the theoretical predictions for 68%, 69% and 70% solutions obtained using equation 3.1.



Figure 4.3: Change in viscosity with varying glycerol concentration at 30°C using both the 1P and 2P techniques. Again, 0.20 μ m beads were used for all experiments and the solid line represents the theoretical predictions based on equation 3.1.

	Viscosities (cP)			% Deviation from Theoretical	
Concentrations (%)	Theoretical	1P	2P	1P	2P
30	1.86	1.99	2.09	6.99	12.37
50	4.22	4.41	4.01	4.50	4.98
70	14.37	14.86	15.10	3.40	5.08
90	112.05	108.01	118.32	3.52	5.60

Table 4.2: Comparison between theoretical and experimental viscosities of different concentrations of glycerol at 30°C for 1P and 2P.

4.1.2 Poly(Ethylene Oxide)

For PEO, two different concentrations were used: 4% and 6%. To test whether the number of tracks analyzed would affect the derived viscous and elastic moduli, the data analysis was performed by gradually increasing the number of tracks analyzed. The results are presented in figures 4.4 (for 1P) and 4.5 (for 2P), respectively.



Figure 4.4: Graph of elastic (left) and viscous (right) moduli using 1P for a 6% PEO solution with 0.20 μ m beads at 30°C with increasing number of tracks. For 1P, the average viscoelatic moduli do not vary much with increasing number of tracks.

The viscous and elastic moduli for both PEO concentrations were calculated using 1P and 2P (with the same data set being used for each method). This experiment was repeated three times and the average and standard deviation were calculated. The moduli calculated using 2P are within one standard deviation of the rheometer results whereas for 1P they are significantly



Figure 4.5: Graph of elastic (left) and viscous (right) moduli derived using 2P for a 6% PEO solution with 0.20 μ m beads at 30°C with increasing number of tracks. There is a better agreement between 2P and rheometer measurements for the higher number of tracks.

different for both the 4% (figure 4.6) and 6% (figure 4.7) solutions.



Figure 4.6: Comparison between 1P, 2P and rheometer results for the elastic and viscous moduli of 4% PEO solutions containing 0.20 μ m beads at 30°C. Each data point is an average of 3 different measurements, and the error bars represent the standard deviation. There is very good agreement between 2P and rheometer, but 1P values are higher.

Finally, the viscous and elastic moduli at 37°C were measured for the 4% solution. The 2P and rheometer results are shown in figure 4.8.


Figure 4.7: Comparison between 1P, 2P and rheometer results for the elastic and viscous moduli of 6% PEO solutions containing 0.20 μ m beads at 30°C. Each data point is an average of 3 different measurements, and the error bars represent the standard deviation. There is very good agreement between 2P and rheometer, but 1P values are different.



Figure 4.8: Comparison between 1P, 2P and rheometer results for the elastic (left) and viscous (right) moduli of 4% PEO solutions containing 0.20 μ m beads at 37°C. Each data point is an average of 3 different measurements, and the error bars represent the standard deviation. There is good agreement between 2P and rheometer, but 1P values are different.

4.1.3 MCF-7

For the MCF-7 experiments cells were prepared and beads were internalized as described in section 3.2.3. The first step was to determine whether either 1P or 2P could be used to measure the viscous and elastic moduli of untreated MCF7 cells and whether the results were

reproducible. Figure 4.9 gives a comparison between the moduli derived using the 1P and 2P methods on cells for three separate trials of the experiment (one week apart). Again, the same dataset were used for both 1P and 2P. In each case, 60 fields-of-view were captured.



Figure 4.9: Elastic (left) and viscous (right) moduli of untreated MCF7 cells. Three trials of the experiment were done, one week apart. Data analysis done using 1P (top) and 2P (bottom). In each case, an average modulus over 60 fields-of-view was used.

Then, changes in viscous and elastic moduli of MCF-7 cells were determined over a 48 hour time window with and without paclitaxel treatment. This experiment was repeated twice and a similar pattern was observed in both cases for 1P. However, for the first experiment, only 20 videos were captured and not enough beads were available for 2P analysis. The results for the second group of experiments are shown in figures 4.10 and 4.11. The 2P data analysis

was only performed for the treated cellsbecause not enough tracks were available for 2P. An indication that the number of tracks is too small is when the output for the elastic modulus calculation is zero.



