Biophysical Characterization and Theoretical Analysis of Molecular Mechanisms Underlying Cell Interactions with Poly(N-isopropylacrylamide) Hydrogels

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Biophysical Characterization and Theoretical Analysis of Molecular Mechanisms Underlying Cell Interactions with Poly(N-isopropylacrylamide) Hydrogels

by

Michael C. Cross

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Physics College of Arts and Sciences University of South Florida

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Keywords: atomic force microscopy, isopropylacrylamide, tissue engineering, flory rehner theory, bioprinting

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DEDICATION

To my wife and partner, Megan.
An incredible journey continues.
ACKNOWLEDGMENTS

My journey, “bumbling around in the dark,” and “in the pursuit of high-quality ignorance,” would not have been possible without the significant, substantive, and supportive contributions of so many. At a critical juncture in my doctoral program, Drs. Nathan Gallant, Garrett Matthews, and Ryan Toomey each welcomed the opportunity to work with me. Working together, they created an environment in which I was able to flourish. My sincere gratitude goes to my major professor, Dr. Garrett Matthews, for allowing me wide autonomy and providing strategic support in my research efforts. His intellectual curiosity and gentle guidance have been nothing short of inspiring. I have thoroughly enjoyed my interactions with him. I would like to thank my unofficial, “co-major” professor, Dr. Nathan Gallant, for his suggestions and support. He treated me as one of his own despite being in different colleges. Most importantly, he identified one of my primary career goals and provided the opportunity to pursue training in that area. My gratitude also extends to my other unofficial, “co-major” professor, Dr. Ryan Toomey. Wherein I learned the most productive writing occurred over a glass of wine and the most illuminating data analysis included a mug of beer. His patience and assiduous encouragement through my struggles reflected his dedication to my success. I would like to thank my committee members, Drs. Sarath Witanachchi and Myung “Paul” Kim for supporting my dissertation defense. Others to which I owe a debt of gratitude for their advice and support are Drs. Casey Miller, Shekar Bhansali, Lawrence Morehouse, Richard Pollenz, Mr. Bernard Batson, Mr. Charles Jackson, and Ms. Lyra Logan. This dissertation would not be possible without the generous support of the Sloan Foundation, the McKnight Foundation, the NSF Bridge-to-Doctorate Program, and the NSF Innovation Corps. Finally, I wish to thank my wife and partner, Megan, for her incredible support and encouragement throughout the years.
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<table>
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<tr>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
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<tr>
<td>FRT</td>
<td>Flory-Rehner theory</td>
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<td>MFT</td>
<td>Mean field theory</td>
</tr>
<tr>
<td>PNIPAAm</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean square</td>
</tr>
<tr>
<td>SCF</td>
<td>Self-consistent field theory</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<td>VPT</td>
<td>Volume phase transition</td>
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## LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
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<tr>
<td>$f$</td>
<td>Free energy</td>
</tr>
<tr>
<td>$g$</td>
<td>Osmotic pressure</td>
</tr>
<tr>
<td>$\phi_i$</td>
<td>Flory-Huggins solvent-polymer average interaction parameter</td>
</tr>
<tr>
<td>$\phi_0$</td>
<td>Volume fraction of polymer at reference state (typically synthesis)</td>
</tr>
<tr>
<td>$N_m$</td>
<td>Number of segments bounded on both ends by crosslinks</td>
</tr>
<tr>
<td>$\chi$</td>
<td>Flory-Huggins solvent-polymer two-body interaction parameter</td>
</tr>
<tr>
<td>$n_i$</td>
<td>Mole amount of species $i$ in system</td>
</tr>
<tr>
<td>$N_0$</td>
<td>Total number of molecules in system</td>
</tr>
<tr>
<td>$V$</td>
<td>Total volume of system</td>
</tr>
<tr>
<td>$V_0$</td>
<td>Volume of system at reference state</td>
</tr>
<tr>
<td>$v$</td>
<td>Unit volume of a mean field theory cell</td>
</tr>
<tr>
<td>$v_0$</td>
<td>Reference unit volume (water molecule)</td>
</tr>
<tr>
<td>$l$</td>
<td>Length of a mean field theory cell</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Ratio of chain extension</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature (Kelvin)</td>
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ABSTRACT

