MEASURING THE MICROSCALE MECHANICAL PROPERTIES OF FIBRIN FIBERS AND CANCER CELLS

BY

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TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ iv

LIST OF TABLES .......................................................................................................... v

LIST OF ABBREVIATIONS ........................................................................................ vi

ABSTRACT .................................................................................................................. vii

Chapter

I. INTRODUCTION ...................................................................................................... 1

II. THE MECHANICAL PROPERTIES OF DRY, ELECTROSPUN FIBRINOGEN
    FIBERS .................................................................................................................. 14
    Published in Materials Science and Engineering: C, March 2012

III. THE MECHANICAL PROPERTIES OF SINGLE FIBRIN FIBERS FORMED
    FROM BLOOD PLASMA ....................................................................................... 40

IV. THE DIFFUSION OF EGFP AND MSH2 IN BREAST CANCER CELLS AS
    MEASURED BY RASTER IMAGE CORRELATION SPECTROSCOPY ... 61
    Submitted to New Journal of Physics, October 2013

V. A COMPARISON OF RICS & FRAP FOR MEASURING DIFFUSION OF
    SMALL PROTEINS IN LIVE CELLS ....................................................................... 86

VI. CONCLUSION ........................................................................................................ 107

APPENDIX .................................................................................................................. 122

SCHOLASTIC VITAE ............................................................................................... 131
# LIST OF FIGURES

1. Schematic of Electrospinning Setup ................................................................. 4
2. Schematic of Manipulation of Fibrinogen Fibers ............................................ 5
3. Experimental Flowpath for RICS. ................................................................. 7
4. Electrospinning Apparatus ............................................................................ 20
5. Electrospun Fibrinogen Fiber Manipulation ............................................... 21
6. Fibrinogen Fiber Extensibility ..................................................................... 25
7. Fibrinogen Fiber Elastic Limit ..................................................................... 27
8. Incremental Stress–Strain Curves and Fiber Relaxation ............................ 29
9. Fibrin Fiber Manipulation .......................................................................... 48
10. Fibrin Fiber Stress–Strain Curves and Relaxation ...................................... 50
11. Single Fiber Modulus and Fibrinogen Glycation ...................................... 52
12. Fibrin Fiber Modulus as a Function of Diameter ....................................... 53
13. Fiber Extensibility as a Function of Fibrinogen Concentration ............... 54
14. DIC and Fluorescence Image of Cancer Cells ............................................. 67
15. Image of RICS Analysis ............................................................................. 70
16. Results of RICS Computer Simulations ..................................................... 72
17. Summary of RICS Diffusion Measurements ............................................ 75
18. Example FRAP Recovery Curve ................................................................. 93
19. Example Fits to RICS Data ....................................................................... 94
20. Diffusion Coefficients Measured Using FRAP and RICS ......................... 98
21. Histogram of Bimodal Distribution of Diffusion Coefficients ................. 100
LIST OF TABLES

1  Summary of Cancer Cells Used in this Study......................................................... 8
2  Comparison of the Mechanical Properties of Electrospun Fibrinogen, Native
    Fibrin, Electrospun Collagen and Native Collagen Fibers .............................. 31
3  Description of Human Mammary Epithelial Cells ............................................. 65
4  Comparison of Cytoplasmic and Nuclear Viscosity Live Cells ......................... 78
5  Comparison of Mechanical Properties of Natural and Synthetic Fibers for Tissue
    Engineering ..................................................................................................... 109
6  Comparison of Cellular Viscosity Measured by Small Particles in Live Cells .. 115
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscope</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine Pituitary Extract</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged Coupled Device</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>FCS</td>
<td>Fluorescence Correlation Spectroscopy</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>FRAP</td>
<td>Fluorescence Recovery After Photobleaching</td>
</tr>
<tr>
<td>HFP</td>
<td>1,1,1,3,3,3-Hexafluoro-2-Propanol</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser Scanning Microscope</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Media</td>
</tr>
<tr>
<td>NOA-81</td>
<td>Norland Optical Adhesive</td>
</tr>
<tr>
<td>P53</td>
<td>Tumor Protein 53</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic Acid</td>
</tr>
<tr>
<td>RICS</td>
<td>Raster Image Correlation Spectroscopy</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Cell Growth Factor</td>
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ABSTRACT

Justin Sigley

MEASURING THE MICROSCALE MECHANICAL PROPERTIES OF FIBRIN FIBERS AND CANCER CELLS

Dissertation under the direction of

Martin Guthold, Ph.D., Associate Professor of Physics

and

Keith Bonin, Ph.D., Professor and Chair of Physics

Microscale material properties dictate the macroscale behavior of biological systems. Fibrinogen, one of the most abundant proteins in blood, is converted into fibrin fibers that perform the essential mechanical task of stemming the flow of blood. Fibrinogen fibers can be fabricated by a technique called electrospinning. We studied the mechanical properties of dry, electrospun fibrinogen fibers using a combined atomic force/fluorescence microscopy technique. The mechanical properties of these electrospun fibers are important due to their potential use in tissue engineering and their biocompatibility. The same atomic force/fluorescence microscopy technique is used to measure the mechanical properties of fibrin fibers formed from patient plasma. The mechanical properties of blood clots have been related to diseases such as cardiovascular disease and diabetes, but the mechanisms responsible for their mechanical properties are not well understood. The glycation of fibrinogen, a marker for glycemic control in diabetic patients, did not affect the mechanical properties of individual fibrin fibers. The
modulus of the fibers was found to be directly related to the diameter of the fibers and provides evidence for a non-uniform density of protofibrils within the fiber.

Cancerous and non-cancerous cells have different mechanical properties arising from biochemical alterations as normal cells transform to cancer cells. This transformation may affect the mobility of proteins and small molecules within the cell. We used a novel technique called Raster Image Correlation Spectroscopy (RICS) to measure the diffusion coefficients of fluorescent proteins in living cells. We found the diffusion coefficients of these proteins are not affected by neoplastic transformation in the cytoplasm, but the mobility of the fluorescent proteins is altered in the nucleus of the cells. This suggests neoplastic transformation alters the intra-nuclear structure on a length scale similar to the sizes of the proteins measured. We cross-validated the RICS results with Fluorescence Recovery After Photobleaching (FRAP) experiments. The RICS and FRAP results agree in 87% of the measurements. We then demonstrated the accuracy of RICS measurements by performing a RICS analysis on quantum dots undergoing a programatically controlled 2D random walk. Direct confirmation of RICS results provides progress toward a gold standard for measurements of the dynamics of molecules in live cells.
CHAPTER I

INTRODUCTION
In the last few decades, biophysics has emerged as a powerful discipline combining theories and measurements from physics to the study of biological systems. It is becoming increasingly apparent that molecular and microscale material properties dictate the macroscale behavior of biological systems. Two systems where this is especially true are fibrin fibers and cancer. In this dissertation, I will present measurements of the mechanical properties of fibrin(ogen) fibers and human breast cancer cells. I describe the novel methods used to extract this information and how these measurements can be used to further our understanding of these systems as well as provide insight into possible future diagnostics and prophylactics.

Fibrin fibers are the main constituent of blood clots. In the body, fibrin fibers are formed when thrombin cleaves the fibrinopeptides A and B on fibrinogen exposing positively charged binding sites (knobs) A (Gly-Pro-Arg-Val) and B (Gly-His-Arg-Pro). The positively charged knobs fit into the negatively charged “a” and “b” pockets. These interactions cause the fibrin monomers to form in a half-staggered orientation into a two-stranded protofibril [1], [2]. Once the protofibrils grow to an average length of 15 monomers, they laterally aggregate to form fibers that branch into the three dimensional mesh network that stems the flow of blood [1], [3]. The mechanical properties of individual fibrin fibers have a critical influence on the occurrence of heart attacks, strokes, and embolisms [4]. Increased coagulation activity and disturbed fibrinolysis are predictors for future vascular events [5].

Experiments have been performed to study the mechanical properties of whole fibrin clots such as modulus, elasticity, and creep [6]–[9]. However, the small size of fibrin fibers has made it difficult to study the mechanical properties of individual fibers.
In order to properly model the whole clot, the properties of the individual fibers constituting the clot are needed. Some work has recently been done to elucidate these properties [10], [11]. One area that can greatly benefit from measurements of single fiber properties is tissue engineering. Due to their low immunogenicity, biodegradability, and general biocompatibility, fibrin(ogen) fibers may be good candidates for tissue engineering scaffolds. The performance of biomedical devices depends on the mechanical properties of their components [12]. Furthermore, these devices will often be stored in a dry environment but used in a wet environment. Thus, it is important to study their mechanical properties in both of these environments. We also want these devices to be low cost and easy to manufacture. For those reasons, electrospinning has become an increasingly popular technology used for the production of nano-scale fibers for use in tissue engineering. In electrospinning, polymers are dissolved in a solution which is extruded through a blunt-tip needle in an electric field (Figure 1). As the electric field is increased between the needle and a grounded collector plate, the force of the electric field on the polymer solution becomes greater than the surface tension of the solution and a Taylor cone forms expelling the solution from the tip [13]. As the solvent dries, charges within the solution stream migrate to the surface of the fiber. The electrostatic repulsion caused by small bends in the fiber, cause it to whip and elongate until the fibers are collected on a grounded substrate [14]. Electrospinning has been used to fabricate biomaterials for bone, ligament, blood vessel, peripheral nerve, skin, cartilage, muscle, heart, and heart valve [15]–[22].
Figure 1. A schematic of the electrospinning setup. A syringe pump is used to regulate the flow of polymer solution through the blunt tipped needle. The needle is charged to a high potential using a voltage supply. The Teflon tubing is used to position the highly charged needle away from the metal syringe pump so that the fibers are collected on the grounded copper plate opposite the needle instead of the metal syringe pump.

In Chapter II, I describe a combined atomic force/fluorescence microscope technique that is used to measure the mechanical properties of dry, electrospun fibrinogen fibers. The fibers are created on a striated substrate with 13.5 μm wide gaps between 6.5 μm wide ridges. The technique uses standard AFM cantilevers to laterally stretch fibers with a force in the range of $10^{-2}$ to $10^4$ nN, making it applicable to the study of many nanoscopic fibers [23]. An inverted fluorescence microscope is used to monitor the entire manipulation process (Figure 2).
The lateral force applied to the fiber is measured by the torsion of the cantilever given by the deflection of a laser beam reflected off of the back of the cantilever [24], [25]. The modulus, extensibility, elastic limit, and relaxation times of these fibers are measured and a molecular mechanism is proposed to account for these properties. These results are then compared to measurements of other fibers that have been measured using the same technique.

The AFM technique is used again in Chapter III to measure the mechanical properties of fibrin fibers formed from patient blood plasma. To my knowledge this is the first time anyone has studied the individual fibrin fibers formed from plasma instead of a purified fibrinogen system. This approach more closely resembles the polymerization process that takes place in vivo and allows for the study of the effects of diseases on the mechanical properties of fibrin fibers. In this study we decided to study the effects of diabetes on the properties of single fibrin properties.
Diabetes is a risk factor for cardiovascular disease (CVD) increasing one’s risk by 2 – 4 times [26] with 68% morbidity in diabetic patients being due to CVD [27]. The relationship between diabetes and CVD is not well understood. Some CVD patients are found to have alterations to the properties of fibrin clots and research has suggested a relationship between macrovascular disease and diabetic control [28] prompting this study. Several studies have been done on whole clots in CVD patients and diabetic patients, but many of these studies have conflicting results [26], [29]–[33]. I measured the mechanical properties of single fibrin fibers formed from patient plasma and compared these results to single fibers made using the normal purified system. In both cases the fibrin polymerizes after activation of thrombin and is stabilized by factor XIII. I also used the results to hypothesize that fibrin fibers are not a homogenous collection of protofibrils but instead vary in density as the fiber diameter grows. A molecular mechanism for this process is proposed.

The second part of this dissertation is related to the mechanical properties of human breast cancer cells at the nanometer scale. Advances in biophysics are providing opportunities to establish connections between cell mechanics and biological function [34]. Changes in the mechanical properties of cells as they transform from healthy to cancerous, may give insight into the biochemical alterations the cells are going through which can lead to better diagnostics and treatments. Several studies have been done to measure the global mechanical properties of cells [35]–[40] which often relate changes in the cell’s disease state to changes in the cytoskeletal structure. Instead, I studied the dynamics of proteins within cells to determine if changes to the mechanical properties of cells due to cancer were able to affect the motion of those proteins.
There are several techniques that have been developed to measure the intracellular dynamics of small molecules such as particle tracking [41] and fluorescence recovery after photobleaching (FRAP) [42], and in this study I used both raster image correlation spectroscopy (RICS) and FRAP. RICS can accurately measure molecular diffusion on the microseconds-to-seconds timescale [43]–[46] in living cells using a laser scanning confocal microscope. It takes advantage of the inherent time information stored in a raster scanned image as the confocal scanning laser moves across the sample. If a fluorescent molecule being imaged moves to a nearby pixel, there will be a correlation in the fluorescence intensity fluctuations of these pixels related to the time it takes for the laser to move to the next pixel. These fluctuations are measured for each pixel in the image and averaged over many images to create a spatial correlation function from which diffusion coefficients can be extracted (Figure 3).

**Figure 3.** A schematic of the experimental flowpath for RICS. The pixels within a raster scanned image are shifted with respect to each other and the intensities multiplied to create an autocorrelation function. That function is fit to a spatial correlation function and the diffusion coefficient is determined by the fit.
In these studies, I measured the diffusion coefficient of fluorescent proteins in four different types of human mammary epithelial cells which simulate progressive stages of neoplastic transformation. The cell types included are healthy HME, immortal HME+LT+hTERT, tumorigenic HMLER, and metastatic MDA-MB-231 cells. A summary of the cell types used is included in Table I.

**Table I.** A summary of the human mammary epithelial breast cancer cells used.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Immortal</th>
<th>Tumorigenic</th>
<th>Metastatic</th>
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<tbody>
<tr>
<td>HME</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>HME + LT, TERT</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>HMLER</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
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</table>

Genetic instability is the underlying driver of cancer initiation and progression [47]. DNA repair processes play an important role in genetic fidelity. One of these repair processes, called mismatch repair, recognizes and fixes insertion and deletion errors that occur during replication. The protein that identifies mismatched bases is a heterodimer called MutSα which consists of the proteins MSH2 and MSH6 [48]. These proteins must find their way to the DNA within the nucleus making them susceptible to changes within the cellular environment that may impede their movement. In Chapter IV, I used RICS to measure the diffusion coefficients of enhanced green fluorescent protein (EGFP) and EGFP-MSH2 constructs.

While these techniques used to measure dynamics of molecules are providing new insight into the biological function of cells, the interpretation of results can be difficult.
All these techniques require a kinetic model to be fit to the data to extract quantitative information. It has become increasingly recognized that the choice of kinetic model can cause very different results for the same or similar studies [49], [50]. A standard set of protocols for these techniques has not yet been developed. Therefore, to increase confidence in such measurements, these techniques need to be cross-validated with other orthogonal techniques. In Chapter V, I cross-validated the RICS measurements from Chapter IV with FRAP measurements. While the results are in agreement in most cases, there are a few measurements where the results do not agree. To determine which results were the most accurate, I performed an experiment that imitates RICS experiments, however, I had direct control over the motion of the particles being imaged. This technique provided verification of the RICS results in experiments best modeled by simple diffusion, but may be expanded to model more complicated dynamics. The accuracy of RICS results may now be directly determined and therefore used to cross-validate other techniques.

REFERENCES


CHAPTER II

THE MECHANICAL PROPERTIES OF DRY, ELECTROSPUN FIBRINOGEN FIBERS

Justin Sigley, Stephen Baker, Christine Helms, Joel Stitzel, Joel Berry, Keith Bonin, Martin Guthold

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ABSTRACT

Due to their low immunogenicity, biodegradability and native cell-binding domains, fibrinogen fibers may be good candidates for tissue engineering scaffolds, drug delivery vehicles and other medical devices. We used a combined atomic force microscope (AFM)/optical microscope technique to study the mechanical properties of individual, electrospun fibrinogen fibers in dry, ambient conditions. The AFM was used to stretch individual fibers suspended over 13.5 μm wide grooves in a transparent substrate. The optical microscope, located below the sample, was used to monitor the stretching process. Electrospun fibrinogen fibers (diameter, 30–200 nm) can stretch to 74% beyond their original length before rupturing at a stress of 2.1 GPa. They can stretch elastically up to 15% beyond their original length. Using incremental stress–strain curves the viscoelastic behavior of these fibers was determined. The total stretch modulus was 4.2 GPa while the relaxed elastic modulus was 3.7 GPa. When held at constant strain, fibrinogen fibers display stress relaxation with a fast and slow relaxation time of 1.2 s and 11 s. In comparison to native and electrospun collagen fibers, dry electrospun fibrinogen fibers are significantly more extensible and elastic. In comparison to wet electrospun fibrinogen fibers, dry fibers are about 1000 times stiffer.