Figure 4.10: Elastic (top) and viscous (bottom) moduli obtained using 1P for MCF-7 cells with bead (0.10 μ m diameter) uptake through endocytosis. The graphs on the left are for the untreated cells (control), and those the right are for the treated cells. Average was taken over 60 fields-of-view, which corresponds to about 600 beads. Errorbars represent the standard error.

To test whether microinjection could be used as an alternative technique to endocytosis, the treated MCF-7 cell experiment was repeated using microinjected beads instead. The measurements were only done up to the 24 hour time-point in this case because not enough beads were available for analysis at the 48 hour timepoint. The results are shown in figure 4.12.



Figure 4.11: Elastic and viscous moduli obtained using 2P for MCF-7 cells treated with paclitaxel as a function of time after exposure to drug. Bead (0.10 μ m diameter) uptake was by endocytosis. Sixty fields-of-view were used, corresponding to about 600 beads. Here, no standard error could not be calculated since all videos were analyzed simultaneously.



Figure 4.12: Viscous and elastic moduli obtained using 1P for MCF-7 cells microinjected with 0.19 μ m diameter fluorescent beads and treated with paclitaxel. Video captured up to the 24 hour timepoint only due to not enough beads being available for analysis at the 48 hour timepoint.

4.2 Discussion

4.2.1 Glycerol

Overall, the glycerol experiments showed that the current experimental setup can accurately measure the viscous mdulus and viscosity of a purely viscous, newtonian fluid (simplest case) over a broad range of concentrations and over a range of temperatures relevant to cell experiments. In the first experiments, the viscous modulus obtained using 1P and 2P were compared to the results obtained using a rheometer. From figure 4.1, all three measurements are in agreement with one another. The maximum percentage difference between the viscosity from experiment and the theoretical predictions is at most 5.15% in the 2P case (Table 4.1). As mentioned previously, 2P microrheology is more susceptible to noise and sample drift. In addition, more beads are needed for 2P. Those can lead to bigger errors in the estimation of the MSD and subsequently, the viscous modulus. That might explain the bigger standard deviation. Nevertheless, a 5.15% percentage difference is still acceptable.

The next set of experiments were designed so as to determine whether the viscosity of glycerol could be accurately measured over a range of concentrations and temperatures. Again, as shown in figures 4.2 and 4.3, both 1P and 2P have very good accuracy over the range of concentrations and temperatures examined. From table 4.2, the percentage deviations from the theoretical values are all less than 10%, with the exception of 2P viscosity for 30% glycerol. In such a dilute solution, the MSD of the polystyrene beads is larger. Hence, they tend to drift in and out of the focal plane more often. As a result, there are less tracks that meet the minimum length requirement. In addition, the tracks tend to be shorter. That might explain why the error is bigger in this case. Moreover, the exposure time used to capture videos was the same in all glycerol experiments. At lower concentrations, this might lead to a bigger uncertainty in the

position of the bead at a certain time-point (since the bead is moving during the finite exposure time). This leads to a greater dynamic error as explained by [35]. This source of error might also be relevant to the other concentrations, but the effect is not as pronounced.

Another source of error might be due to the temperature fluctuations. The temperature in the rheometer could be more accurately controlled. It was estimated that in the rheometer, the temperature was 30 ± 0.1 °C. Hence, it was to be expected that the percentage deviation would be smaller. However, the temperature for the microrheology experiments was controlled by heating the objective, which in turn heated the PC-20 chamber holding the glycerol sample. As a result, the temperature uncertainty might have been higher (up to $\pm 1^{\circ}$ C). Moreover, it is also possible that the fluorescent light caused a small temperature increase at the focus. It was, however, not possible to measure the extent of this uncertainty. Finally, there is also an uncertainty in the actual concentration of glycerol to account for when comparing to theoretical values. In the sample preparation stage, the bead solution (1% bead concentration) is added to the glycerol. The mass of bead solution added was taken into account when calculating the final glycerol concentration. However, it is still possible that even though the aim was to obtain, for example, a 70% solution of glycerol, a 70.5% solution was made instead. This will result in a change of the viscosity from 14.37 cP to 14.93 cP, a 4% change. This should also be taken as a source of uncertainty.