So-called, “Dynamic biomaterials” comprised of stimuli-responsive hydrogels are useful in a wide variety of biomedical applications including tissue engineering, drug delivery, and biomedical implants. More than 150,000 peer-reviewed articles (as of 2016) have been published on these materials, and more specifically, over 100,000 of these are on the most widely studied, poly(N-isopropylacrylamide). This thermoresponsive polymer in a crosslinked hydrogel network undergoes a large volume phase transition \(\frac{V}{V_0} = 10^{-100}\) within a small temperature range \(T = 1 - 3K\) making it particularly useful for tissue engineering applications because of the ability to control the topographical configuration of cells into tissue modules which can be applied in multiple layers to form three-dimensional constructs. Nevertheless, applications with poly(N-isopropylacrylamide) hydrogels are hindered by two key obstacles: 1. there is presently no quantitative prediction of mechanical properties over the volume phase transition and 2. the mechanisms of cell attachment and detachment remain controversial and unclear.

Current polymer-solution theory, first postulated by Paul Flory and Maurice Huggins in 1942, successfully predicts hydrogel swelling for non-stimuli-responsive polymers based on an empirically derived interaction parameter. However, for stimuli-responsive polymer hydrogels, this theoretical framework fails to quantitatively predict swelling and mechanical properties of the polymer. Currently, only qualitative agreement with experiment has been shown.

Cell-cell and cell-matrix interactions are mediated through proteins collectively known as cell adhesion molecules. For cell-matrix interactions, these are generally the transmembrane protein, integrin, and the serum protein, fibronectin. It is widely accepted that nearly all molecular mechanisms of cell-matrix interactions are dependent on recognition of the peptide sequence Arg-Gly-Asp. However, much less is known about mechanical mechanisms involved in cell-cell and cell-
Obstacles to the advancement of these applications are 1) unclear mechanisms of cell release and 2) extended exposure of cells to hypothermic conditions. The author, in collaboration with others, has published work demonstrating reduced cell exposure to hypothermic conditions during tissue module release and elucidated a mechanism of tissue module release: mechanical strain. The central hypothesis of work in this proposal is that tissue module release occurs due to a mechanical strain-rate coinciding with critical force needed overcome the dynamic bond strength of cell adhesion molecules. Advances in this area could improve biomaterial design and accelerate the field of regenerative medicine by reducing or eliminating the need for allograft transplants.

This dissertation project, then, seeks to address these two obstacles through biophysical characterization methods and analysis including: atomic force microscopy, scanning electron microscopy, laser-scanning confocal microscopy, phase-contrast microscopy, and mass-balance analysis. It is hypothesized that, (1) mechanical properties of PNIPAAm hydrogels are quantitatively predicted based on crosslinker ratio in the water-rich phase, (2) release of cells from micropatterned PNIPAAm hydrogels occurs when the lateral strain in the surface exceeds $\epsilon > 0.25$, and (3) the molecular mechanism of rapid cell release from micro-patterned PNIPAAm hydrogels is mediated by the transmembrane protein integrin and its extracellular matrix receptor, fibronectin. Results from these studies could be useful for improving the design of biomaterials based on PNIPAAm hydrogels for applications in tissue engineering.
CHAPTER 1
INTRODUCTION

The focus of this study is biophysical characterization of the thermo-responsive polymer, poly(N-isopropylacrylamide) (PNIPAAm) for applications in tissue engineering. In a crosslinked hydrogel network, PNIPAAm exhibits a volume phase transition (VPT) which consists of a large volume change (10-100 fold) over a narrow temperature range ($\Delta T = 1-3K$) [1,2]. The VPT phenomena is particularly useful for biomedical applications in tissue engineering [3–5] and drug delivery [6–8]. These so-called, “dynamic biomaterials” fabricated with PNIPAAm enable the ability to control the topographical configuration of cells into tissue modules [9], cell sheets [10–12], and avoid the use of scaffolds in tissue reconstruction [13,14]. A central obstacle to the advancement of these broad applications is fundamental: theoretical predictions of the VPT in PNIPAAm hydrogels show qualitative but not quantitative agreement [15]. Another significant challenge includes understanding the mechanisms of cell attachment and detachment from PNIPAAm surfaces [16,17].

Results from this dissertation project address aspects of these obstacles towards advancing knowledge in the area of biomaterials and tissue engineering. Specifically, there are three hypothesis upon which this investigation is based:

- Mechanical properties of PNIPAAm hydrogels are quantitatively predicted based on crosslinker ratio in the water-rich phase.