INTRODUCTION

With a concentration of a few g/l, fibrinogen is among the most abundant proteins in blood plasma; it is also non-immunogenic and relatively easy to purify. Its major physiological role is to form a meshwork of nanoscopic fibrin fibers – the major structural component of a hemostatic blood clot – in the event of vascular injury. Fibrinogen also
plays a role in platelet adhesion and wound healing [1]. These physiological and biological properties of fibrinogen may make fibrinogen fibers a good candidate for use in biomedical devices, such as tissue engineering scaffolds, drug delivery vehicles, cell substrates, wound pads, sutures, and others [2]. Some of these devices are used in wet (aqueous) conditions; others may be used in dry and/or wet conditions. Most of these devices, such as drug delivery vehicles, wound pads and sutures, may transition from a dry (manufacture, storage) to a wet environment (application). It is, thus, important to investigate their properties in dry (ambient) and wet conditions.

The performance of biomedical devices does not only depend on the physiological and biological properties of their components, but also on the mechanical properties of their components. Clearly, the device components need to endow the device with sufficient mechanical integrity and it may be important to maintain this integrity in dry and wet conditions. In other applications, such as drug delivery vehicles, wound pads, or sutures it may be beneficial to have a strong, stable material in dry conditions, that then changes to a softer, pliable, biocompatible, digestible material in the body. The mechanical properties of devices can have a strong influence on the biological function of the device. For example, the differentiation of cells depends on the mechanical properties of the substrate; stem cells grown on hard, medium hard and soft substrates differentiated into bone, muscle and nerve cells, respectively [3], [4], [5].

The performance of a device can also depend on the device topography at the nanoscopic and microscopic levels. For example, cells grow better on substrates mimicking the dimensions and porosity of the extracellular matrix, rather than on flat, featureless substrates [2].
Thus, biomedical devices often need to fulfill the following requirements: they need to mimic the fibrous, porous topography of the extracellular matrix at the microscopic level; they need to have specific mechanical properties at the microscopic, and macroscopic levels; and they need to be fashioned from biocompatible materials. Additionally, they should be stable under storage, and it may be beneficial if they change properties in the body under wet conditions.

Electrospinning technology offers the potential to control material, structural, and mechanical properties of biocompatible scaffolds and devices. In electrospinning, an electric field created by a high voltage source causes a jet of polymers in a volatile solvent to elongate into ever thinner fibers, speeding evaporation so that nanometer diameters can be achieved. The fibers are drawn to a lower voltage surface or collection plate [6], [7]. Electrospinning has been used to fabricate biomaterials for bone, ligament, blood vessel, peripheral nerve, skin, cartilage, muscle, heart, and heart valve [8], [9], [10], [11], [12], [13], [14], [15].

Electrospun fibers, and fibers in the extracellular matrix, have a diameter on the order of a hundred nanometers. Until recently, it has been difficult to determine the mechanical properties of these nanoscopic fibers, since a suitable methodology was missing. We have developed a combined atomic force microscope (AFM)/optical microscope technique to determine the mechanical properties of individual nanoscopic fibers in buffer or ambient conditions [16], [17], [18]. Using standard cantilevers, this technique can measure forces in the $10^{-2}$ to $10^4$ nN range and should, thus, be applicable to many native biological fibers, electrospun fibers, or other nanoscopic fibers [19]. Here, this technique was used to determine the mechanical properties of single, dry electrospun
fibrinogen fibers. These results complement our experiments on electrospun fibrinogen fibers in wet (aqueous) conditions [17]. We report extensibility, elasticity, stiffness, and relaxation behavior. We found that electrospun fibrinogen fibers are easy to make, stable (in dry and wet environments), and they are more extensible and elastic than electrospun collagen fibers [20], [21] and may, thus, become the fiber of choice for some biomedical devices.

METHODS AND MATERIALS

Substrate Preparation

Preparation of the striated substrate is based on soft lithography and micromoulding in capillaries [22]. Briefly, a PDMS (polydimethylsiloxane) stamp was prepared by pouring dimethylsiloxane plus catalyst (Sylgard, Dow Corning Corp, Midland, MI) onto an SU-8-silicon master grid (gift from Prof. Superfine, University of North Carolina, Chapel Hill) in a Petri dish. The polymer was cured at 70°C for 1 h. The PDMS stamp was removed from the master and pressed into a 10 μl drop of Norland Optical Adhesive-81 (NOA-81, Norland Products, Cranbury, NJ) on top of a 60 mm×24 mm, #1.5 microscope cover slide (Thomas Scientific, Swedesboro, NJ). The NOA-81 was cured for 70 s with UV light (365 nm setting, UVP 3UV transilluminator, Upland, CA) and the stamp was removed. The substrate pattern had 6.5 μm wide ridges separated by 13.5 μm wide and 6 μm deep channels.

Formation of Electrospun Fibrinogen Fibers

Fibrinogen fibers were electrospun based on the procedures developed by Wnek et al. [23]. A solution of 100 mg/ml lyophilized bovine fibrinogen, (Sigma-Aldrich
Chemical Co.), 9 part 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma Aldrich), and 1 part minimum essential medium (MEM, 10× MEM, Gibco, Invitrogen cell culture) was prepared and placed in a 1 ml, 4 mm diameter syringe (Becton-Dickinson, Franklin Lakes, New Jersey). The syringe was equipped with a 20 gauge blunt needle (Howard Electronic Instruments, Kansas) attached to Teflon tubing (Small Parts Inc.). The Teflon tubing connected to a 3 mm piece of 20 gauge hypodermic tubing (Small Parts Inc.). The syringe was placed in a syringe pump (PHD 2000 Infusion Syringe Pump, Harvard Apparatus, Holliston, Massachusetts), the hypodermic tubing was maintained at a voltage of 22 kV (Spellman High Voltage Electronics Corporation) and the solution was dispensed at a rate of 2 ml/h toward a grounded substrate a distance of 16 cm away. Fibers were spun for 5–10 s onto each substrate which consisted of a striated cover slide taped to the front of a grounded copper plate. Fiber preparation was done in a large plexiglass box with access to open air (ambient conditions and air pressure), at room temperature (23 °C). As is typical in electrospun fibers, the solvent evaporates as the fibers form and stretch in the electric field, resulting in dry fibers on the substrate. The fiber sample was removed from the electrospinning apparatus and was stored in a small plastic box in ambient conditions until further use. We did not determine the residual hydration level of the fibers that might occur due to the ambient humidity in the laboratory. However, since all fibers were treated and stored the same way, and since our results were reproducible across several measurements, we assume that the residual water content was very low and consistent across fibers. A schematic of the setup, and an SEM image of electrospun fibers on the striated substrate are shown in Figure 4.
Figure 4. Electrospinning. (A) Schematic of the electrospinning apparatus. The syringe pump is used to regulate the flow of the solution through the blunt needle tip. The tip is held at 22 kV and the pump rate is 2 ml/h. Fibers are spun onto a glass slide prepared with ridges and attached to a grounded copper plate. (B) SEM image of the electrospun fibrinogen fibers suspended over the grooves of a striated substrate. Scale bar is 7.00 μm.

Combined Microscopy and Manipulation

Fibrinogen fiber manipulations and force acquisitions were performed using a combined atomic force and inverted optical microscopic technique [16], [17], [24]. The AFM (Topometrix Explorer, Veeco Instruments, Woodbury, NY) rests on a custom-made stage on top of an inverted microscope (Zeiss Axiovert 200, Göttingen, Germany) (Figure 5A). The fibrinogen sample is sandwiched between the AFM and optical microscope. The stage is designed to allow for independent movement of the objective, AFM cantilever and electrospun fibrinogen sample. Illumination for the sample is provided by the camera light inside the AFM.
Figure 5. Fibrinogen fiber manipulation. (A) Schematic of fibrinogen fiber manipulation. The fiber is suspended over the grooves of a striated substrate. The AFM tip, located above the sample, pulls on the fiber while the optical microscope, located below the sample, acquires images and movies of the manipulation. Figure adapted from [17]. (B) Top view schematic of fiber manipulation. $L_{\text{initial}}$ is the initial length of the fiber, $L'$ is the length of the stretched fiber and $s$ is the distance the tip has traveled. $L'$ can be found trigonometrically from $L_{\text{initial}}$ and $s$, and the strain can be calculated from these quantities (see text). Image adapted from [17]. (C) Optical microscopy movie frames of a fiber being stretched and broken. The fiber is on the left in the first photograph and the cantilever (large, dark object) and AFM tip (marked by an asterisk) are visible on the right. The broken fiber is to the right of the cantilever in the fourth image. Scale bar is 14 μm.

The AFM cantilever tip (NSC35/AlBS, force constant 14 N/m, MikroMasch, Wilsonville, OR) was placed in the center of a groove next to a fiber for manipulation. The tip, controlled by a nanoManipulator (3rd Tech, Chapel Hill, NC), was then used to laterally stretch the fiber at a rate between 306 and 395 nm/s. Images and movies of the manipulation were collected by a Hamamatsu EM-CCD C9100 Camera (Hamamatsu Photonics KK, Japan) and IPLab software (Scanalytics, Fairfax, VA) (Fig. 2C).
Meanwhile, the tip travel distance, s, elapsed time and left–right photodiode signal, I_l, were recorded by the nanoManipulator software.

**Stress and Strain Calculation**

Stress and strain values were calculated as previously reported [16], [17]. The left–right photodiode signal, I_l, was recorded during a manipulation to determine the lateral force via, \( F_l = K_C \cdot I_l \). The lateral force spring constant \( K_C \), was determined from cantilever beam mechanics,

\[
K_C = \frac{E t^3}{6 l^2 (h + t/2)} \cdot S_n,
\]

where \( E \) is the Young’s modulus of silicon \((1.69 \times 10^{11} \text{ N/m}^2)\), \( w, t, \) and \( l \) are the cantilever width, thickness, and length respectively, \( S_n \) is the normal sensor response of the cantilever, and \( h \) is the height of the tip. The length and width of the cantilever and height of the tip were determined using the optical microscope and the thickness was calculated using the resonance frequency of the cantilever, \( f = 0.276 \cdot \frac{E t^3}{\sqrt{\rho (\pi h^3 t^3 + 2.383 wt^2 l^4)}} \), where \( \rho = 2330 \text{ kg/m}^3 \) is the density of silicon.

The stress was determined by dividing the force applied to the fiber by the cross-sectional area of the fiber, \( A \). The cross-sectional area, \( A = \pi \cdot (D/2)^2 \), was determined by AFM imaging the fiber on the top of the ridge (adjacent to the groove where it was manipulated). Imaging was done in noncontact mode and the diameter, \( D \), was extracted from the topography data; assuming a circular cross-section. Stress, \( \sigma = \frac{F_{fiber}}{A} \), was calculated assuming a constant fiber radius (engineering stress) and using the trigonometric relationship between the lateral force measured by the AFM tip, \( F_l \), and the force applied to the fiber \( F_{fiber} \);

\[
F_{fiber} = \frac{F_l}{2 \sin \beta},
\]

where \( \beta = \arctan \frac{s}{L_{initial}} \) (see Figure 5B). The strain of the fiber was determined using the initial length of the fiber, recorded by the
optical images, and the distance of the top travel, $s$, recorded by the nanoManipulator. The engineering strain, $\varepsilon_{Eng} = \frac{L'-L_{\text{initial}}}{L_{\text{initial}}}$, where $L'$ is the length of the stretched fiber and $L_{\text{initial}}$ is the initial length of the fiber (Figure 5B) and the true strain, $\varepsilon_{\text{true}} = \ln\left(1 + \varepsilon_{Eng}\right)$, were calculated. We choose to use true strain over engineering strain because we believe that it is a more accurate representation of the actual mechanical properties of these fibers at high strains.

RESULTS

Fibrinogen fibers were electrospun from a 100 mg/ml fibrinogen solution, onto a striated substrate for mechanical testing. The striated substrate had 13.5 μm wide and 6 μm deep grooves and 6.5 μm wide ridges. We then viewed the fibers under the optical microscope, and selected single fibers for manipulation that spanned the ridges, as shown in Figure 5B and C. As the fibers were pulled, we observed the manipulation with the optical microscope to ensure that each fiber stayed attached to the ridges. We have found that fibrinogen fibers are very sticky. They attach non-specifically and very strongly to the cured optical adhesive substrate, without any further treatment. Slippage may occasionally occur at the interface between the fibers and the substrate; however, these slippage events can be easily detected in the AFM force data traces and/or in the optical microscopy images and movies. We are familiar with data that do show slippage, since other, non-biological fibers, such as electrospun PCL fibers, often do show slippage (data not shown). Data in which the fiber detached from either ridge were not included in the analysis. The average diameter of the manipulated fibers, as determined by AFM, was 95 nm, with a range from 30 nm to 200 nm.
Fiber Extensibility

We first determined the maximum extensibility, \( \varepsilon_{\text{max}} \), of electrospun fibrinogen fibers, i.e. the strain at which the fiber ruptures, by stretching the fibers parallel to the ridges until they broke (Figure 5C). The fibers were pulled at a rate between 306 and 395 nm/s. As the fibers were stretched the force required to stretch the fibers increased (Figure 6). In the figure the force increases until the fiber reaches a strain of 57%, at which point the fiber ruptures and the force drops to zero. The average maximum extensibility, \( \varepsilon_{\text{max}} \), was found to be 74 ± 22% before rupturing at a stress of 2.1 ± 1.5 GPa (all values presented as average ± standard deviation). Figure 6C, shows a histogram of the maximum extensibility. The change in slope of the graph at ~35% strain (Figure 6A, B) indicates that the modulus of electrospun fibrinogen fibers decreases with increasing strain; this phenomenon is known as strain softening. All manipulated fibers showed strain softening and the average change in slope, taken as the initial slope divided by the slope after softening, was 2.9 ± 1.1. This change in slope occurred at ~20% strain.
Figure 6. Extensibility. (A) Lateral force vs. true strain for $\varepsilon_{\text{max}}$. The fiber is continuously stretched until it breaks. Strain softening is visible at 34% strain, as the slope of the curve decreases. The extensibility or breaking strain of this fiber is 57%. Lateral force is shown in units of nA to understand the general shape of the curve. (B) Lateral force is shown in nN. True strain was plotted starting at 10% strain because this is where true strain and engineering strain differ, and because the error in $F_{\text{fiber}} = F_{i}/2\sin\beta$ can become very large for small $\beta$ (small strains). (C) Maximum extensibility for dry fibrinogen fibers ($n = 47$). The average maximum extensibility was $74 \pm 22\%$. 
**Fiber Viscoelastic Properties**

Next, the elastic limit of electrospun fibrinogen fibers was determined. The elastic limit, as described here, is the maximum strain after which the fiber is still able to return to its original length once the applied force is removed. We first pulled the fiber to a small strain and then retracted the AFM tip to allow the fiber to return to its initial position. If permanent deformation did not occur the fiber was pulled to a greater strain and returned again. This process was repeated, increasing the strain at each step, until permanent deformations could be seen in the fiber. After visual inspection of permanent fiber deformation, the stress–strain curves were also used to verify the strain at which the fiber deformed (Figure 7). In Figure 7A, the first three pulls, blue, green, and red, respectively, do not reach the elastic limit and the fiber returns to its original position and shape. This can be seen from the plot because the stress returns to zero as the true strain reaches zero. The black curve, however, shows that the elastic limit for this fiber has been exceeded, since the stress goes to zero (no tension) before the strain reaches zero. That is, in this curve, the fiber had some slack due to permanently induced deformations. Fig. 4B shows a histogram of the elastic limit; the average elastic limit is $\varepsilon_{\text{elastic}} = 15 \pm 4\%$. 
Figure 7. Elastic limit. (A) Stress vs. true strain for the elastic limit ($\varepsilon_{\text{elastic}}$) of dry fibrinogen fibers. The fiber was pulled four times with the first pull being shown in blue. Since the stress and strain return to zero at the same point when the fiber is allowed to relax, $\varepsilon_{\text{elastic}}$ has not been reached. Pulls 2 (green) and 3 (red) show the same kind of relaxation. Pull 4 (black) shows that the elastic limit has been reached because the return stress reaches zero before the strain reaches zero. (B) Elastic limit distribution for dry fibrinogen fibers ($n = 47$). The average elastic limit was $15 \pm 4\%$.

We also determined the total and elastic modulus for dry electrospun fibrinogen fibers. Figure 8 shows an example of an incremental stress as a function of true strain curve for dry electrospun fibrinogen fibers. Figure 8A shows a plot of the true strain as a function of time. For this example, the fiber was pulled to a strain of $\varepsilon = 67\%$ and held for 21 s. It was then pulled to a strain of $\varepsilon = 80\%$ and held for 31 s. Lastly it was pulled to a strain of $\varepsilon = 102\%$ and held for 34 s. The fiber ruptured after further pulling. Figure 8C, D shows a plot of stress as a function of time for the same fiber manipulation. The plots together show that as the fiber is held at constant strain the stress decays, but it does not decay to zero stress. This is indicative of viscoelastic behavior.

Fig. 5B shows the stress as a function of true strain for the same fiber manipulation. The red curve is the raw data, the black solid line is the total modulus
determined by the slope between adjacent stress peaks, and the black dashed line is the elastic component of the total modulus determined by fitting an exponential curve to the stress decay. The mean value for total modulus of dry electrospun fibrinogen fibers was 4.2 ± 3.4 GPa and the elastic modulus was 3.7 ± 3.1 GPa.