4.2.2 Poly(Ethylene Oxide)

PEO was used as an example a non-Newtonian, viscoelastic phantom. The objective of the first experiment was to determine whether the viscous and elastic moduli depended on the number of beads used for analysis. In the 1P case, the number of beads did not significantly alter the viscous and elastic moduli results, especially at the low frequencies. For the viscous modulus,

however, there is bigger deviation at the higher frequencies. Since higher frequencies correspond to smaller lag times, the MSDs considered are smaller. As a result, a small uncertainty in the bead location will result in a large percentage error in the MSD (compared to the lower frequencies).

In the 2P case, the number of tracks analyzed were important. If there are not enough beads, one can see that there is a greater deviation at the two edges of the curve. For example, consider figure 4.6 and 4.7. At the two edges of teh curves for the 2P measurements, the standard deviation is larger. At the higher frequencies, if there are only a few beads, the standard error in the bead MSD is greater, leading to a bigger curvature in the viscous and elastic modulus calculated. At the lower frquencies (or higher lag times), the curvature is most probably due to an inadequate number of beads being analyzed since beads tend to drift out of the focal plane. About 3000 tracks were needed to obtain good agreement between the 2P values and the rheometer. However, even with as little as 1000 different tracks, there was a very good agreement between the viscous and elastic moduli obtained from the rheometer and two-point microrheology.

The next step was to see whether the viscous and elastic moduli could be measured accurately at two different concentrations. Two observations can be made by considering figures 4.6 and 4.7. First, data analysis using 1P microrheology does not produce accurate results, but it does suggest that the solution is viscoelastic. Moreover, the small size of the errorbars indicate that the method is precise. This is in agreement with data published by Williams et al. [43] who also did measurements with PEO solutions. Poly(ethylene oxide) is an inhomogeneous polymer solution. Hence there are regions of high polymer strand concentration (corresponding to high viscoelasticity) and low polymer strand concentration (low viscoelasticity). The MSD in 1P is determined from the motion of single beads. Hence, beads in dilute regions will have large MSDs whereas beads in concentrated regions will have a low MSD. The average

MSD from these results cannot accurately predict the bulk behavior of the solution but only measure local mechanical properties. On the other hand, 2P microrheology can accurately and precisely measure viscous and elstic moduli of both PEO concentrations; the 2P results agree with the rheometer measurements. In both cases, the values obtained using 2P are within one standard deviation of the rheometer values. Both techniques can however be used to observe changes in the viscous and elastic moduli, i.e. the viscous and elastic moduli measured using both 1P and 2P were higher in the 6% solution compared to the 4% solution.

The viscous and elastic moduli of the 4% solution at 37°C also agreed with the rheometer measurements. At 37°C, the uncertainty in the temperature is higher in the microrheology experiments. There is a bigger temperature gradient between the PC-20 chamber and the room. Therefore, it is to be expected that the agreement between the rheometer and microrheology values would be less. Nevertheless, even at 37°C there is a very good agreement between the rheometer and 2P. A better way of controlling the temperature might improve experimental results. For example, the PC-20 chambers could be heated from all sides instead of just heating the objective. In addition, a temperature controller system with a feedback loop can be used to maintain a constant temperature with a smaller uncertainty.

4.2.3 MCF-7

Both 1P and 2P can be used to determine the viscoelastic nature of MCF-7 cells (figure 4.9). However, as demonstrated by the experiments on PEO solutions, the 1P results are not accurate, while the 2P method can generate accurate results. Nevertheless, 1P data analysis can still be used to detect whether the viscous and elastic moduli change when either the temperature or concentration of the solution was increased. After repeating the measurement three times, one can see that the experimental results cannot be reproduced with good accuracy. For the 1P elastic modulus there is a significant difference among the three days at the lower frequencies. However as the frequency is increased, all lines seem to converge to a single value. For the viscous modulus, though, the differences between the three days are more consistent and the three lines do not converge. In the 2P case, two distinct lines are obtained on the two days in which enough data was available for analysis. The differences that are observed can also be attributed to some other factors. Again, the constant temperature is not easy to maintain, despite the use of the temperature controller. Using thermocouple measurements, it was estimated that the deviation from the set temperature was approximately 1°C. In addition, the natural variability within cell cultures has to be taken into account. The measurements were taken a week apart, meaning that the cells were passaged three times before the next set of experiments.