- Release of cells from micro-patterned PNIPAAm hydrogels occurs when the lateral strain in the surface exceeds $\epsilon > 0.25$.

- The molecular mechanism of rapid cell release from micro-patterned PNIPAAm hydrogels is mediated by the transmembrane protein integrin and its extracellular matrix receptor, fibronectin.
A key outcome of this work will be that designs of biomaterials for tissue module printing with release times in seconds will eliminate dependence on cell metabolism, integrin-turnover, serum protein desorption, and biomaterial chemical properties. Another important outcome will be an enhanced understanding of the cell-substrate adhesion process required for the development of new biomaterials that will be useful for drug-testing platforms, regenerative medicine, and ultimately, bioengineered tissue autografts. Complementary to these application-focused outcomes, this work advances theoretical treatment of thermoresponsive biomaterials with quantitative relation of molecular thermodynamic properties to macroscopic bulk measurements.

1.1 Review of the Literature

1.1.1 Biomedical Applications

End-stage organ failure occurs in several diseases including those of the liver, heart, kidney, and lungs. Orthotopic transplantation, a treatment used in organ failure, is greatly limited by the availability of donor organs. Bioengineered organ grafts hold great promise for tackling the challenges of donor shortages and immunosuppression associated morbidity [18–20]. Regeneration of organs or damaged tissues is made problematic by the intricate three-dimensional (3D) structure required for proper function. An engineered organ will need to precisely mimic the physical properties and biological activity of the target organ. Such 3D organization and control over the physical properties are beyond the scaffolding techniques currently developed for tissue engineering.

In vitro formation of complex tissues and organs is dependent on how well cell organization can be controlled. Micro-tissues, a two-dimensional (2D) layer of cells, are useful in both therapeutic and discovery-based tissue engineering strategies [21]. These cell systems also require defined cell geometries, which can be achieved through topographical control. Contact printing of cell sheets is one approach to mimic in vivo native cellular environments by precisely configuring multicellular constructs into 3D patterns essential for the formation of functional tissues. Laser-assisted bio-printing [22], cell-sheet printing [5, 13], and inkjet-based printing [23, 24] are examples of
bottom-up bioengineering technologies utilized for organotypic tissue formation [25].

Though most studies in this field focus on the advancement of clinical treatments, several of which are promising [26–28], the varied test conditions and approaches have resulted in a body of literature with conflicting data and unclear mechanisms [29]. However, what is clear is that contact printing of cells is dependent on (1) cell attachment to an intermediate, (2) release of cells from the intermediate, and (3) cell viability following release. A unifying theme for these three areas is the mechanical interaction between the cells and intermediate. Characterization of the cell-matrix mechanical interactions in contact printing will be a necessary step towards elucidating underlying mechanisms of cell release and will advance the field.

1.1.2 Dynamic Biomaterials

This class of materials, reversibly actuated by defined stimuli, consists of stimuli-responsive polymers, permanently cross-linked hydrogels, reversible hydrogels, micelles, modified interfaces, and conjugated solutions [30–32]. The most widely utilized polymer, PNIPAAm, is thermoresponsive and its properties were first described in detail by Heskins & Guillet in 1968 [33]. In aqueous solutions, the hydrogel exists in a polymer-rich phase above the VPT temperature, and in a water-rich phase below this temperature. More recently, additional methods of actuation have been developed. These actuation stimuli fall into three primary categories: (1) chemical, (2) physical, and (3) biochemical [34].

Dynamic biomaterials have been synthesized through methods such as nitroxide-mediated polymerization [35], atom transfer radical polymerization [36], reversible addition-fragmentation chain transfer polymerization [37], and ‘click’ chemistry [38]. The methods noted are able to maintain and introduce functional groups in both polymers and block copolymers while providing a defined molecular weight distribution.

These materials are useful in biomedical applications, primarily drug delivery and tissue engineering [39]. In drug-delivery, a therapeutic can be diffused directly into a hydrogel and released
with external stimulus. For tissue engineering and regenerative medicine, they serve as scaffolds, implants, and culture surfaces.