We did not observe any trend in mechanical properties related to fiber diameter, indicating that electrospun fibrinogen fibers may have a relatively homogeneous cross section. For natural fibrin fibers, we did observe a diameter-dependence of the Young's modulus – the Young's modulus decreased with increasing diameter [18], [25] – which indicates that natural fibrin fibers are denser in the center than at the periphery of the cross section.

The incremental stress versus time curves can also be used to determine the stress relaxation times. Figure 8D shows a typical relaxation curve. The relaxation times are determined by fitting a double exponential curve, \( y = a \exp(-t/\tau_1) + b \exp(-t/\tau_2) + c \), to the relaxation curve. For dry electrospun fibrinogen fibers, the fast relaxation time was \( \tau_1 = 1.2 \pm 0.4 \) s and the slow relaxation time was \( \tau_2 = 11 \pm 5 \) s. A single exponential fit can only fit either the slow or fast relaxation and therefore did not fit the data at either the beginning or the end of the relaxation curve.
**Figure 8.** Incremental stress–strain curves and relaxation. (A) True strain versus time for a fiber that was pulled incrementally. The fiber was stretched to a given length and held at a constant strain while allowing the fiber to relax. The plateaus on the graph indicate where the AFM tip was stopped and the fiber was held at constant strain during the manipulation. (B) Stress versus true strain for dry electrospun fibrinogen fibers that were pulled incrementally. The red curve is the raw data for the fiber, the black solid line is the total modulus determined by the stress before relaxation, and the black dashed line is the elastic modulus component of the total modulus determined by fitting an exponential curve to the relaxation. (C) Stress versus time for a fiber that was pulled incrementally. The stress was shown to decay exponentially during the time when the fiber is held at a constant strain, showing viscoelastic behavior for dry electrospun fibrinogen fibers. (D) Relaxation curve for dry electrospun fibrinogen fiber. The curve was fit with a double exponential function which produced two relaxation times. The fast relaxation time for this fiber was $\tau_1=1.5$ s and the slow relaxation time was $\tau_2=20$s.
DISCUSSION

The physiological function of fibrinogen is to form a mesh of fibrin fibers – the major structural component of hemostatic blood clots – in the event of vascular injury. The fibrin network also provides a scaffold for binding of tissue cells such as monocytes, fibroblasts, and endothelial cells while fibrin degradation products attract monocytes and neutrophils [26]. The biocompatibility of fibrinogen and its role in tissue repair and proliferation make fibrin(ogen) fibers a natural choice for medical devices, including tissue engineering, drug delivery, or wound healing applications. Moreover, fibrinogen can be easily electrospun into nanoscopic fibers. Electrospinning is done in ambient conditions, and thus, the fibers in the initial device are dry. The device may then be further processed (e.g. loaded with drugs, fashioned into a new shape) and stored until use. Subsequent use will then often involve exposing the device to the aqueous environment of the body. It is, thus, important to determine the properties of the fibers comprising medical devices in dry and wet conditions.

We determined the mechanical properties of individual, dry, electrospun fibrinogen fibers. The fibers had an average diameter of 95 nm, the same order of magnitude as natural fibrin fibers and fibers of the extracellular matrix. Electrospun fibrinogen fibers are relatively stiff and remarkably extensible and elastic, when compared to other natural, electrospun, or even synthetic fibers of similar stiffness (Table II and [27]). For example, dry electrospun collagen fibers ($\varepsilon_{\text{max}} = 33\%$; $\varepsilon_{\text{elastic}} = 1–2\%$) [20], native collagen fibers ($\varepsilon_{\text{max}} = 12–68\%$; $\varepsilon_{\text{elastic}} = 3–8\%$), the fibers in the spokes of spider webs (Araneus MA silk, $\varepsilon_{\text{max}} = 27\%$) and nylon fibers ($\varepsilon_{\text{max}} = 15–50\%$; $\varepsilon_{\text{elastic}} = 8\%$) are less extensible.
Table II. A comparison of the available, mechanical properties of electrospun fibrinogen, native fibrin, electrospun collagen and native collagen fibers. $\varepsilon_{\text{max}}$, maximum extensibility; $\varepsilon_{\text{elastic}}$, elastic limit; $E_0$, relaxed (elastic) Young's modulus; $E_\infty$, (total) Young's modulus; $\tau_1$, fast relaxation time, $\tau_2$, slow relaxation time; $h$, strain hardening factor (ratio of total Young's modulus at high strain to total Young's modulus at low strain). A number $h > 1$ indicates strain hardening, and a number $h < 1$ indicates strain softening.

<table>
<thead>
<tr>
<th>Fiber type</th>
<th>$\varepsilon_{\text{max}}$</th>
<th>$\varepsilon_{\text{elastic}}$</th>
<th>$E_0$(MPa)</th>
<th>$E_\infty$(MPa)</th>
<th>$\tau_1$ (s)</th>
<th>$\tau_2$ (s)</th>
<th>$h$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry, electrospun fibrinogen fibers</td>
<td>110%$^a$</td>
<td>16%$^a$</td>
<td>4200</td>
<td>3700</td>
<td>1.2</td>
<td>11</td>
<td>0.3</td>
<td>This study</td>
</tr>
<tr>
<td>Wet, electrospun fibrinogen fibers</td>
<td>130%</td>
<td>-</td>
<td>17.5</td>
<td>7.2</td>
<td>3.0</td>
<td>55</td>
<td>~0.5</td>
<td>[17]</td>
</tr>
<tr>
<td>Wet, crosslinked fibrinogen fibers</td>
<td>147%</td>
<td>50-75%</td>
<td>8.0</td>
<td>4.0</td>
<td>2.1</td>
<td>49</td>
<td>1.9</td>
<td>[18]</td>
</tr>
<tr>
<td>Wet, uncrosslinked, fibrinogen fibers</td>
<td>226%</td>
<td>60-120%</td>
<td>3.9</td>
<td>2.0</td>
<td>2.9</td>
<td>54</td>
<td>3.21</td>
<td>[18]</td>
</tr>
<tr>
<td>Dry, electrospun collagen fibers</td>
<td>33%</td>
<td>&lt;2%</td>
<td>-</td>
<td>10,000-200$^b$</td>
<td>-</td>
<td>-</td>
<td>&lt;0.1$^c$</td>
<td>[20], [21]</td>
</tr>
<tr>
<td>Wet, crosslinked electrospun collagen fibers$^d$</td>
<td>-</td>
<td>-</td>
<td>260-70$^b$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[21]</td>
</tr>
<tr>
<td>Native tendon collagen fibers</td>
<td>12%</td>
<td>-</td>
<td>-</td>
<td>7,500-160</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[28], [29], [30]</td>
</tr>
</tbody>
</table>

$^a$ For better comparison, engineering strain, $\varepsilon_{\text{Eng}} = (L' - L_{\text{initial}})/L_{\text{initial}}$, is reported in the table for all fibers. In the text of this paper we report true strain $\varepsilon_{\text{True}}=\ln(1+\varepsilon_{\text{Eng}})$. For dry, electrospun fibrinogen fibers, $\varepsilon_{\text{max, true}} = 74\%$, $\varepsilon_{\text{elastic, true}} = 15\%$ (as in manuscript text), and $\varepsilon_{\text{max, Eng.}} = 110\%$, $\varepsilon_{\text{max, Eng.}} = 16\%$ (as in table).

$^b$ The modulus of electrospun collagen fibers strongly depends on the radius, with thinner fibers having a larger modulus; fiber diameter ranged from about 200 nm to 800 nm [20] and 150 nm to 500 nm [21].

$^c$ Electrospun collagen fibers show extreme strain softening and $h$ may be significantly smaller than 0.1.

$^d$ Uncrosslinked, electrospun collagen fibers are not stable in aqueous buffers.

Natural, wet uncrosslinked fibrin fibers have a modulus of 4 MPa [18], and they have been shown to be extraordinarily extensible ($\varepsilon_{\text{max}} = 226\%$ for uncrosslinked fibers) [16]. Wet, electrospun fibrinogen fibers retain some of the mechanical properties of the native fibrin fibers as they have a similar modulus of 17.5 MPa and large extensibility ($\varepsilon_{\text{max}} = 130\%$) [17]. Thus, upon drying, electrospun fibrinogen fibers become nearly 1000 times stiffer, but only somewhat less extensible. Wet and dry fibrinogen fibers may be
useful in applications that require extensible and elastic nanofibers. Thus, these fibers may be able to fill a unique niche in medical or material science applications.

We would like to propose a molecular mechanism why electrospun fibrinogen fibers become so much stiffer upon drying. The 1000-fold increase in stiffness suggests that the removal of water stiffens or fixes molecular elements that are soft and flexible in the presence of water. Very recent experiments suggest that a key element providing fibrin fibers with its low modulus, elasticity and extensibility is the long, flexible, mostly unfolded alpha-C region [31], [32]. This region may also play a significant role in influencing the mechanical properties of electrospun fibrinogen fibers. We would like to propose that water is critical to keep this region solvated and unfolded, and that removal of water may severely reduce the mobility and extensibility of this region. Thus, the mechanical properties of fibrin and fibrinogen fibers are dominated by the soft, extensible alpha-C region in the presence of water; in the absence of water this region becomes stiffer (or is fixed), and other elements, for example the alpha-helical coiled coils may play a more dominant role.

Fibrinogen is a highly water-soluble molecule. It occurs at micromolar concentrations in blood; yet, it does not polymerize until thrombin activates it by removing fibrinopeptides A and B to expose binding sites A and B. Given this high water solubility, it is surprising that electrospun fibrinogen fibers are stable in aqueous buffers, even without any activation or crosslinking, as was observed in our previous study [17]. In the following paragraph we would like to discuss this surprising stability of electrospun fibrinogen fibers. There are some lines of evidence indicating that fibrinogen largely maintains its native structure under electrospinning conditions. For example, the
CD spectra of fibrinogen in aqueous buffer and in electrospinning solvent are similar; and the mechanical properties of native fibrin fibers and electrospun fibrinogen fibers are similar. The A:a interactions, which direct the half-staggered assembly of fibrin into proto-fibrils, are missing in the electrospun fibrinogen fibers, raising the question ‘what holds the electrospun fibrinogen fibers together in aqueous buffers?’ There must be additional or strengthened interactions in electrospun fibrinogen fibers. We speculate that the interactions between the alpha C-regions, which exist in fibrin fibers, might also be present in electrospun fibers. In soluble fibrinogen, this region is folded back and tucked into the central domain. Electrospinning conditions may untuck these regions allowing them to interact with each other. This could be tested by spinning fibers from a mutant fibrinogen that do not have this region. Electrospun collagen fibers, a different protein without such a flexible region, are not stable in aqueous buffer (without crosslinking). Additional interactions between the fibrinogen molecules in the fibers may be provided by the salt and amino acids contained in the minimum essential medium (MEM) of the spinning buffer. In fact, it is difficult to spin fibrinogen fibers without MEM.

Previous researchers have reported on the use of electrospun fibrinogen as a candidate for tissue scaffolds. McManus et al. electrospun fibrous mats of fibrinogen and evaluated their bulk mechanical behavior under dry and wet conditions [33]. They found that wet conditions increased extensibility but modulus and peak stress were significantly reduced. In a later study, it was found that human bladder smooth muscle cells readily migrated into electrospun mats of fibrinogen and initiated remodeling through collagen production [34].
From a biomechanical viewpoint, electrospun fibrinogen fibers offer composition and elasticity features that favor cell attachment and the potential for remodeling. The RGD amino acid sequence (Arg-Gly-Asp) is the universal cell recognition motif and can be found at positions Aα95–97 and Aα572–574 on the fibrinogen molecule, while RGD independent integrin binding sites occur on the fibrinogen γ-chain and RGD independent cell interactions have been reported [35]. Many cell types such as endothelial cells, smooth muscle cells, keratinocytes, fibroblasts, and leukocytes and growth factors such as fibroblasts growth factor-2 (FGF-2) and vascular endothelial cell growth factor (VEGF) have been shown to bind to fibrin(ogen) [35], [36], [37]. Cellular adhesion and proliferation along with the mechanical properties of the fibers are likely to be important components of engineered load-bearing structures like blood vessels and bone where cell attachment and subsequent mechanotransduction are essential to tissue remodeling. Native tissues, like arteries for example, exhibit a highly elastic material behavior at lower physiologic pressures (elastin dominates) and a stiffening behavior at higher pressures (collagen dominates) [38]. Fibrinogen could well serve as the elastic component for engineered arteries. In the case of engineered valves for pediatric patients, the degradability of fibrinogen may be important so that the valve can remodel as the patient grows.

Single fiber data, as presented in this paper, will inform the design of electrospun medical devices and tissue scaffolds, and will, thus, ultimately allow intelligent and guided design of scaffolds for medical devices.
ACKNOWLEDGMENTS

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CHAPTER III

THE MECHANICAL PROPERTIES OF SINGLE FIBRIN FIBERS FORMED FROM BLOOD PLASMA

Justin Sigley, Wei Li, Christine Helms, Marlien Pieters, Martin Guthold

The following manuscript has been prepared for journal submission. Justin Sigley collected all plasma fibrinogen data, performed all analysis, and drafted the manuscript. Wei Li collected the purified fibrinogen data and Christine Helms helped with initial preparation of the manuscript. Marlien Pieters supplied the plasma samples and edited the manuscript. Martin Guthold acted in an advisory and editorial role during data collection and manuscript preparation.
ABSTRACT

The mechanical and structural properties of blood clots may be related to certain cardiovascular and other diseases. The studies uncovering these relationships were typically done on whole, macroscopic clots. On the other hand, most experiments at the microscopic, single fibrin fiber level have been performed on clots formed from purified fibrinogen. Here, we used a combined atomic force microscope (AFM)/fluorescence microscope technique to determine the mechanical properties of individual fibrin fibers formed from blood plasma instead of purified fibrinogen. Blood samples were taken from uncontrolled diabetic patients, as well as age, gender, and BMI-matched healthy individuals. The patients then underwent treatment to control blood glucose levels before end blood samples were taken. The fibrinogen glycation of the diabetic patients was reduced from 8.81 to 4.96 mol glucose/mol fibrinogen. The healthy individuals had a mean fibrinogen glycation of 3.98 mol glucose/mol fibrinogen. The total and elastic modulus, as well as extensibility and relaxation times of individual fibrin fibers were measured. We found fibrinogen glycation had no significant effect on modulus, extensibility, or relaxation times. Several studies have attributed increased clot modulus to an increased branchpoint density due to a high blood fibrinogen concentration. We found individual fibrin fibers also have an increased modulus which depends on fiber diameter and is controlled by blood fibrinogen concentration. The diameter dependence of the modulus suggests fibrin fibers polymerize as a dense core of protofibrils which decreases in density as the fiber grows to a maximum thickness.
INTRODUCTION

Fibrin fibers, the structural constituent of blood clots, perform the mechanical task of stemming the flow of blood. Fibrin clot formation is initiated by thrombin, which cleaves fibrinopeptide A & B. This converts the fibrinogen to fibrin monomers by revealing the complementary binding sites located on the central E domain and end D domains. The fibrin monomers non-covalently polymerize to form dimers, trimers, tetramers, and protofibrils in a half-staggered structure with a periodicity of 22.5 nm. The protofibrils laterally aggregate upon reaching a sufficient length of 0.6 – 0.8 μm [1]. Electron microscopy has shown these protofibrils twist helically while maintaining the 22.5 nm periodicity [2]. The twisting restricts the possible lateral contacts as the energy to stretch an added protofibril exceeds the binding energy thus limiting the final diameter of a fiber. Factor XIIIa then stabilizes the network through the formation of cross-links.

In the past sixty years, there has been continuing effort to study the mechanical properties of clots. Understanding the mechanical properties of clots is not only essential for the treatment of clotting disorders and disease, but can also provide insight into clot formation [3]. Rheometry has been used to study the properties of whole clots, [4][5] while single molecule experiments provide molecular information [6]. Single fiber properties that connect the molecular mechanisms with the macroscopic scale properties have also been studied using atomic force microscopy (AFM) [7]. Most investigations have been performed on clots formed by the addition of thrombin to purified fibrinogen; however, the elasticity of individual fibrin fibers in plasma clots has been studied using confocal microscopy and optical tweezers experiments [8].
The use of plasma clots enables one to directly measure the properties of clots from patients who suffer from specific clotting disorders and diseases such as cardiovascular disease (CVD). Diabetes is a risk factor for CVD increasing ones risk by 2-4 times [9] with 68% of morbidity in diabetic patients being due to CVD [10]. According to the Center for Disease Control, diabetes affects 25.8 million people or 8.3% of the population in the United States [10]. With this number expected to rise, the need to understand this disease and its implications has never been greater. The relationship between CVD and diabetes is not well understood, although alterations to the properties of fibrin fiber clots are often found in CVD patients. Research has suggested a relationship between macrovascular disease and diabetic control [11]. In one study, hyperglycemia was shown to increase atherosclerotic plaque [12]. Boyne, however, lists many factors and risks that often occur simultaneously with diabetes and lead to increased CVD risk such as hypertension, weight gain, lipid dysmetabolism, and elevated fibrinogen concentrations [11].