After establishing the possibility of using either 1P and 2P to obtain a viscous and an elastic modulus for MCF-7 cells, they were treated with paclitaxel and the changes in mechanical properties were determined over a 48 hour time period. First, consider the one-point microrheology results. For the elastic modulus, a gradual increase was observed over time in the treated cells and this was not seen in the control. This increase is especially noticeable at the lower frequencies. For the viscous modulus, there is again, a small increase, but it is not significant, as can be seen when compared to the untreated cells. However, it is not as apparent as the elastic modulus change. As shown by Pelling et al. [32], during early apoptosis, the cytoskeleton is constantly reorganizing. Since mechanics of apoptosis are highly dependent on the cytoarchitecture, Pelling demonstrated that there are changes in the elastic modulus at short timescales (<1 s), whereas the changes in the viscous modulus are only at the higher timescales (>60 s). Even though he performed his experiments using a combination of atomic force and confocal microscopy (thus probing different regions of the cell at lower frequencies), our work shows similar results. The microrheology experiments only probe at the short timescales (i.e. higher frequencies) and show a relative increase in the elastic modulus but not the viscous. It is possible that if lower frequencies are probed, similar results to the ones observed by Pelling might be obtained. When 2P data analysis was attempted, a different pattern from 1P was observed; There was no gradual increase in either viscous or elastic moduli. However, a change in the slope of the graph was still measured. With PEO, about 3000 tracks were needed to obtain accurate 2P results. In the case of MCF-7 cells, about 500 tracks from 60 different fields-of-view were used for 2P analysis. Given especially that cells have greater heterogeneity than PEO, it is likely that more beads are needed to see a similar pattern to that in figure 4.10. There are several ways in which the number of beads being tracked could be increased. For the above experiments, the cell plating density at the time of the experiments was about 20 % in the culture dishes. If this density is increased, this will lead to more beads being captured per field of view. Moreover, increasing the frame size from 400x400 to something larger will also increase the number of cells being imaged. However, this will lead to a decrease in framerate. In the end, a compromise must always be found between increasing the frequency and capturing enough beads for analysis.

Before performing any experiment, the cells are serum and insulin starved to stop cell division. However, it was not possible to determine in which part of mitosis each cell was in when treated. It might be possible that cells in different stages of the cell cycle have different mechanical properties. For example, during prophase, the nucleus becomes denser as the chromosomes form. Similar changes might also occur in the cytoplasm. This might explain some of the differences in figure 4.9. It could be that for different experiments, cells in different stages of cell division were used for analysis. Even then, a significant change in the average viscoelastic properties was obsserved when using 1P analysis. Moreover, during apoptosis, the cell membrane permeability increases. Such an increase would result in an influx of water and other molecules into the cytoplasm, which might lead to a decrease in viscosity. However, this was only observed at the lower frequencies in our experiments.

Chapter 5

Conclusion and Future Work

5.1 Conclusions

The techniques of one-point particle tracking microrheology (1P) and two-point microrheology (2P) were used in this thesis to measure the changes in mechanical properties that occur when MCF-7 cells undergo cell death. The hypothesis was that both one-point particle tracking microrheology and two-point microrheology could be used to measure the mechanical properties (viscous and elastic moduli) of inhomogeneous viscoelastic materials, breast-cancer cells (MCF-7). The first step was to validate the experimental procedure with a purely viscous (glycerol) and a viscoelastic (PEO) solution. For glycerol, I demonstated that both 1P and 2P can be used to accurately measure the changes in viscous modulus and viscosity that occur with increasing temperature and concentration. Using a PEO solution, I was also able to show that although 1P can be used to determine whether the viscosity and elasticity change when the temperature and/or concentration is increased, it does not do so accurately. To obtain accurate measurements of the viscous and elastic moduli of PEO, the 2P analysis method had to be used. It was estimated that about 3000 distinct particles were required to obtain values for the viscous and elastic moduli in agreement with rheometer measurements.

For the MCF-7 cells, the first objective was to confirm whether either method could be used to detect the viscoelastic nature of the cells on a consistent basis. The experiment was hence carried out three times over the span of three weeks. It was observed that even though the value for the moduli obtained on each of the three days were of the same order of magnitude, and followed the same trend, there were still some differences, especially at the lower frequencies. The differences were mostly attributed to natural variability within the cell line (for example, due different cell size and densities), uncertainties in the temperature as well as errors in the experimental procedure, as explained in the discussion section of chapter 4. Then the cells were treated with paclitaxel and the viscoelastic moduli calculated using both the 1P and 2P methodologies were calculated. Using 1P, it was observed that at the low frequencies, there is an increase in the elastic modulus, but not in the viscous modulus. These changes were not observed in the control experiments. For 2P, no corresponding trends were observed. Thus the hypothesis that 2P could be used to observe the changes that occur in the cell's mechanical properties as it undergoes apoptosis is inconclusive and further studies are required. The 1P results are also consistent with the results obtained by Pelling et al. [32] using AFM to calculate the moduli. It was shown that more beads are likely required for the 2P method to give more accurate results.