1.1.3 Cell Interactions with Dynamic Biomaterials

One regenerative medicine application involves an approach where cells are harvested as a sheet [5]. This is implemented by grafting thin films of PNIPAAm on to tissue culture surfaces [40]. The method has been used to transplant human myocardial tissues fabricated from induced pluripotent stem cells into rats and shown to remain viable as long 6 months [41]. It was utilized via a commercial product, Nunc™ Dishes with UpCell™ Surface (Thermoscientific, USA), to create multiple layers of adipose-derived stem cells for wound healing and resulted in significantly smaller injury areas and potentially higher vascular density [42]. Finally, renal cell sheets were produced from primary cells and demonstrated increase in relative levels of erythropoietin, a hormone controlling red blood cell production, both in vitro and in vivo [43].

The release is purported to occur by metabolic changes in the cells and hydrophilicity of the polymer which is induced by the thermally responsive nature of PNIPAM films [44, 45]. Culture temperatures are lowered to between 277 K and 293 K which result in the release of cell sheets within 15 to 90 minutes [46]. However, this method results in loss of cell shape and spatial organization. Another key challenge with this technique is prolonged exposure to hypothermic temperatures which can influence cell health and ultimately cell function [47].

1.2 Research Aims

1.2.1 Aim One

The goal of this aim is to advance understanding of how PNIPAAm hydrogel mechanical properties affect cell-material interactions for the purpose of improving biomaterial designs in tissue engineering applications. In concert with results from previous studies identifying the mechanical dependence of the transmembrane protein integrin $\alpha_5\beta_1$ [48], results from this project could con-
tribute to clarifying and resolving existing findings.

This project characterizes micro-patterned PNIPAAm hydrogels with scanning electron microscopy, atomic force microscopy, and phase-contrast microscopy to determine the effect of defined topographical features on cell release. The central hypothesis for this aim is that release of collections of cells in the form of a tissue module occurs due to a mechanical strain-rate coinciding with critical force needed to overcome the dynamic bond strength of cell adhesion molecules with the PNIPAAm hydrogel surface.

This hypothesis is tested with the following experiments

- Characterization of PNIPAAm Surface Topography
- Molecular Basis of Release
- Cell Viability After Release

Advances in this area could improve dynamic biomaterial design. More specifically, the use of micropatterned PNIPAAm hydrogels as scaffolds for 3D cell bioprinting could accelerate the field of regenerative medicine by reducing or eliminating the need for allograft transplants.

1.2.2 Aim Two

The goal of this aim is to answer the question, “can mechanical properties of PNIPAAm hydrogels be predicted with a general form of Flory-Rehner theory?” Current theory can predict non-stimuli-responsive polymer hydrogel swelling in a solution based on an empirically derived interaction parameter. However, for stimuli-responsive hydrogels, theoretical models to predict swelling and mechanical properties of the polymer currently show only qualitative agreement with experiment [49,50]. Although substantial effort has been committed to characterizing mechanical properties of PNIPAAm [51–57], reconciliation of theory with experiment remains elusive [15].

In this study, predictions of Flory-Rehner theory are compared to experimental results for mass fraction and elastic modulus of PNIPAAm hydrogels. Mass-balance measurements and atomic
force spectroscopy are used to determine the mass fraction and obtain force curves of poly(N-isopropylacrylamide) hydrogels with crosslinker ratios 1:200, 1:100, 1:67 and 1:40. The volume fraction and Young’s modulus are calculated from these experimental measurements to determine quantitative relationships within the Flory-Rehner theoretical framework. The hypothesis for this project is that mechanical properties of PNIPAAm hydrogels are quantitatively predicted based on crosslinker ratio in the water-rich phase.

This hypothesis is tested with the following experiments:

- Volume Swelling Analysis
- Elastic Modulus Analysis
- Experiment & Theory in the Water-rich Phase
- Experiment & Theory in the Polymer-rich Phase

Results from this study are useful in predicting the mechanical properties of the hydrogel using empirically derived parameters similar to Hansen solubility parameters [58]. These could be highly applicable in a manufacturing context and in the development of biomedical applications using PNIPAAm hydrogels.

1.3 Dissertation Outline

- Chapter 2 gives an overview of polymer theory, relevant derivations, and relationships with hydrogel mechanical properties. It also covers recent work on molecular mechanisms of the VPT in PNIPAAm. This chapter serves to introduce foundational relationships discussed throughout the dissertation. Namely, volume fraction, interaction parameters and mechanical properties of hydrogels.