Studies have been done on whole blood clots in CVD patients that show the clots have increased modulus, decreased permeability, and decreased lysis when compared to control clots [13]. Other studies have looked at clots in diabetic patients as well as fibrinogen incubated with glucose in the lab in an attempt to understand the connection between CVD and diabetes. Many of these studies have conflicting findings. A few studies have reported increased resistance to fibrinolysis in samples with increased glycation [9][14]. Another study, however, found decreased resistance to fibrinolysis as glycation increased [15]. Some studies showed a shorter lag phase in polymerization and decreased permeability in diabetic clots [14][16], while a few other studies showed no
difference in polymerization, kinetics, clot porosity, and clot density in diabetic samples compared to control samples [15][17]. Crosslinking of the fibrinogen monomer by FXIII has also been studied in relation to diabetic control. One study reported altered alpha multimer formation [15], while another reported no difference in crosslinking [14].

In the present investigation, we measured the mechanical properties of individual fibrin fibers formed from citrated plasma using a combined fluorescence/atomic force microscopy method. Our goal was to elucidate the mechanism causing increased whole clot modulus in CVD patients and its possible connection to diabetes. Our results show increased glycation does not seem to alter the modulus or extensibility of single fibrin fibers in a predictable way. We did however, find that total fiber modulus varies with fiber diameter suggesting that fibers are not made of a homogeneous and isotropic collection of protofibrils. We propose a model for fibrin fiber formation in which fibers become less dense at increasing diameters resulting in lower modulus. We corroborate this model with measurements made on plasma fibrin clots as well as purified fibrin clots.

METHODS

Plasma Collection, Determination of Fibrinogen Concentration and Glycation

Citrated blood was collected from patients by our collaborator Marlien Pieters. Within 30 minutes of collection, the blood was centrifuged for 15 minutes at 2000 g at 4°C. The plasma was extracted and stored at -80°C until fiber sample preparation. The Pieters lab measured the fibrinogen concentration and fibrinogen glycation. Fibrinogen glycation was measured as previously described [17] using a two-reagent enzymatic
To determine if diabetes had an effect on the mechanical properties of single fibrin fibers, diabetic and non-diabetic control patients were selected. Patients had to be uncontrolled (HbA1C > 9%) on maximum-dose combination oral hypoglycemic medication, BMI > 25 kg/m², 40-65 years of age, and blood pressure sufficiently controlled (< 140/90 mmHg) to be included in the study. Blood was collected, and then the patients underwent a three step intervention program. First, patients were taught how to monitor glucose, coordinate insulin use with meals, and manage hypoglycemic events with glucagon. Secondly, patients received 10 IU (equivalent to 0.347 mg) of insulin daily in addition to the current treatment of maximum dose oral hypoglycemic treatment. Sulphonylureas were stopped and insulin use was adjusted individually until 4 out of 5 subsequent fasting values were less than 7.2 mM. Lastly, short acting insulin was used to control post-prandial glucose levels (<10 mM). Non-diabetic control subjects with matching age, gender, and BMI were included. Baseline oral glucose tolerance tests were done to rule out diabetics.

Substrate Preparation

Striated coverslides were prepared for plasma samples as previously described [18]. Briefly, optical adhesive (NOA-81, Norland Products, Cranbury, NJ) was placed on a coverslide. A rectangular polydimethylsiloxane (PDMS) stamp was pressed into the adhesive to create a 1.5 x 1.5 cm well for containing buffer. In the center of the well, a drop of optical glue was placed and a second PDMS stamp was used to create a striated
surface with 8 µm wide ridges and 12 µm wide grooves. The optical glue was then cured under a 365 nm UV light for 1.5 minutes.

**Fibrin Sample Preparation**

To form fibrin fibers, 14 µl of citrated plasma was combined with 0.1 U/ml thrombin and 20 mM CaCl$_2$ and pipetted onto the striated coverslide. The reaction ran for 1 hour in a moist atmosphere at room temperature. This time period was chosen to allow completion of fiber formation including full stabilization of fibrin by factor XIIIa. After an hour, the slide was rinsed with calcium free buffer at pH 7.4 to stop the clotting reaction. A pipette tip was used to manually remove excess fibers from the top of the sample. The sample was then rinsed with buffer. The fibers were labeled with 24 nm carboxyl coated fluorospheres (Invitrogen, Carlsbad, CA) and rinsed with buffer once again.

Purified fibrin samples were formed similar to the plasma fibrin samples. In this case 0.3 U/ml thrombin and 9 U/ml FXIII was added to purified fibrinogen in concentrations varying from 0.8 mg/ml to 6 mg/ml. After clotting, the mixtures were rinsed with calcium free buffer and labeled with 24 nm fluorospheres.

**Manipulations**

A unique prospect of this study was the use of an AFM to measure the mechanical properties of single fibrin fibers in a hydrated plasma clot. Fibrin clots were visualized on an inverted fluorescence microscope (Figure 9) and consist of straight fibrin fibers spanning grooves in our striated substrate. Fibers that span a groove and have no branch points within that groove were chosen for manipulation. This does not introduce bias
since the average fiber length between branch points in a plasma fibrin clot has been measured using confocal microscopy to be larger than the width of the grooves in our substrate. [19][8]

Fiber modulus, extensibility, and relaxation behavior were measured as previously described [20]. Manipulations were performed using a combined atomic force microscope (AFM) (Topometrix Explorer, Veeco Instruments, Woodbury, NY) and inverted fluorescence microscope (Axiovert 200, Zeiss, Gottingen, Germany). The fiber sample was placed between the AFM and optical microscope using a customized stage which allows the sample to be moved independently of either microscope. Illumination of the sample is provided by a camera light in the AFM. Fibers were stretched with the AFM cantilever (CSC-38, MikroMash, Willsonville, OR) at a rate of ~320nm/s (Figure 9). Cantilever deflection, distance and time data were collected by NanoManipulator software (3rd Tech, Chapel Hill, NC). Images were collected during manipulation by a Zeiss AxioCam and Axiovert software. Fiber diameter was determined by AFM imaging the fiber on top of the ridge adjacent to where the fiber was manipulated.
Figure 9. Fibrin fiber manipulation. (A) Schematic of fibrin fiber manipulation. The fiber is suspended over the grooves of a striated substrate. The AFM tip, located above the sample, pulls on the fiber while the optical microscope, located below the sample, acquires images and movies of the manipulation. Figure adapted from [21]. (B) Top view schematic of fiber manipulation. L_{initial} is the initial length of the fiber, L' is the length of the stretched fiber and s is the distance the tip has traveled. L' can be found trigonometrically from L_{initial} and s, and the strain can be calculated from these quantities (see text). Image adapted from [21]. (C) Optical microscopy movie frames of a fiber being stretched and broken. The fiber is on the left in the first photograph and the cantilever (large, dark object) and AFM tip (marked by an asterisk) are visible on the right. The broken fiber is to the right of the cantilever in the fourth image. Scale bar is 14 μm.
RESULTS AND DISCUSSION

Statistical Analysis

Means and standard deviations were calculated using the standard equations. To determine statistical significance between samples, a two tailed t-test was used with an α level set at 0.05.

Fiber viscoelastic properties

In the present investigation, plasma fibrin clots were measured from 14 different plasma samples. The samples include 4 control (non-diabetic) patients, and 5 diabetic patients. Blood was collected from the diabetic patients before intervention (uncontrolled diabetics) and after intervention (controlled diabetics). Clots were made multiple times from the same plasma samples over the duration of the study. We did not observe any significant difference in the viscoelastic properties of fibers from the same plasma sample over the course of the investigation. The samples were all collected from females between the ages of 44 and 65 with a median age of 58. Fibrinogen concentration ranged from 3.53 to 5.62 mg/ml. Fibrinogen glycation ranged from 2.99 to 11.81 mol glucose/mol fibrinogen. Prior to intervention, uncontrolled diabetic patients had an average glycation of 8.81 ± 3.40 mol gluc/mol fibrg which decreased to an average of 4.96 ± 2.37 after intervention. The glycation of all diabetic patients decreased after intervention. Non-diabetic control patients had an average fibrinogen glycation of 3.98 ± 1.00.

We determined the total and elastic modulus for plasma fibrin fibers as a function of glycation and fibrinogen concentration. Figure 10 shows an example of an incremental stress as a function of strain curve. The fiber is stretched then held at a constant strain for
a period of time before being pulled again. The process is repeated at higher and higher strains until the fiber finally ruptures. The plots show that as a fiber is held at constant strain, the stress decays. This is indicative of viscoelastic behavior. By fitting an exponential curve to the stress decay, we can measure the total modulus, elastic modulus, and relaxation times for the fibers.

**Figure 10.** Incremental stress–strain curves and relaxation. (A) Strain versus time for a fiber that was pulled incrementally. The fiber was stretched to a given length and held at a constant strain while allowing the fiber to relax. The plateaus on the graph indicate where the AFM tip was stopped and the fiber was held at constant strain during the manipulation. (B) Stress versus strain for fibrin fibers that were pulled incrementally. The red curve is the raw data for the fiber, the black solid line is the total modulus determined by the stress before relaxation, and the black dashed line is the elastic modulus component of the total modulus determined by fitting an exponential curve to the
relaxation. (C) Stress versus time for a fiber that was pulled incrementally. The stress was shown to decay exponentially during the time when the fiber is held at a constant strain, showing viscoelastic behavior. (D) Example relaxation curve for fibrin fiber. The curve was fit with a double exponential function which produced two relaxation times. The fast relaxation time for this fiber was $\tau_1=1.5$ s and the slow relaxation time was $\tau_2=20$s.

The total modulus of the fibrin fibers varied from 1.0 MPa to 33.4 MPa and the elastic modulus of the fibers varied from 0.6 MPa to 17.3 MPa. While measuring stress decay, we found the fibers have a fast relaxation time of 2.3 s and a slow relaxation time of 34 s. We observed an increase in single fiber total and elastic modulus as the fibrinogen concentration increased, however fibrinogen glycation did not seem to have any predictable effect on the modulus or relaxation times of single fibers (Figure 11).
Figure 11. (A) Single fiber modulus does not depend on fibrinogen glycation. We also do not see any dependence on fast (B) or slow (C) relaxation times with glycation. Error bars are standard errors of the mean.
We did however observe a negative exponential correlation between fiber modulus and diameter (Figure 12). We repeated these modulus measurements on fibrin fibers made from purified fibrinogen and found the same negative exponential correlation between fiber modulus and diameter.

**Figure 12.** Plots of fiber modulus as a function of diameter for blood plasma fibrin (A) and purified fibrin (B). There is a significant (p < 0.01) negative exponential relationship between fiber modulus and fiber diameter (notice log scaling). The exponent for plasma fibrinogen fibers is -1.63 (N = 206, R^2 = 0.47) while the exponent for purified fibrin fibers is -1.50 (N = 116, R^2 = 0.19).
We also stretched fibrin fibers to their breaking point to determine extensibility. The fibrin fibers on average stretched between 1.5 to 2.5 times their initial lengths before breaking. We did not observe a significant correlation between fiber extensibility and fibrinogen concentration or glycation (Figure 13).

Figure 13. Fiber extensibility as a function of fibrinogen concentration (A) and glycation (B). We did not observe any significant trend between extensibility and fibrinogen concentration or glycation. Error bars are standard error of the mean.
Whole clot modulus has been shown to increase in clots with small diameter fibers [4]. This is generally attributed to the increased density of branch points observed in clots with small diameter fibers. However, in the current study, we also observe an increased modulus as fiber diameter decreased in the absence of branch points. For proper clot modeling, we must also take into account the increased clot modulus due to the increased modulus of the single fibers constituting the clot. Here we propose a model to explain the modulus of single fibers decreasing as the diameter of the fiber grows.

One can begin to model single fibrin fibers as a paracrystalline array of rod-shaped protofibrils. In such an array we make the assumption that each rod-shaped protofibril is identical along the long axis such that the stretching force of a fibrin fiber is directly proportional to the number of protofibrils. If we assume a fibrin fiber forms a hollow tube of protofibrils, the stretching force required to reach a specific strain would increase linearly as a function of the radius, \( F(R) \sim R \). There has been no experimental evidence however that fibrin fibers form tubes. More commonly, fibrin fibers are assumed to have a uniform density of protofibrils. In this case, the stretching force of an individual fiber would increase by the radius squared, \( F(R) \sim R^2 \). Our experiments suggest a model between these two extremes to be a better indicator of fibrin growth. In the diabetic plasma system and a purified system, we measured the force to increase with the radius to the 1.6 power and 1.5 power respectively. In this model, the fiber would start with a dense core that becomes less dense as more protofibrils aggregate to the outside of the fiber. The helical twist of the growing fiber restricts the possible lateral contacts for additional protofibrils as the energy to stretch an added protofibril exceeds...
the binding energy. This suggests the fibers and protofibrils still in solution should reach a dynamic equilibrium at a maximum fiber radius.

The fibers measured in the current study had slightly increased total modulus and elastic modulus of 11.2 MPa and 6.1 MPa respectively compared to an average total modulus of 8.0 MPa and elastic modulus of 4.0 MPa as measured in a previous study in our lab [7]. The final concentration of fibrinogen used previously was 1.0 mg/ml. In the current study the average concentration of fibrinogen in the plasma was between 3.5 – 5.6 mg/ml. The total modulus of fibrin fibers made from purified fibrinogen at these same concentrations, was measured to be 14.7 MPa. A study of 552 patients measured the average blood fibrinogen level to be 2.4 mg/ml [22]. The relatively high fibrinogen concentrations in our study may be due to the genetic makeup of the patients which were all black female Africans from South Africa. Another study reported similarly high fibrinogen concentrations in the blood of African males and females from the Northwest Province of South Africa [23]. The increased fibrinogen concentration leads to smaller fiber diameters and subsequently increased modulus [4].

CONCLUSION

The AFM provides three distinct advantages over other methods. First, we can more accurately determine the diameter of individual fibers. Second, due to the striated substrate, we have a well-defined fiber end. Each fiber is connected to the clot network so well-defined fiber ends are less likely to appear in vivo, however eliminating the movement of anchor points greatly reduces the error in elastic moduli measurements. Lastly, due to the large range of forces the AFM can apply, we are able to measure
extensibility of single fibers. This method can also be extended to other biologically relevant networks such as extracellular matrix, microtubules, and actin filaments.

We set out to measure the mechanical properties of single fibrin fibers in clots formed from diabetic patients to determine if fibrinogen glycation had an effect on these mechanical properties. While we determined there was no significant correlation between fibrinogen glycation and fibrin fiber modulus, extensibility, and relaxation; we did however notice a trend between fiber modulus and fiber diameter. High fibrinogen blood concentrations form clots with a high density of branchpoints and small diameter fibers [4]. We have shown that the branchpoint density is not the only important factor in determining whole clot modulus. The modulus of individual fibrin fibers which comprise the clot also significantly contribute to the bulk mechanical properties of the clot.

Many studies of the mechanical properties of whole fibrin clots can be found in the literature, however, relatively few studies have been done on the individual components making up these clots. Proper modeling of whole clots is needed for accurate predictions of the effect of diseases on the mechanical properties of clots. It should be possible to calculate the bulk mechanical properties of a whole fibrin clot by knowing the properties of the individual components. Our bottom-up approach provides a foundation for determining initial parameters for modeling whole fibrin clots or other complex polymeric systems. A distinct advantage of this approach is the ability to determine the differences in the mechanical properties of networks with very dissimilar structures.
REFERENCES


CHAPTER IV

THE DIFFUSION OF EGFP AND MSH2 IN BREAST CANCER CELLS AS MEASURED BY RASTER IMAGE CORRELATION SPECTROSCOPY

Justin Sigley, John Jarzen, Karin Scarpinato, Martin Guthold, and Keith Bonin

The following manuscript has been submitted to the New Journal of Physics. Stylistic variations are due to the requirements of the journal. Justin Sigley collected all data, performed all analysis, and drafted the manuscript. John Jarzen and Karin Scarpinato supplied the initial Weinberg cells and transfection plasmids. Martin Guthold and Keith Bonin acted in an advisory capacity throughout data collection and analysis and with Karin Scapinato, acted in an editorial capacity during manuscript preparation.
ABSTRACT

We applied Raster Image Correlation Spectroscopy (RICS) to measure the diffusion of enhanced green fluorescent protein (EGFP) and the mismatch repair protein MSH2 in live cells. Normal, immortal, tumorigenic, and metastatic mammary epithelial cells were transfected with EGFP and EGFP-tagged MSH2. The diffusion coefficients were then measured in the cytoplasm and nucleus to determine if neoplastic transformation has an effect on the mobility of MSH2. The diffusion of these proteins in the cytoplasm was unaffected by neoplastic transformation. In the nucleus, however, we measure higher EGFP diffusion rates compared to the rates in the cytoplasm in all cell types except for tumorigenic cells. Compared to EGFP alone, the diffusion rate of EGFP-MSH2 is 7 – 8 times smaller in the nucleus of immortal and metastatic cells. This suggests neoplastic transformation alters the intra-nuclear structure on a small enough length scale to affect the mobility of small proteins. We have demonstrated that RICS can be a useful tool to characterize dynamic processes in the search for better cancer diagnostics and therapeutics.