5.2 Future Work

Both 1P and 2P microrheology are very useful tools in the study of cell mechanics. In this work, the tracked particles were embedded within the cell by endocytosis. However there are other methods to insert tracers into cells. It would be important to compare the results obtained using various methods of embedding cells, such as microinjection and ballistic injection. The

ballistic injection method is particularly interesting in that it has all the advantages of microinjection, without most of its drawbacks. The number of particles available for tracking is much larger. Thus, more accurate 2P results might be obtained. Also, the damage to the cell is less than when using a microinjection needle. It will also be interesting to compare the same results to those obtained when using endogenous particles. By using different types of particles, one can obtain a more complete picture of the cell's properties.

Another important step would be to measure how the properties change depending on location within the cell (such as the nucleus and cytoplasm). For example, by probing different regions in the cytoplasm of Swiss 3T3 fibroblast cells, Tseng et al. [39] showed that there is a high degree of heterogeneity. Right now, all particles are embedded within the cytoplasm. Using the ballistic injection method, it will be possible to embed particles in all regions of the cell. Then, by restricting the particle tracking to specific locations, a mechanical map at different times after treatment could be obtained for different regions within the cell. It will therefore be important to develop the necessary techniques to make such measurements possible. Developing such a mechanical map will also shed more light on the question of which part of the cell is predominantly responsible for the increase in ultrasound backscatter when apoptotic cells are imaged at high frequencies.

Currently, the range of frequencies which can be probed is limited (1-100 rad/s). If we want to apply the results from these experiments to models of ultrasound backscatter from cell, we need to develop technology that allows us to capture videos at much higher framerates (i.e higher frequencies). A simple solution would be to acquire a CCD camera which can capture images at higher-speeds without compromising resolution or contrast. It might also be possible to extrapolate the results from the low frequency measurements to higher frequencies, something which has not been done so far using passive microrheology techniques. There are also some other improvements that can be done to the experimental setup. The temperature distri-

bution at the bottom of the culture dish is currently unknown and this leads to uncertainties in the actual temperature at the time of measurements. Using an infrared camera to determine the temperature profile will help reduce such an experimental error. Moreover, it is not known whether the fluorescent light is having any effect on the temperature. Again, an infrared camera will help determine that. Also, one of the requirements for optimal cell culture is to have a 5% CO_2 environment while capturing the videos. This is not possible using the current experimental setup. Creating a chamber around the culture dish on the microscope stage and perfusing that chamber with 5% carbon dioxide might improve the robustness of our experimental results. In our cell experiments, it was also very hard to image late apoptotic cells since these are non-adherent. Some materials such as polyethyleneimine and poly-L-lysine have been used to enhance cell adhesion. It is important to obtain more data on the properties of cells in late apoptosis (or necrosis) since one hypothesis is that the more significant changes in the mechanical properties occur during that stage. Moreover, right now, it is not possible to tell whether the cells are undergoing apoptosis or necrosis. Using markers that tag for specific types of cell death might help us differentiate between the two and further refine our experimental results. It is also not possible to determine which cells are in the early or late apoptotic stage. At each time-point, an average measurement is taken, under the assumption that the number of dying cells increases with time. To further refine the experimetal procedure, the cells can be tagged with fluorescent markers such as Annexin V and propidium iodide (PI). Annexin V, which fluoresces green, binds to phosphatidylserine, which is translocated to the outer cell membrane during early apoptosis whereas PI stains nectotic cells with red fluorescence. Finally, the focus in our laboratory is on breast cancer. The only cell line studied so far has been the MCF-7 cells. Thus, for completeness, it will be very important to perform similar studies on different cell lines such as AML, ZR-75-1 (breast carcinoma showing oestrogen dependence) or MDA-MB-231 (virulent and rapidly-growing ductal carcinoma) cells.

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