INTRODUCTION

Advances in biophysics are providing opportunities to establish connections between cell mechanics and biological cellular functions. Cancer research is one area where this is especially true. Recent reports indicate that cancerous and non-cancerous cells have different physical and mechanical properties arising from biochemical alterations as normal cells transform to cancerous cells [1]. There is, however, significant ambiguity about how these properties change. One source of ambiguity is the type of cell
being studied. For example, human bladder cells [2], mammary epithelial cells [3], pancreatic epithelial cells [4], [5], and mouse fibroblast cells [6] have shown increased deformability in cancerous cells compared to normal cells; while human hepatocytes [7], myeloid and lymphoid leukemia cells [8], and Lewis lung carcinoma mouse cells [9] have shown decreased deformability with cancer. The mechanical properties of tissues may differ from the properties of individual cells. Stiffness maps from benign human breast biopsies show uniform stiffness profiles characterized by a single peak in contrast to malignant tissues which have broader peaks resulting from tissue heterogeneity and a characteristic low-stiffness peak representative of cancer cells [10]. Another reason for different results is the methods being used to measure the mechanical properties of cells. Atomic force microscopy has been used to test local membrane properties [8], [11], while micropipette aspiration [4], [12] and microplate stretchers [13] measure global cell properties.

Other techniques have been developed to probe the intracellular environment. Particle tracking provides a very localized measurement of cellular viscosity without having to take into account the contribution of the plasma membrane [14]. Fluorescence recovery after photobleaching (FRAP) has been used to measure protein mobility in live cells [15], however the high powered bleaching pulse used in FRAP may directly alter the diffusion dependent kinetics in a cell by altering local viscosity [16]. A relatively new method called raster image correlation spectroscopy (RICS), can accurately measure molecular diffusion in the microseconds-to-seconds timescale [17] in living cells using a laser scanning confocal microscope. In relation to cancer, this technique has been used to measure the diffusion of P53 in live HeLa cells treated with DNA damaging agents [18].
Genetic instability is the underlying driver of the neoplastic process providing an important role in both initiation and progression of cancers [19]. As such, DNA repair processes play a central role in the prevention of cancer by correcting damage caused by exogenous and endogenous sources. The mismatch repair process corrects base substitution mismatches and insertion-deletion mismatches resulting from replication errors and recombination events. The replication of DNA damaged in this way leads to the incorporation of wrong bases opposite the damaged bases in daughter cells. These mutations cannot be corrected after incorporation and thus the mutations accumulate over time. The first step in the mismatch repair process is recognition of the mismatch. This is performed by a heterodimeric protein complex consisting of MutSα homologous proteins MSH2 and MSH6. These proteins must find their way to the DNA within the nucleus and be able to selectively bind to their target which involves specific conformational changes [20]. All these activities are susceptible to changes in the cellular environment. In the current study, we use RICS to measure the diffusion of enhanced green fluorescent protein (EGFP) and EGFP-MSH2 constructs in human mammary epithelial cells engineered to mimic different stages of neoplastic transformation.

METHODS

Cell Culture and Transfection

For the current study, we used human mammary epithelial (HME) cells that were engineered by the Weinberg laboratory to simulate progressive stages of neoplastic transformation [21]. These cells include low-passage normal HME cells (Lonza), HME cells expressing hTERT and large T antigen (LT) (HME +LT, TERT), and HMECs
expressing hTERT, LT, and H-rasV12 (HMLER). We also included the human metastatic breast cancer cell line MDA-MB-231. The expression of hTERT lengthens and maintains telomeres in DNA strands allowing previously senescent cells to exceed the Hayflick limit. Increased telomerase activity alone was found to be sufficient to immortalize post-M0 HME cells but not pre-M0 HMEs [22]. Expression of hTERT and deactivation of the pRB and p53 pathways by LT were sufficient to create immortal HMECs that did not form subcutaneous tumors in mice [21]. HMECs expressing hTERT, LT, and H-rasV12 were shown to form tumors in nude mice with 52% efficiency [21]. MDA-MB-231 cells were obtained from the ATCC (MDA-MB-231 ATCC HTB-26). A summary of the cell types is listed in Table III.

Table III. A summary of the human mammary epithelial cell types used.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Immortal</th>
<th>Tumorigenic</th>
<th>Metastatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HME</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>HME + LT, TERT</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>HMLER</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

Cells were cultured in 35 mm poly-d-lysine coated glass bottom dishes (MatTek) at 37°C in 5% CO₂ in MEGM Mammary Epithelial Cell Growth Medium (Lonza) with bovine pituitary extract (BPE) at a final concentration of 52 µg/ml. Cells were passaged at about 70 – 90% confluence. Culture medium was replaced every 2 days. For
fluorescence imaging, cells were transfected with EGFP and EGFP-MSH2 plasmids using Lipofectamine LTX (Life Technologies). For each 35 mm dish, 200 μl of Opti-MEM reduced serum media (Life Technologies), 10 μl Lipofectamine LTX, 2.5 μl Plus Reagent, and 2.5 μg of DNA plasmid were added. Cells were transfected at 50-80% confluence and imaged within 1 – 3 days after transfection.

**Microscopy**

Fluorescence imaging was done on a Zeiss LSM 710 laser scanning confocal microscope mounted on an AxioObserver Z.1 inverted microscope using a 63x 1.4 NA oil-immersion objective. Cell dishes were mounted in an enclosed Pecon PS1 incubation system (Carl Zeiss) to maintain the local environment at 37°C with 5% CO₂ and high humidity to prevent media evaporation while imaging.

For RICS imaging, stacks of 100 frames were collected for each sample with a scan area of 128 x 128 pixels. Pixel size was set to 0.03 - 0.05 μm. The pixel dwell time was set to 100.85 μs corresponding to a line time of 28.04 ms. This relatively slow scan speed was found to give the greatest signal to noise. The 488 nm line of a 35 mW argon laser was used for EGFP excitation. The power of the laser was set to 0.5% using the included Zen software. Images were collected in the cytoplasm and nucleus of all four cells types. Cells undergoing mitosis and rounded cells were excluded from the study. Figure 14 shows an image of HME + LT, TERT cells with expression of EGFP-MSH2 and MDA-MB-231 cells with expression of EGFP.
RICS Theory and Analysis

Raster Image Correlation Spectroscopy (RICS) is a single-molecule fluorescence imaging correlation spectroscopy [17], [23]–[25] that takes advantage of the inherent time information stored in a raster scanned image as the confocal scanning laser moves across the sample. Basically the intensity of one pixel is measured, and then the intensity at a neighboring pixel is measured immediately after. If the fluorescent molecule being imaged has moved to this neighboring pixel, there will be a correlation in the intensity fluctuations of these pixels related to the time it takes for the laser to move to the next pixel. This is done for each pixel in the image to generate the spatial correlation function defined in (1).

\[
G_S(\xi, \psi) = \frac{\langle I(x,y)I(x+\xi,y+\psi) \rangle_{xy}}{\langle I(x,y) \rangle_{\xi,y}^2}
\]  

(1)
Here $I(x,y)$ is the intensity corresponding to the pixel located at the $(x,y)$ position of the image, $\xi$ and $\psi$ are the spatial increments in the x and y directions, and the angle brackets indicate averages over the x and y directions. The RICS correlation function $G(\xi,\psi)$ for freely diffusing molecules can be expressed as the product of 3 factors as shown in (2).

$$G(\xi,\psi) = G_D(\xi,\psi)S(\xi,\psi)G_T(\xi,\psi)$$

(2)

The first factor describes the effect of diffusion on the intensity of one pixel versus its neighboring pixels while taking into account the difference in time between the horizontal and vertical line times. The diffusion factor is given in (3).

$$G_D = \frac{\gamma}{N} \left( 1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{\omega_0^2} \right)^{-1} \left( 1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{\omega_z^2} \right)^{-1/2}$$

(3)

Here, $D$ is the diffusion coefficient in $\mu m^2/s$, $\gamma$ is a factor to account for beam geometry, $N$ is the number of molecules in the focal volume, $\tau_p$ and $\tau_l$ are the pixel and line times in seconds, and $\omega_0$ and $\omega_z$ are the distances from the central peak intensity of the beam spot - the point spread function (PSF) - where the intensity has fallen by $1/e^2$ in the radial and axial directions in $\mu m$. The values for $\omega_0$ and $\omega_z$ are 0.314 and 1.57 $\mu m$ respectively and were determined by performing RICS with fluorescein which has a known diffusion coefficient of 300 $\mu m^2/s$ in water [26], [27].

The second factor of the RICS correlation function describes the apparent broadening of the Gaussian PSF due to diffusion and is given in (4).

$$S(\xi,\psi) = \exp \left( -\frac{\left( \frac{\xi^2 + \psi^2}{\omega_0^2} \right)^2}{1 + \frac{4D(\tau_p \xi + \tau_l \psi)}{\omega_0^2}} \right)$$

(4)
Here \( \delta r \) is the pixel size in microns. The final factor of the RICS correlation function accounts for fluctuations in brightness of a single molecule over time. This is commonly referred to as the blinking term and is given in (5).

\[
G_T(\xi, \psi) = 1 + Ae^{-(\tau_p \xi + \tau_r \psi) / \tau}
\]  

(5)

Here \( A \) and \( \tau \) relate to the changes in fluorescence intensity and the characteristic time of the blinking. This term is negligible when measuring fast dynamics or in the pure diffusion case.

Analysis of the images was done using Matlab and SimFCS (Laboratory for Fluorescence Dynamics) as described by [23]. Figure 15 below shows a sample image for RICS analysis, the autocorrelation function of the image, and sample fits to the autocorrelation function for molecules diffusing at 0.4 and 4.0 \( \mu m^2/s \). The autocorrelation function becomes narrower both in the horizontal and vertical directions as the diffusion rate increases, and for the diffusion values illustrated in Fig. 4, the narrowing in the vertical direction is quite apparent.
Figure 15. A: One frame of a sample RICS image showing streaks due to particles diffusing at 4 \( \mu \text{m}^2/\text{s} \). B: RICS autocorrelation function of A. C-D: Fit to the autocorrelation function (lower surface) and residues (upper surface) for particles diffusing at 0.4 \( \mu \text{m}^2/\text{s} \) (C) and 4.0 \( \mu \text{m}^2/\text{s} \) (D). The autocorrelation function becomes narrow in the horizontal direction and vertical direction as the diffusion rate increases.

RICS Simulations

Computer simulations of RICS experiments were performed using SimFCS. For each simulation, a single population of particles diffused through a box with 12.8 \( \mu \)m sides. The point spread function was simulated using a 3-D Gaussian with independent radial and axial waists. The radial waist was set to 0.314 \( \mu \)m to match our confocal microscope. The axial waist was set to five times the radial waist. The simulations generated stacks of image files which were then analyzed as described above in SimFCS.
In these simulations, we adjusted the diffusion rate of the particles to 0.4, 0.8, 2.0, or 4.0 \( \mu \text{m}^2/\text{s} \), and the total number of particles to 5, 10, 22, 36, 50, 100, 500, and 1000. To determine if the pixel dwell time had a significant impact on the measured diffusion coefficient, we adjusted the simulated pixel dwell time between, 50, 100, 200, and 400 \( \mu \text{s} \) for the simulations with particles diffusing at 0.4 \( \mu \text{m}^2/\text{s} \).

RESULTS

We performed RICS simulations using SimFCS to explore the effect of the fluorophore concentration on the measured diffusion coefficient. The results of these simulations are shown in Figure 16. The total number of particles ranged from 5 to 4000. We simulated particles with diffusion coefficients ranging between 0.4 and 4.0 \( \mu \text{m}^2/\text{s} \). All simulations showed a decrease in the measured diffusion as the number of particles increased beyond 20. In simulations with greater than 100 particles, the measured diffusion seems to asymptotically approach 0.4 times the theoretical diffusion. One explanation for this observation in real cells could be particle-particle interaction. However in our simulations the concentration of particles is low enough that particle interactions are negligible. Another possible explanation is that at high enough particle densities, as particles are left behind by the scanning laser, new particles will replace the old ones in the focal volume. To the laser this would appear as the first particle staying in the beam longer giving rise to a slow diffusion bias. Three different scan speeds of the laser were also chosen for particles diffusing at 0.4 \( \mu \text{m}^2/\text{s} \) in the simulations. All three
scan speeds were able to accurately predict the diffusion rate - however the fast scan speeds have a larger error compared to the slow scan speeds.

Figure 16. Results of RICS simulations with varying number of particles and diffusion coefficients. The diffusion coefficients given in the legend are in units of \( \mu m^2/s \). The ratio of the measured diffusion coefficient to the theoretical diffusion is plotted versus the number of particles. At high particle densities, the measured diffusion decreases to 0.4 times the theoretical diffusion.

Four different cell types corresponding to different stages of neoplastic transformation were studied. In the cytoplasm and nucleus of each of these cell types, we measured the diffusion dynamics of 27 kDa EGFP and 133 kDa EGFP-MSH2 constructs. RICS images sometimes contain large immobile structures that tend to dominate the spatial correlation function. To filter out these immobile features, we subtracted the average image of the cell calculated using a moving average of ten frames in each 100
frame stack. The focal volume was calibrated as described in [23] using fluorescein dye which has a known diffusion constant in water of 300 \( \mu \text{m}^2/\text{s} \) to give a \( \omega_0 \) value of 0.314 \( \mu \text{m} \). This calibration method assumes a Gaussian shaped confocal laser beam.

We performed fluorescence loss in photobleaching (FLIP) experiments to determine if the EGFP and EGFP-MSH2 constructs were able to freely diffuse through the cell and cross the nuclear membrane. In these experiments, the entire nucleus of an HMLER cell was repeatedly bleached while monitoring the fluorescence intensity of a region of interest in the cytoplasm. The fluorescence intensity in the cytoplasm continuously decreased as the fluorescent proteins diffused into the nucleus and were bleached. When the bleaching pulse was turned off, the fluorescence intensity quickly recovered in the nucleus indicating there is no significant barrier to EGFP or EGFP-MSH2 localization to the nucleus (see Appendix).

A summary of the diffusion measurements as a function of location and cell types is given in Figure 17. For each data point, between 19 and 52 measurements were taken (median = 29) for a total \( N = 515 \). From these results several interesting observations can be made. First, in the cytoplasm of the cells there is no significant difference in the diffusion rate between the EGFP and EGFP-MSH2 constructs. This is expected as EGFP does not show specific binding to cellular structures or other large protein complexes in the cytoplasm and MSH2’s known interactions are primarily in the nucleus. If we assume spherical particles, one would expect the EGFP to have a diffusion rate 1.6 times the diffusion of EGFP-MSH2 due to the size of the proteins, however this is not a statistically significant difference. Second, we measured no statistically significant difference in diffusion rates in the cytoplasm among the different cell types using the Student’s t-test.
(p > 0.05). Previous studies have shown the p53 gene to be implicated in cytoskeletal reorganization by interaction through intermediate filaments and actin [28], [29]. Similarly, other studies have suggested Ras and Ras-related GTPases exhibit some control over actin reorganization [30], [31]. This cytoskeletal reorganization affects the macroscopic properties of the whole cell [2], [3], [32]; however, at the length scales (<10 nm) measured by the current study, there is no significant change in diffusion. Third, there is a significant increase in the diffusion rate of EGFP in the nuclei of HME, HME+LT+hTERT, and MDA-MB-231 cells. Micro-pipette aspiration has shown isolated nuclei of articular chondrocytes to be 3-4 times stiffer and twice as viscous as the cytoplasm [33], however microinjected FITC dextrans of molecular size up 580 kDa were found to be fully mobile in the nucleus. In contrast, our results suggest that at small length scales, the nucleus is less viscous than the cytoplasm, or at least contains open compartments within the nuclear matrix where free diffusion may occur for smaller proteins.
Figure 17. Summary of the diffusion measurements in all 4 cell types (normal, immortal, tumorigenic, metastatic) for the 4 different combinations of diffusing protein (free EGFP and EGFP-MSH2 complex) and cell region (cytoplasm and nucleus). Error bars are standard error of the means and the total N = 515.

Lastly, the most striking result is that the diffusion of EGFP in HMLER cells is about 4 times slower in the nucleus compared to HME cells and about 15 times slower compared to HME+LT,hTERT and MDA-MB-231 cells. The EGFP should not specifically bind to any location in the nucleus so the slower diffusion rate must be due to the rearrangement of the nuclear matrix to create a higher viscosity than that observed in the other cells. A previous study of nuclear proteins in breast cancer cells implicated the protein P114 to strongly bind DNA fragments to matrix attachment regions within the nucleus [34]. The P114 binding activity was observed in human breast carcinomas but not in normal or benign breast cells. This could create transient nuclear microdomains leading to the slower diffusion rate we observed. Another study showed the protein P300 induced nuclear morphological changes that correlated to cancer prognosis in prostate cells [35]. P300 induces histone acetylation, which changes the chromatin structure. The authors also suggest that expression of P300 is mediated by lamin A and lamin C, which
are the constituent components of the nuclear matrix. Mutations in lamina have also been linked to colorectal cancer [36]. The major difference between the HMLER cells and the others is the high amount of mutated Ras in the HMLER cells. The P114 protein is part of the Rho-GTPase EGF family, which exerts some control over the Ras/Rho-GTPase proteins, and altered expression of lamina has been observed in Ras transformed cells. This makes the Ras family of proteins an attractive marker for cancer or a possible therapeutic target.

All cell types measured except for the HMLER cells showed a statistically significant decrease (p < 0.05) in diffusion rates in the nucleus with EGFP-MSH2 compared to EGFP alone. The EGFP-MSH2 is about 100 kDa larger than EGFP. Assuming the proteins are roughly spherical, we would expect the diffusion to decrease by a factor of 1.6 for the larger protein. The EGFP-MSH2 may still be able to dimerize with MSH6 to form an EGFP-MutSα complex. Again assuming spherical particles, one would expect a factor of 2 decrease in diffusion based on size alone. In HME cells, we do see this factor of 2 decrease. In contrast, the diffusion of EGFP-MSH2 in the nucleus of HME+LT,hTERT and MDA-MB-231 cells was found to be 7 – 8 times slower than EGFP alone. A fraction of this EGFP-MutSα may transiently bind to DNA, which would slow diffusion, however this would not explain why the HMLER cells show no difference in diffusion and the HME cells only show a factor of 2 difference in diffusion in the nucleus. There are also other proteins that the MSH2 may be interacting with to slow down the diffusion. A more likely explanation is anomalous sub-diffusion. Anomalous sub-diffusion governs Brownian motion on length scales smaller than a crossover length which depends on the concentration of immobilized obstacles and the
percolation threshold [37], [38]. Our results suggest that in the HME+LT,hTERT and MDA-MB-231 cells, the nuclear organization is altered such that a doubling in size of our diffusing protein leads to a drastic decrease in the diffusion coefficient. The concentration of obstacles in the nucleus of these cells creates barriers on the MutSα length scale (10 – 20 nm). In contrast, the nucleus of HME cells contains barriers interacting at larger length scales since we only see a factor of 2 decrease in diffusion, and the nucleus of HMLER cells contains barriers interacting at smaller length scales since we measure much slower diffusion. It is unclear from the current study whether MutSα mismatch recognition activity is altered by the transformation of the cells or the inclusion of EGFP, though EGFP-MSH2 continues to interact with its partner, MSH6 [39].

The viscosity of the cytoplasmic and nuclear region of the cells can be estimated from the translational diffusion coefficient by the Stokes-Einstein relation \( \eta = \frac{k_B T}{6\pi r D} \), where \( \eta \) is the viscosity, \( k_B = 1.38 \times 10^{-23} \text{ m}^2\text{kg/sK} \) is the Boltzman constant, \( T \) is the temperature (which in our study is 310 K), \( r \) is the radius of the particle, and \( D \) is the diffusion coefficient. In the cytoplasm of all of our tested cell types, using EGFP or EGFP-MSH2, we calculate a viscosity of between 24 and 100 mP·s. In the nucleus of our cells with EGFP-MSH2, we calculate a similar viscosity range of between 24 and 75 mP·s. In the nucleus of HME+LT+TERT and MDA-MB-231 cells where we observed faster diffusion of free EGFP, we calculate a viscosity of 4.6 mP·s. These results are similar to other reported values in the literature in different cells types and using different methods of measurement (see Table IV).
Table IV. A comparison of the viscosity in living cells as measured by small particles.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Cell</th>
<th>Location</th>
<th>Technique</th>
<th>Viscosity (cP)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP/MSH2</td>
<td>HME</td>
<td>Cytoplasm</td>
<td>RICS</td>
<td>24 – 100</td>
<td>Current study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.6 - 61</td>
<td></td>
</tr>
<tr>
<td>Porphyrin dimer</td>
<td>CHO</td>
<td>Cytoplasm</td>
<td>Fluorescent ratiometric</td>
<td>50</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>3T3</td>
<td>Cytoplasm</td>
<td>molecular rotor</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Various sizes of</td>
<td>HeLa</td>
<td>Cytoplasm</td>
<td>Reanalysis of previous results</td>
<td>2.0 – 44</td>
<td>[37]</td>
</tr>
<tr>
<td>PEG/PEO</td>
<td></td>
<td></td>
<td>obtained by FRAP/FCS</td>
<td>0.88 - 24</td>
<td></td>
</tr>
<tr>
<td>ERCC1/XPF-GFP</td>
<td>3T3</td>
<td>Nucleus</td>
<td>FRAP</td>
<td>5</td>
<td>[38]</td>
</tr>
<tr>
<td>EGFP</td>
<td>ASTC-a-1</td>
<td>Nucleus</td>
<td>FCS</td>
<td>2.55</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Nucleus</td>
<td></td>
<td>2.04</td>
<td></td>
</tr>
</tbody>
</table>

What is clear from the current study and other recent reports is that cancer can alter the skeletal structure of both the cytoplasm and nucleus. The structure of these domains influences diffusion and molecular transport, which has broad ranging implications in processes like transcription, signaling, and cell metabolism. As we have shown in this study, the relative length scale of the molecule being studied to the surrounding barriers is critical to the diffusive behavior of the molecule. As the size of molecules increases or the surrounding environment of the cell becomes more crowded, anomalous sub-diffusion dominates. The next important question to ask then is what is the biological significance? Brownian motion may seem more efficient because particles are able to diffuse faster. There are, however, instances where sub-diffusion may prove more beneficial. For example, sub-diffusion may increase efficiency of certain processes.
by keeping reactants localized. This may be especially true in the cell nucleus where correct chromosomal positioning is necessary for proper transcription. As single-molecule tracking techniques continue to develop and improve, we can begin to understand how protein and small molecule dynamics in the cell are altered by and in return alter the functions of the cell. Future research in this area must consider the environment of the cell on a submicron level in order to reveal the underlying mechanisms for processes controlling dynamic biological systems.

CONCLUSION

FRAP and fluorescence correlation spectroscopy (FCS) have been used to study protein mobility in cancer cells, but each suffers from a few disadvantages. FRAP usually requires the concentration of labeled molecules to be on the micromolar level. If one is interested in the binding dynamics of these molecules with a limited number of binding cites, the entire process may be hidden by the large number of freely diffusing molecules. Second, the intense bleach pulse used in FRAP may cause a cellular response that changes the properties one is trying to measure [16]. FCS has the disadvantage of being limited to measuring dynamics that occur on the time scale of milliseconds. Here we used the novel technique of RICS. It can probe a broad range of time scales, enabling the study of fast diffusion or slower transient binding dynamics at small length scales where the dynamics of individual proteins becomes important. It is clear that genes, and the pathways they control, play a crucial role in the genesis and progression of cancer. We have shown here that RICS is a useful tool to characterize the dynamic processes that
may affect gene expression in the search for new and better cancer diagnostics and therapeutics.

We have measured the diffusion of small proteins in the cytoplasm and nucleus of four different cell lines representing an extensive spectrum of neoplastic transformation. Many studies have shown the effects that cytoskeletal reorganization has on the mechanical properties of whole cells and how these changes relate to cancer. In the current study we were able to determine that cytoskeletal reorganization did not affect the diffusion of proteins through the cytoplasm. In the nucleus we found the diffusion of EGFP is about 15 fold slower in tumorigenic cells compared to the other cell types tested. We also see that the addition of MSH2 to EGFP slows the diffusion in the nucleus of the other cell types. In the HME nucleus, the diffusion rate is slowed by a factor of 2 which is expected for a particle following Brownian motion. In the HME+LT+TERT and MDA-MB-231 nuclei, the EGFP-MSH2 diffuses 7 – 8 times slower compared to EGFP alone. We suggest the process of neoplastic transformation alters the nuclear skeleton on a small enough length scale to affect the mobility of proteins and other small molecules. Whether this anomalous sub-diffusion is advantageous for the cell is subject to debate.
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REFERENCES


CHAPTER V

A COMPARISON OF RICS & FRAP FOR MEASURING DIFFUSION OF SMALL PROTEINS IN LIVE CELLS

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The following manuscript has been written for journal submission. Justin Sigley performed all experiments and data analysis and drafted the manuscript. Anita McCauley helped with initial setup of the confocal microscope and edited the manuscript. Martin Guthold and Keith Bonin acted in an advisory and editorial capacity during data collection, analysis, and preparation of the manuscript.
ABSTRACT

There are several different approaches to measuring the dynamics of small molecules in live cells, but the accuracy of these measurements remains uncertain. Cross-validation of two techniques is often used to ensure accuracy, but other approaches are needed to rule out mutual errors. We have used raster image correlation spectroscopy (RICS) and fluorescence recovery after photobleaching (FRAP) to measure the diffusion coefficients of EGFP and EGFP-MSH2 constructs in human mammary epithelial cells mimicking four stages of neoplastic transformation. The RICS and FRAP results agree in 87% of the measurements. We then demonstrate the accuracy of the RICS measurements by performing a RICS analysis on 6 nm quantum dots undergoing a programmatically controlled 2D random walk. Direct confirmation of RICS results provides more confidence in our cross-validation with FRAP measurements, and provides progress toward a gold standard in molecular dynamics measurements in live cells.

INTRODUCTION

Recent advances in microscopy have enabled scientists to quantify dynamic processes in live cells at the molecular level [1]–[3]. Better microscopy systems, such as confocal microscopy, and the discovery of fluorescent proteins which can be genetically encoded into living cells, have been especially critical for the study of protein dynamics in live cells [4], [5]. Several techniques have been developed to take advantage of these technologies. One commonly used technique to measure molecular dynamics is Fluorescence Recovery after Photobleaching (FRAP). In FRAP, the fluorescent molecules in a specified region of the cell are photobleached, and the rate at which the
bleached molecules are replaced by unbleached ones from outside the bleached region is measured by taking a time series of images during fluorescent recovery of the bleached region. A series of kinetic models can then be applied to the recovery of fluorescence in the bleached area to obtain estimates of diffusion and/or binding rates. These kinetic models are often constructed to simulate different aspects of the FRAP experiment such as the photobleach, diffusion, binding kinetics, and cellular geometry. In recent years, it has become increasingly recognized that the choice of kinetic model can give very different diffusion or binding results even though the analysis was based on the same or similar studies [1], [6]. Another source of these divergent results is the bleaching and imaging protocols used in the various studies [7]. Much work has been done to correct for errors in previous FRAP analysis due to differing imaging protocols [8]. This includes proper correction for observational photobleaching, detector blinding, and initial photobleach profiles. However, without a standard set of protocols for FRAP collection and analysis, cross-validation by other techniques is required.

A relatively new technique that can measure diffusion and binding rates on similar timescales to FRAP is Raster Image Correlation Spectroscopy (RICS) [9]. RICS has a few distinct advantages over FRAP. First, FRAP measures the behavior of an ensemble of molecules requiring micromolar concentrations of fluorescent particles [10]. In contrast, RICS measures the dynamics of single particles which can be measured at concentrations much closer to endogenous levels. Second, the intense bleaching pulse used in FRAP has been shown to increase local intracellular viscosity altering the mechanics that one is trying to measure [11]. In this study we measure the diffusion coefficients of enhanced green fluorescent protein (EGFP) and EGFP-MSH2 constructs
in human mammary epithelial (HME) cells using both FRAP and RICS techniques. MSH2 is one of the homologous proteins that make up the heterodimeric protein MutSα which is implicated in DNA mismatch repair recognition [12]. We have included MSH2 in this study to determine if the FRAP and RICS techniques can measure a slower diffusion coefficient due to a larger protein that is known to interact with other large proteins and DNA. The two techniques show good agreement in more than 87% of the taken measurements providing good cross-validation. We have also devised a validation method for RICS based on computationally controlled movement of quantum dots that enables us to measure diffusion coefficients in a highly controlled manner independent of cell or fluorescent probe effects.

METHODS

Cell Culture and Transfection

For the current study, we use human mammary epithelial (HME) cells that were engineered by the Weinberg laboratory to simulate progressive stages of neoplastic transformation [13]. These cells include low-passage normal HME cells (Lonza), HME cells expressing hTERT and large T antigen (LT) (HME +LT, TERT), and HMECs expressing hTERT, LT, and H-rasV12 (HMLER). We also included the human metastatic breast cancer cell line MDA-MB-231. These four cell types were selected because they represent four different stages (or degree) of neoplastic transformation: normal, immortal, tumorigenic, and metastatic. Cells were cultured in 35 mm poly-d-lysine coated glass bottom dishes (MatTek) at 37°C in 5% CO₂ in MEGM Mammary Epithelial Cell Growth Medium (Lonza) with bovine pituitary extract (BPE) at a final concentration of 52 μg/ml.
Cells were passaged at about 70 – 90% confluence. Culture medium was replaced every 2 days. For fluorescence imaging, cells were transfected with EGFP and EGFP-MSH2 plasmids using Lipofectamine LTX (Life Technologies). For each 35 mm dish, 200 μl of Opti-MEM reduced serum media (Life Technologies), 10 μl Lipofectamine LTX, 2.5 μl Plus Reagent, and 2.5 μg of DNA plasmid were added. Cells were transfected at 50-80% confluence and imaged within 1 – 3 days after transfection.

**Microscopy**

Fluorescence imaging for both FRAP and RICS was done on the same Zeiss LSM 710 laser scanning confocal microscope mounted on an AxioObserver Z.1 inverted microscope using a 63x 1.4 NA oil-immersion objective. Cell dishes were mounted in an enclosed Pecon PS1 incubation system (Carl Zeiss) to maintain the local environment at 37°C with 5% CO₂ and high humidity to prevent media evaporation while imaging. The 488 nm line of a 35 mW argon laser was used for all fluorescence imaging. For FRAP acquisition, 1500 images at an image size of 512 x 50 pixels were acquired. The zoom factor was set to 4 to yield a pixel size of 0.088 μm. A single bleaching pulse was performed on the 300th scan in a circle of 1.58 μm radius. Rapid observational photobleaching occurs during the first few image scans so 300 prebleach images were taken to ensure that observational photobleaching was in a regime that could accurately be described by an exponential decay function. The laser output was set to 0.5% for observation and 100% for the bleaching pulse.

For RICS image acquisition, stacks of 100 frames were collected for each sample with a scan area of 128 x 128 pixels. Pixel size was set to 0.03 - 0.05 μm. The pixel dwell time was set to 100.85 μs corresponding to a line time of 28.04 ms. A faster scanning rate
was initially used, but produced measurements with low signal-to-noise ratios. We systematically reduced the scan speed until the signal-to-noise ratio plateaued. We found 100.85 μs was the fastest scan speed that did not decrease our signal-to-noise ratio. Again the 488 nm line of a 35 mW argon laser was used for EGFP excitation. The power of the laser was set to 0.5% using the included Zen software. The laser beam waist was measured to be 0.314 μm in the radial direction and 1.57 μm in the axial direction and was determined by performing RICS on fluorescein which has a known diffusion coefficient of 300 μm²/s in water [14], [15]. RICS and FRAP images were collected in the cytoplasm and nucleus of all four cell types. Cells undergoing mitosis and rounded cells were excluded from the study.

We also performed two sets of RICS measurements on 6 nm diameter COOH functionalized CdSeS/ZnS quantum dots (Sigma). For these experiments, a 1 μg/ml solution of the quantum dots in water was applied to a glass microscope slide and dried in ambient conditions. For one set of RICS experiments, the quantum dots were imaged while the slide was held stationary in the confocal microscope. For the other set of experiments, we attached the slide to a MP-285 Motorized Micromanipulator (Sutter Instruments). The Micromanipulator and slide were controlled by a custom Matlab program to simulate a 2D random walk with a diffusion coefficient of 15.8 μm²/s. The slide was imaged during this walk using the same protocols for RICS imaging described above and the resulting images analyzed in SimFCS.

FRAP Data Processing

All FRAP data processing was performed by a custom written Matlab program. In the raw FRAP data, the intensity from three regions of interest (ROI) were collected. The
first ROI is the bleach and recovery spot. The second ROI is a nearby region in the cell dish containing no cells. This region supplies a background value that is subtracted from the measured FRAP data. The third ROI is a region in a nearby cell that has fluorescence but is not connected to the cell being measured. This ROI was used to determine if detector blinding occurred. Detector blinding is a temporary loss of detector sensitivity that can occur after the bright bleach pulse. We did not observe detector blinding in any of our samples. After the initial FRAP curve was collected, a second set of images was taken with identical settings to the FRAP collection except without the bleach. This image series is used to correct for observational photobleaching and was collected at least 1 minute after the original FRAP curve to ensure the fluorescence had completely equilibrated. An exponential decay function was fit to the observational photobleaching. The FRAP curve was then divided by the exponential function. Finally the corrected FRAP curve was logarithmically averaged so as not to bias the plateau region of the curve when fitting with a FRAP model (Figure 18).
EGFP is not expected to specifically bind to any cellular structures or other large protein complexes in the cytoplasm or nucleus of the cells used in these experiments, so we used a simple diffusion model to fit the FRAP data. We also tried fitting the FRAP data to a full reaction-diffusion model as described in [16] however the fits were insensitive to changes in the pseudo-on rate ($k^*_{on}$) and the off-rate ($k_{off}$), and an f-test of the chi-squared values of the fits showed there was no statistical improvement to the fits when using a full model compared to a simple diffusion model. For a circular bleach spot, a closed form solution for the simple diffusion FRAP model [17] is given by

$$f_{rap}(t) = e^{-\frac{\tau_D}{2t}}\left[I_0\left(\frac{\tau_D}{2t}\right) + I_1\left(\frac{\tau_D}{2t}\right)\right],$$

(1)

where $\tau_D = \omega_0^2/D_r$, $\omega_0$ is the bleach spot radius, $t$ is time, $D_r$ is the diffusion coefficient, and $I_0$ and $I_1$ are modified Bessel functions.
RICS Data Processing

RICS is a single-molecule fluorescence imaging correlation spectroscopy [2], [4], [9], [18] that takes advantage of the inherent time information stored in a raster scanned image as the confocal scanning laser moves across the sample. Basically the intensity of one pixel is measured, and then the intensity at a neighboring pixel is measured immediately after. If the fluorescent molecule being imaged has moved to this neighboring pixel, there will be a correlation in the intensity fluctuations of these pixels related to the time it takes for the laser to move to the next pixel. This is done for each pixel in the image to generate a spatial correlation function. We then fit a RICS correlation function for freely diffusing molecules to the spatial correlation function as described previously [2]. Figure 19 shows an example of RICS curves for two different diffusion coefficients.

**Figure 19.** Example fits to RICS data (bottom surfaces) and their residuals (top surfaces). The spatial correlation fit on the left is for a particle with a diffusion of 0.4 μm²/s while the fit on the right is for a particle with a diffusion coefficient of 4.0 μm²/s. As the diffusion coefficient increases, the spatial correlation fit gets narrower along the x-direction (laser scanning direction) and y-direction.
RESULTS

FRAP Bleaching Pulse Intensity

A bleach depth of about 50% is desired to acquire FRAP curves with a good signal to noise ratio. Deep bleaches can be obtained by either increasing the power of the bleaching laser or by making several bleaches in the same region. There is an issue with performing several bleaches in the same region. For highly dynamic processes, such as diffusion, there will be an exchange of particles into and out of the bleach region while the bleaching pulse is taking place. This exchange of particles can complicate the interpretation of the recovery curves. Instead we chose to increase photon flux by increasing the power of the laser while keeping the time to bleach the same. We found that as we increased the power of the laser, we measured longer recovery times indicating slower diffusion. For a few of our experiments, the diffusion rate was more than two orders of magnitude smaller than what we measured with RICS. There are two factors that we think may play a role in the flux dependent recovery. First, the higher energy of the laser pulse may be exciting a long lived non-emissive triplet state in the EGFP [19]. Second, the intense bleaching pulse may perturb the local area of the cell in such a way that longer recovery times are observed [7], [11]. To test this hypothesis, we repeatedly bleached a portion of the cell, and then monitored the fluorescence intensity of the whole cell over a period of five minutes (see Appendix). We found the total fluorescence intensity in the cell decreased while the bleaching was taking place, however five minutes after the bleaching, the fluorescence intensity of the whole cell had recovered to the prebleach intensity. This suggests the EGFP was being excited to a non-emissive state with a lifetime on the order of minutes. We decreased the bleaching laser intensity until
this effect disappeared. A balance must be struck in choosing the laser intensity such that there is a good bleach depth without impacting the mechanics one is trying to measure.

The Effect of Fluorophore Concentration

FRAP measurements are commonly acquired in relatively bright cells with a fluorophore concentration in the micro-molar range [16]. In contrast, RICS measurements are usually performed in cells with a fluorophore concentration in the nano-molar range [20]. We measured the fluorescence intensity of solutions with known concentrations of EGFP and compared that to the cellular intensities during RICS and FRAP experiments to approximate the concentration of EGFP within the cells to be between 30 and 500 nM. In order to directly compare FRAP and RICS, we attempted to measure diffusion coefficients using RICS in cells with fluorophore concentrations in the micro-molar range. However at these concentrations, the fluorescence intensity fluctuations due to particles coming into and out of the laser beam are indistinguishable from the background. We had difficulty fitting a RICS correlation function to the autocorrelation data in these cells, but in the bright cells where we could fit a RICS function, we measured an average diffusion coefficient of $0.24 \pm 0.13 \mu m^2/s$. This is similar to the vibration or noise of our system which we measured to be $0.45 \pm 0.09 \mu m^2/s$ by performing RICS on fixed quantum dots. We used time lapse photography to measure the thermal drift of our system using the same quantum dots to be much smaller at $1.0 \pm 0.5 \text{nm/s}$. We found RICS measurements in cells with a fluorophore concentration greater than 500 nM are difficult to acquire and do not provide accurate values.

Next we performed FRAP experiments in cells with low fluorophore concentrations (<500 nM). There was increased noise in the FRAP measurements due to
a reduced bleach depth, however the diffusion coefficient remained statistically unchanged in dim cells compared to bright cells.

Comparison of RICS and FRAP Measurements

Figure 20 shows a comparison of diffusion coefficients of EGFP and EGFP-MSH2 constructs measured using RICS and FRAP in HME, HME+LT, hTERT, HMLER, and MDA-MB-231 cells. We find the FRAP and RICS diffusion measurements agree in 14 out of the 16 cases (4 cell types, 2 proteins, and 2 locations within the cell).
Figure 20. Diffusion coefficients measured using FRAP (A) and RICS (B). Diffusion measurements using both methods agree within error for greater than 87% of the samples. Error bars are standard errors of the mean and the total number of cells measured for RICS was 515 and for FRAP was 352.

The two cases where the FRAP and RICS measurements are not in agreement are when measuring freely diffusing EGFP in the cytoplasm of MDA-MB-231 cells (marked by * in Figs. 3(A) and (B)) and the nucleus of HME+LT,hTERT cells (marked by ** in Figs. 3(A) and (B)). The FRAP results are what we expected to measure. MDA-MB-231 cells are metastatic so must be able to break off from the primary tumor site and squeeze
through the basement membrane to get into the bloodstream or lymph system. After travelling to a new location in the body, the metastatic cells must then squeeze back through the blood/lymph vessel walls to implant into new tissue. Studies have shown metastatic cells are more deformable compared to healthy cells [21]–[25]. Our results reflect this on a small scale. The deformable metastatic cells are presumably less viscous allowing for the rapid diffusion of small molecules and proteins within the cell. We also expected to observe slower diffusion of the EGFP-MSH2 compared to the EGFP alone. There are two reasons for this. First, the EGFP-MSH2 is a much larger protein compared to EGFP alone and as such diffuses more slowly. Second, MSH2 is known to interact with many other proteins which would further slow the effective diffusion coefficient. MSH2 also binds to DNA, however in the time scale of the current study (seconds for FRAP and ms for RICS) most MSH2 bound to DNA would appear immobile and therefore be background subtracted.

Another difference between the FRAP and RICS measurements is that we observed a bimodal distribution of diffusion measurements when performing FRAP experiments on EGFP-MSH2 in the cytoplasm of HME+LT,hTERT cells. A histogram of this distribution is provided in Figure 21. The first peak of the histogram gives an average diffusion coefficient of $2.38 \pm 1.05 \, \mu m^2/s$ which matches our RICS measurements. However the second peak of the histogram gives a diffusion coefficient of $9.74 \pm 0.96 \, \mu m^2/s$. This is the only sample where we observed a bimodal distribution. A couple of the other FRAP measurements did give higher diffusion coefficients, but these were beyond two standard deviations of the average and occurred only once or twice per sample group. They were therefore excluded from the study as outliers.
Figure 21. Histogram of the bimodal distribution of diffusion coefficients measured by FRAP of EGFP-MSH2 in the cytoplasm of HME+LT,hTERT cells. The peak with smaller diffusion coefficients matched our RICS measurements.

While cross-validation of most of our FRAP measurements by RICS experiments is a good indicator that we are accurately measuring the real diffusion rates in cells, there is still the possibility that we are measuring the same incorrect diffusion rates by these two techniques. This is true of all cross-validation techniques. A better way to determine accurate measurements is to design an experiment that removes almost all of the variables that could be misinterpreted in one’s results. For the case of diffusion rates in cells as measured by EGFP, this means eliminating the variability between cell types, local environment, temperature effects, and photobleaching effects, while maintaining the imaging or data collection protocol.

Since the RICS technique works by passive observation, that is we do not have to manipulate the sample intensity to perform a measurement, we can design an experiment that allows us to eliminate many of the variables associated with live cell imaging. We used a micromanipulator to move a microscope slide containing immobile 6 nm quantum dots in a 2D random walk simulating a diffusion rate of 15.8 $\mu$m$^2$/s. Quantum dots were
used in this experiment due to their high quantum efficiency and low susceptibility to photobleaching. The quantum dots are also very similar in size to the EGFP and EGFP-MSH2 used in the live cell experiments. Using this setup, we are able to precisely control and record the position of the quantum dots. This setup also enables us to perform RICS measurements using the exact same protocols that were used when collecting data on live cells. After performing a RICS analysis on the images obtained with this method, we measured a diffusion coefficient of $12.3 \pm 0.7 \, \mu m^2/s$. We do have a systematic error where the RICS measurements are giving us a diffusion coefficient 22% lower than the known “diffusion coefficient” of the quantum dots. We suspect this systematic error is due to the time it takes for the Micromanipulator to change directions. There is a sub-millisecond pause when the Micromanipulator finishes a movement and then is given a new movement command. During this time, the sample is not moving and therefore may systematically decrease the measured diffusion coefficient. This systematic error is smaller than the standard deviation we often see in our live cell measurements due to the heterogeneity of the cells.

**DISCUSSION**

FRAP has been extensively used over the last few decades to measure molecular dynamics in cells. Much effort has gone into creating a gold standard protocol for taking FRAP measurements [1], [7], [16], [26]. Cross-validation by a separate technique is important to provide increased confidence in the efficacy of experimental measurements in vivo. In contrast, RICS is a relatively new technique that is still be developed and applied to study organisms and cells. We have shown here that RICS can be used as a
cross-validation tool for FRAP, but it’s real strength lies in the relative ease with which one can design RICS experiments to measure the diffusion of particles with directly controlled motion. It is for this reason we have more confidence in the RICS measurements in this study. Additionally, RICS is performed in systems with lower concentrations of transfected marker proteins and at lower intensities for scanning than FRAP. FRAP would greatly benefit from a similar experiment and therefore no longer require cross-validation with other techniques. The difficulty lies in the fact that FRAP is an active measurement technique. That is, FRAP requires a direct manipulation of the sample being measured. It will be difficult for an experimenter to design a setup in which there is direct control over the motion of the ensemble of molecules used in FRAP measurements. It is also unclear how our computationally controlled RICS experiment or a similar FRAP experiment could accurately model systems where significant binding is taking place. In our RICS experiment above, the slide could be paused in its random walk to simulate a binding event however this would pause the motion of all particles which does not accurately model the molecular dynamics taking place in live cells with random binding.

CONCLUSION

In this study, we have used FRAP and RICS to cross-validate diffusion coefficients in breast cancer cells. By accounting for fluorescence concentrations and bleaching pulse intensities in FRAP measurements, we were able to obtain diffusion coefficients in FRAP that are close to those obtained with RICS in most cases. While the measurements are similar, there are a number of approximations in both FRAP and RICS
that could alter the measured diffusion coefficients. For FRAP, these include the shape of
the bleaching profile and finite amount of time the bleaching scan takes. For RICS, there
is no observational photobleaching correction. We may also be misinterpreting the results
due to underlying cell processes that were not controlled in these experiments. We
excluded cells undergoing mitosis; however, we were not able to determine which stage
of interphase the cells were in during imaging.

Cross-validation between several orthogonal methods is progress toward
achieving a gold standard for \textit{in vivo} measurements of molecular dynamics. Here we have
presented a method for verifying RICS measurements in simply diffusing systems by
measuring small particles with a programmable diffusion coefficient. This method can be
used to verify results from \textit{in vivo} RICS experiments, which can then be used to cross-
validate other approaches to molecular dynamics measurements.

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REFERENCES


CHAPTER VI

CONCLUSION
The goal of this dissertation was to measure the mechanical properties of two different biological systems and use those mechanical properties to further our understanding of these systems as well as provide insight into future diagnostics and treatments. In Chapters II and III, I examined the mechanical properties of fibrin(ogen) fibers which are the constituent components of blood clots. In Chapters IV and V, I measured the diffusion coefficients of proteins in human breast cancer cells.

ELECTROSPUN FIBRINOGEN FIBERS

Electrospinning is becoming an increasingly popular technique for fabricating biomaterials for tissue engineering use [1]–[5]. Electrospinning is done in ambient conditions to create dry biomaterials. The application of these materials is usually under wet conditions. Thus, it is important to determine the properties of the fibers comprising the materials in both dry and wet conditions. The choice of fiber to use in these biomaterials is also dependent on the biocompatibility of the material. Fibrin(ogen) is a promising material for use in tissue engineering applications. The mechanical properties can be tailored to the specific application. Fibrin(ogen) has also been shown to bind to several cell types including epithelial cells, smooth muscle cells, fibroblasts, leukocytes, and keratinocytes [6]. In order to choose the correct material for a specific tissue engineering application, the mechanical properties of the material must be known. A library of biocompatible materials and their properties will ultimately guide intelligent design of scaffolds and medical devices.

In Chapter II we used a combined atomic force/fluorescence microscopy technique to determine the mechanical properties of dry, electrospun fibrinogen fibers.
These fibers are relatively stiff and remarkably extensible compared to other natural or synthetic fibers. A comparison of electrospun and natural fibrinogen, fibrin, and collagen fibers is given in Table V. Fibrinogen is more extensible and elastic compared to collagen fibers. Electrospun fibrinogen fiber modulus does not depend on diameter; however, other natural fibrin fibers have a modulus that depends exponentially on fiber diameter. Dry, electrospun fibrinogen fibers also have a much greater modulus compared to wet electrospun fibrinogen or natural fibrin fibers. Recent experiments provide evidence that the long, flexible alpha-C region of the fibrin monomer provides fibrin fibers with their low modulus and remarkable extensibility [7], [8]. We propose that solvation is necessary to keep this region unfolded, and thus the removal of water in dry, electrospun fibrinogen fibers may significantly reduce the mobility and extensibility of this region.

<table>
<thead>
<tr>
<th>Fiber type</th>
<th>$\varepsilon_{\text{max}}$</th>
<th>$\varepsilon_{\text{elastic}}$</th>
<th>$E_0$(MPa)</th>
<th>$E_\infty$(MPa)</th>
<th>$\tau_1$(s)</th>
<th>$\tau_2$(s)</th>
<th>h</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry, electrospun fibrinogen fibers</td>
<td>110%</td>
<td>16%</td>
<td>4200</td>
<td>3700</td>
<td>1.2</td>
<td>11</td>
<td>0.3</td>
<td>This study</td>
</tr>
<tr>
<td>Wet, electrospun fibrinogen fibers</td>
<td>130%</td>
<td>-</td>
<td>17.5</td>
<td>7.2</td>
<td>3.0</td>
<td>55</td>
<td>~0.5</td>
<td>[9]</td>
</tr>
<tr>
<td>Wet, crosslinked fibrinogen fibers</td>
<td>147%</td>
<td>50-75%</td>
<td>8.0</td>
<td>4.0</td>
<td>2.1</td>
<td>49</td>
<td>1.9</td>
<td>[10]</td>
</tr>
<tr>
<td>Wet, uncrosslinked fibrinogen fibers</td>
<td>226%</td>
<td>60-120%</td>
<td>3.9</td>
<td>2.0</td>
<td>2.9</td>
<td>54</td>
<td>3.21</td>
<td>[10]</td>
</tr>
<tr>
<td>Dry, electrospun collagen fibers</td>
<td>33%</td>
<td>&lt;2%</td>
<td>-</td>
<td>10,000-200</td>
<td>-</td>
<td>-</td>
<td>&lt;0.1</td>
<td>[11], [12]</td>
</tr>
<tr>
<td>Wet, crosslinked electrospun collagen fibers</td>
<td>-</td>
<td>-</td>
<td>6-260-70</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[12]</td>
</tr>
<tr>
<td>Native tendon collagen fibers</td>
<td>12%</td>
<td>-</td>
<td>7,500-160</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[13]–[15]</td>
</tr>
</tbody>
</table>

Table V. A comparison of the mechanical properties of natural and electrospun fibrinogen, fibrin, and collagen fibers.
We also discuss the surprising stability of electrospun fibrinogen fibers. In the absence of thrombin, there is no A:a interaction to strengthen the fibrinogen fibers. We suggest that the alpha-C region may be important to providing structure to electrospun fibrinogen fibers. In soluble fibrinogen, this region is folded back and tucked into the central domain. Electrospinning conditions may untuck these regions allowing them to interact with each other. A suggestion for future experiments would be to spin mutant fibrinogen that does not contain this alpha-C region and measure its mechanical properties.

This research adds to the library of information on the properties of materials suitable for tissue engineering applications. While fibrin(ogen) has great potential for use in tissue engineering, further research into other polymers or the techniques used to form the materials would be beneficial for the field. Biopolymers such as laminin and elastin as well as synthetic polymers such as poly-capralactone (PCL) and polylactic acid (PLA) may have mechanical properties that fill a niche for a specific tissue engineering application. The techniques used to form the biomaterials also provide further research opportunities. We have shown in this study that electrospinning can significantly alter the properties of the material. Other techniques such as 3D printing may provide another low cost solution to creating biomaterials with specific mechanical properties.

FIBRIN FIBERS FORMED FROM BLOOD PLASMA

In Chapter III, we modified the atomic force/fluorescence microscopy technique to study fibrin fibers formed from patient plasma instead of the purified system. The plasma system contains more coagulation factors and molecules not present in the
purified system making it a more complicated and interactive system to study. However, patient plasma more closely approximates *in vivo* conditions compared to purified systems. It also makes possible the study of the effect of diseases on the formation of fibrin fibers. In this study, we investigated the effect of diabetes on the properties of single fibrin fibers. Patients with diabetes have an increased risk of cardiovascular disease (CVD). Several studies of whole blood clots have found altered mechanical properties in CVD and diabetes patients [16]–[20]. However, several of these studies have conflicting findings while another study showed no difference in several mechanical properties [21]. Blood plasma was obtained from female South African patients and the fibrinogen glycation was measured as a marker for glycemic control. The modulus, extensibility, elasticity, and relaxation times for single fibrin fibers were measured. We found there was no significant dependence on fibrinogen glycation for any of the mechanical properties measured.

Whole clot modulus has been shown to increase in samples with small diameter fibers [22]. This is usually attributed to the number of increased branchpoints observed in these samples. However, we found the modulus of the individual fibers also increased as the diameter of the fibers decreased. We found the modulus to decrease exponentially to the 1.6 power as the diameter increased. We measured similar results in fibrin fibers made from purified fibrinogen where the modulus decreased with a 1.5 power exponential decay. These findings suggest fibrin fibers are not a homogenous collection of protofibrils which is often assumed in whole clot models. Instead we propose the density of fibrin fibers decreases as more protofibrils laterally aggregate to the outside of the fiber. The helical twist of the growing fiber restricts the possible lateral contacts for
additional protofibrils as the energy to stretch an added protofibril exceeds the binding energy.

There are ample opportunities for further research in this field. The study of fibrin fibers formed from plasma instead of the purified system may be used to study other aspects of cardiovascular disease. The effect of age and diet on the mechanical properties of fibrin fibers may provide further insight into the treatment of cardiovascular disease. There is also work to be done on the formation of fibrin fibers. We have suggested a possible mechanism for limiting fibrin fiber growth and the formation of non-homogenous fibers. One way to test this hypothesis is to measure the water content of fibers of various diameters. One would assume less dense fibers would contain more water per volume compared to more dense fibers. Another possible experiment would be to label fibrin monomers with a fluorescent dye and observe the monomers forming into fibers. The density of the fibers may then be measured by fluorescence intensity using a confocal or super-resolution microscopy technique. These results would have important implications for fibrin clot modeling.

DIFFUSION OF EGFP AND MSH2 IN BREAST CANCER CELLS

In Chapter IV, we set out to measure the diffusion of proteins in cells at different stages of neoplastic transformation. According to the Center for Disease Control, cancer is the second leading cause of death in the United States. Advances in biophysics have been providing scientists with new ways to study the disease. Recent reports indicate that cancerous and non-cancerous cells have different physical and mechanical properties arising from biochemical alterations as normal cells transform to cancerous cells [23].
Cancer cells are often found to be more deformable than non-cancerous cells [24]–[28], but does this have any effect on the mobility of small molecules and proteins within the cells? In this study we used a relatively new technique called Raster Image Correlation Spectroscopy (RICS) to measure the diffusion coefficients of EGFP and EGFP-MSH2 constructs in breast cancer cells. The cells used in the study were healthy HME cells, immortal HME+LT+hTERT cells, HMLER cells, and metastatic MDA-MB-231 cells. We picked two fluorescent probes for this study, EGFP and EGFP-MSH2. EGFP was picked as a control protein since we expect there to be no chemical or biological interaction of EGFP with any other cellular structure or protein in the cell. MSH2 is part of the mismatch repair (MMR) process and thus may be involved in cancer genesis or progression. In fact, MMR protein defects are found in a wide variety of cancers as well as in hereditary non-polyposis colorectal cancer [29].

In our RICS analysis we found four interesting results. First, we found no significant difference between the diffusion of EGFP and EGFP-MSH2 in the cytoplasm of the cells tested. Initially we suspected there might be a difference in diffusion coefficients due to the size difference of EGFP and EGFP-MSH2. According to the Stoke’s-Einstein relation, EGFP-MSH2 should diffuse a factor of 1.6 more slowly than EGFP alone. In our data however, the two constructs did not exhibit a statistically significant difference.

The second interesting result we found was that there is no significant difference in diffusion rates in the cytoplasm among the cell types tested. This is a somewhat surprising result since several studies have shown there to be significant reorganization of the cytoskeleton as cells become cancerous. Specifically, overexpression of p53, which is
present in all our cells except for HME cells, has been implicated in cytoskeletal reorganization of actin and intermediate filaments [30], [31]. Similarly, overexpression of Ras related proteins, which are present in our HMLER cells, has been shown to control actin reorganization [32], [33]. The diffusion of EGFP and EGFP-MSH2 is not affected by this cytoskeletal reorganization suggesting that the pore size between cytoskeletal filaments is consistently large enough to not interfere with the mobility of molecules with sizes on the order of tens of nanometers.

The third interesting result from this study is the significant increase in the diffusion rate of EGFP in the nucleus compared to the cytoplasm of all the cell types except HMLER cells. This suggests that at a small length scale, the nucleus is less viscous than the cytoplasm. This however is not the case for HMLER cells which contain high amount of mutated Ras. Altered expression of lamina, which provides mechanical support for the nucleus has been observed in Ras transformed cells [34]. The altered lamina expression may be one possible explanation for the reduced diffusion seen in the HMLER cell nucleus.

The fourth result from this study is that the diffusion of EGFP-MSH2 in the nucleus is significantly slower compared to EGFP alone in all cells except the HMLER cells. We expect the diffusion of EGFP-MSH2 compared to EGFP to be slower due to its size alone; however, this would not account for the factor of 7 – 8 decrease in the diffusion coefficient. Even if we assume the MSH2 is binding to MSH6, its expected interaction partner, this would only account for a factor of 2 difference in the diffusion coefficient. We suspect that the pore size of the nuclear skeleton is therefore on a similar length scale as the size of the EGFP-MSH2 proteins such that steric interactions between
the proteins and nuclear skeleton become more frequent slowing diffusion. Future research can test this hypothesis by measuring the diffusion of non-interacting particles of different sizes, such as FITC labeled dextrans, in the nucleus. I also estimated the viscosity of the cytoplasm and nucleus of these cells and compared these results to measurements made in similar cells using various techniques (Table VI).

Table VI. A comparison of the viscosity in the cytoplasm and nucleus of living cells.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Cell</th>
<th>Location</th>
<th>Technique</th>
<th>Viscosity (cP)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP/MSH2</td>
<td>HME</td>
<td>Cytoplasm</td>
<td>RICS</td>
<td>24 – 100</td>
<td>Current study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.6 - 61</td>
<td></td>
</tr>
<tr>
<td>Porphyrin dimer</td>
<td>CHO</td>
<td>Cytoplasm</td>
<td>Fluorescent ratiometric molecular rotor</td>
<td>50</td>
<td>[35]</td>
</tr>
<tr>
<td>Various sizes of PEG/PEO</td>
<td>HeLa 3T3</td>
<td>Cytoplasm</td>
<td>Reanalysis of previous results obtained by FRAP/FCS</td>
<td>2.0 – 44</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.88 - 24</td>
<td></td>
</tr>
<tr>
<td>ERCC1/XPF-GFP</td>
<td>3T3</td>
<td>Nucleus</td>
<td>FRAP</td>
<td>5</td>
<td>[37]</td>
</tr>
<tr>
<td>EGFP</td>
<td>ASTC-a-1</td>
<td>Nucleus</td>
<td>FCS</td>
<td>2.55</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Nucleus</td>
<td></td>
<td>2.04</td>
<td></td>
</tr>
</tbody>
</table>

The complexity of live cells makes measuring the molecular dynamics within them complicated and prone to misinterpretation. Cross-validation with an orthogonal technique is often used to increase confidence in the measurements [39]. In Chapter V, I cross-validated the RICS measurements described in Chapter IV by performing FRAP measurements in the same cells. By accounting for fluorophore concentrations and
bleaching pulse intensities in FRAP measurements, I was able to obtain diffusion coefficients from FRAP that were similar to RICS measurements in most cases. While the FRAP and RICS measurements are similar, there is still the possibility that they are both giving the same inaccurate diffusion coefficients. Therefore we developed a validation method for RICS based on computationally controlling the movement of quantum dots that enables us to measure diffusion coefficients in a highly controlled manner independent of cell or fluorescent probe effects. Future research in this area could expand upon our programmable RICS experiment to include binding or other more complicated dynamics. For example, an experiment using a spatial light modulator in a holographic optical tweezers setup may be able to independently control the motion of many particles in an imaging plane. The downside to this technique is that the particles would have to be larger than the size of most proteins. FRAP would greatly benefit from a similar experiment, however, it would be difficult to conceive of an experiment where one would have direct control over a large ensemble of molecules without affecting the FRAP measurement.

The research presented in this dissertation determined mechanical properties of two biological systems. We measured the properties of dry, electrospun fibrinogen fibers that make them good candidates for tissue engineering applications. We expanded upon the AFM/fluorescence microscopy technique to measure the mechanical properties of individual fibrin fibers formed from plasma. We determined that glycation of fibrinogen does not alter the mechanical properties of single fibrin fibers. We also showed how the mechanical properties of fibrin fibers provide evidence that the fibers are not
homogenous as often assumed in fibrin clot modeling. We then measured the diffusion coefficients of proteins in human breast cancer cells using a relatively new technique called RICS. This technique can provide insight into how the mechanical properties of cells change through the process of neoplastic transformation. Finally, we cross-validated the RICS results using FRAP and developed a technique to directly verify the accuracy of RICS experiments in which simple diffusion is the dominant dynamic process. Future research on the mechanical properties of biological systems will further our understanding of these systems and provide insight into the diagnosis and treatment of diseases.
REFERENCES


The following supplement is to accompany Chapter IV. It describes two additional experiments that were performed during the course of the study, however were not included in the submitted manuscript. All data was collected and analyzed by Justin Sigley. Martin Guthold and Keith Bonin acted in an advisory capacity throughout data collection and analysis.
FLUORESCENCE LOSS IN PHOTOLEACING

MATERIALS AND METHODS

Fluorescence loss in photobleaching (FLIP) experiments were performed to determine if the EGFP and EGFP-MSH6 constructs were able to freely diffuse through the cell and across the nuclear membrane. For this study, we used HME cells with EGFP and HMLER cells with EGFP-MSH6. Fluorescence imaging was performed on the same Zeiss LSM 710 system described in Chapter IV and V. The 488 nm line of a 35 mW argon laser was used to repeatedly bleach the entire nucleolus region of the cells. The images were 512 x 100 pixels with a pixel size of 75 nm. Images were taken every 0.2 seconds and bleaching was repeated every 1.5 s with the laser power set to 100%. The average fluorescence intensity in the bleach ROI was recorded as well as the average fluorescence intensity in ROI’s in the nucleus, cytoplasm, and background. In one experiment we repeatedly bleached the nucleolus as described above, then stopped imaging for 3 minutes. This bleaching and 3 minute wait time was repeated 4 times (see Figure S2).

RESULTS

Figure S1 shows the results of a FLIP experiment. For this experiment, we repeatedly bleached the nucleolus while monitoring the average fluorescence intensity in several regions of the cell. We also repeated the experiment about 3 minutes later, except without the bleaching. This gives an observational photobleaching curve for each of the ROI’s. The observational photobleaching curves are normalized on the graph such that the
asymptote of the bleaching curve and its corresponding observational photobleaching curve lay on top of each other. For every ROI, the curve taken during the bleaching experiment decays faster than the observational photobleaching curve. For this to occur, fluorophores from ROI’s outside of the nucleolus must be able to travel into the nucleolus where they can be bleached. The total fluorescence intensity of the cell is decreasing faster than observational photobleaching can account for. Therefore, the fluorophores are able to travel throughout the cell including across the nuclear membrane.

**Figure S1.** FLIP curves in HMLER cells with EGFP-MSH6. The x-axis is marked by the frame number which were each taken in 0.2 s increments. The total length of the experiment is 64 s. The total fluorescence intensity of several regions in the cell are decreasing at a faster rate than observational photobleaching. The fluorophores must be able to diffuse throughout the cell.

We also used a FLIP experiment to determine if the bleaching laser power was set too high and thus exciting a long lived non-emissive triplet state in the EGFP. We
repeatedly bleached a portion of the cell and then monitored the fluorescence intensity of
the whole cell over a period of five minutes. We found the fluorescence intensity of the
whole cell decreased while bleaching was taking place, as shown in Figure S1. We then
stopped imaging the cell for 5 minutes and then repeated the bleaching. After waiting five
minutes, the total fluorescence intensity of the cell had recovered to the prebleach
intensity (see Figure S2). This suggests the EGFP was being excited to a non-emissive
state with a lifetime on the order of minutes.

Figure S2. Multiple fluorescence loss in photobleaching curves. The fluorescence
intensity in each area of the cell decreases during photobleaching. Imaging was stopped
for 5 minutes before repeating the photobleaching experiment. After 5 minutes, the total
fluorescence intensity of the cell has returned to prebleach values. The x-axis here is, as
in Fig. S1, the frame number, with 0.2 s between frames.
CELL CYCLE MEASUREMENTS

MATERIALS AND METHODS

In Chapter IV, we make the assumption that the differences we see in diffusion coefficient measurements between different cells are not due to cell phase because the cells are not synchronized into a specific phase of the cell cycle. We avoid measuring cells in mitosis, but the remaining cells should be randomly distributed throughout the rest of the cell phases so our measurements are a representative sample of cells in all phases (except mitosis). To determine if our cells were synchronized or not, we used Premo FUCCI Cell Cycle Sensor (BacMam 2.0) (Life Technologies) fluorescent dyes. The dyes consist of a geminin-GFP that is present in S, G2, and M phase of the cell cycle and a Cdt1-RFP that is present in G1 phase. Cells were cultured as described in Chapter IV. One day before imaging, 20 μl of each dye reagent was added to a 35 mm cell culture dish with HME cells and left overnight as per the manufacturer’s instructions. Twenty fluorescent images were taken on a Zeiss LSM 710 laser scanning confocal microscope mounted on an AxioObserver Z.1 inverted microscope using a 40x 1.3 NA oil immersion objective. Images were taken from random locations throughout the cell dish. Each of the twenty images consisted of a red, green, and DIC channel. A 561 nm diode laser was used for excitation of the red channel and emission was collected between 578 and 680 nm. A 488 nm argon laser was used for excitation of the green channel with emission collected between 492 and 556 nm.

The images were analyzed using ImageJ software (NIH). For analysis, each image channel was opened in ImageJ. The levels were automatically adjusted to increase
brightness. Auto threshold was used to binarize the image and the result was inverted which was required for particle count analysis. The particle analysis plugin was used to count the fluorescent cells with a size set between 50 – 500 pixels.

RESULTS

Figure S3 is a representative image of HME cells stained with the FUCCI dyes. We counted the labeled cells in each image and determined for this dish, about 70% of the labeled cells were in G1 phase. To try to eliminate bias, another person was asked to independently count the cells in ten of the images. He determined about 65% of the labeled cells were in G1 phase. HME cells are expected to be in G1 phase for about 40% of the total cell cycle; however, the cells may be partially synchronized due to growth variables such as confluence and subculturing. We did not observe colonies of synchronized cells, instead the labeled cells were randomly scattered across the sample. Future experiments need to be performed on multiple cell dishes and cell types to determine if the distribution of cells in a specific phase of the cell cycle is random. The heterogeneity in the diffusion coefficient measurements may in part be due to the cells being in various phases of the cell cycle.
Figure S3. Representative image of HME cells stained with the FUCCI cell cycle marker fluorescent dyes. The green dye is geminin-GFP which labels the cells that are in G, S2, and M phases. The red dye is Cdt1-RFP which labels the cells that are in G1 phase. The scale bar is 75 μm.
Figure S4. Representative DIC images of the cell types used in the study. The morphology of normal HME cells (A) and immortal HME+LT+hTERT cells (B) are similar in that the cells form a confluent monolayer. The tumorigenic HMLER cells (C) and metastatic MDA-MB-231 cells (D) do not form monolayers but instead aggregate and clump together. We included isolated cells or cells at the edge of these clumps in the current study.
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