- Chapters 3 and 4 describe biophysical characterization methods used in this work. Chapter 3 discusses hydrogel synthesis techniques and measurements used to determine volume swelling. Chapter 4 begins by outlining basic elements of atomic force microscopy including theory, practice, and applications. Integral to work accomplished in this dissertation, the central focus of this chapter is force curve analysis and measurement of soft materials in solution.
• Chapters 5 and 6 cover results from this dissertation project. Chapter 5 discusses cell interactions with PNIPAAm and related experimental results. Particular attention is given to mechanical effects of the hydrogel resulting from the volume phase transition. Chapter 6 covers predictions of Flory-Rehner theory and experimental results. The Flory-Rehner theoretical framework is assessed with respect to the relationship between volume fraction and mechanical properties of PNIPAAm hydrogels. Special attention is given to assess quantitative predictions.

• Chapters 7 and 8 serve as a basis for future directions of research covered in this dissertation. Chapter 7 is a peer-reviewed and published in-depth review of the literature with a detailed outline of proposals to advance understanding of protein-surface interactions with PNIPAAm hydrogels. Chapter 8 describes preliminary results towards these goals, primarily focused on the interaction strength of the serum protein, fibronectin, and PNIPAAm hydrogels.

• Chapter 9 concludes the dissertation and summarizes the body of work.
1.4 References


CHAPTER 2  
FLORY REHNER THEORY  

2.1 Introduction  

Most polymer hydrogels can undergo a VPT as the temperature of the system is increased. The VPT is a phenomena in which the volume of the hydrogel swells several times its deswollen size. By contrast, the VPT in PNIPAAm hydrogels 1) drastically reduces volume with temperature increase and 2) does this over a relatively narrow temperature range (1-3 K). The mechanism behind this behavior has been extensively studied since Heskins [1] reported on the phase transition in linear PNIPAAm and it continues to remain an important area of investigation because similar hydration behavior is also observed in physiologically relevant proteins [2].

A polymer hydrogel is made up of linear polymer chains chemically linked together. A single linear polymer chain can be imagined as a spring, existing in a relaxed state unless stretched or compressed by an external action. When these linear chains are placed in a water solution with a finite number of molecules, the system will tend toward maximizing the number of available configurations which generally results in the polymer equally distributing throughout the solution (see figure 2.1). Additionally, the chains will have a tendency to form random coils similar to the relaxed state of a spring. When the solution favorably interacts with the polymer chain, it can stretch out, taking on a more extended or rod-like shape. When it does not favorably interact, the chain may coil even tighter, forming globules to minimize the surface area touching water.

In a closed system at constant temperature and pressure, every molecule interacts with every other molecule primarily through electromagnetic fields which is theoretically complex to describe. A simple model to predict the favorability of solution’s interaction with a polymer is therefore preferred. Thus, most models only incorporate the nearest neighbors because the interactions are usually orders of magnitude greater than those beyond. The most prominent model describing
Figure 2.1. Schematic of polymer interactions in a solution (water). Top) Favorable interaction with the water molecules which causes the polymer chains to stretch. Bottom) Unfavorable interaction with water molecules causes chains to form globules and potentially aggregate.
this water-polymer interaction is the Flory-Huggins solution theory [3,4] which further simplifies the complexities of this system by assuming an average interaction per monomer-solvent contact. Essential to this theory is an empirically derived interaction parameter which can be used to determine the change in free energy caused by mixing under constant temperature and pressure.

In a hydrogel, the free floating polymer chains are now linked together which limits how they distribute in the solution (see Figure 2.2). Here, the number of configurations for the water is somewhat reduced and for the polymer, greatly reduced. Because the system tends toward maximizing the number of available configurations, the balance between mixing, stretching, and coiling is different from that of the free-floating polymer. Thus, Flory-Rehner theory (FRT) [5], based on the interaction parameter from Flory-Huggins solution theory was developed with a modification that includes a parameter for the number of links between chains (crosslink density).

Interaction parameters for PNIPAAm chains in solution have been empirically derived [6,7]. In PNIPAAm, each individual unit making up the polymer chain (N-isopropylacrylamide monomer) contains a subgroup that interacts favorably with water (amide) and a subgroup that interacts unfavorably with water (isopropyl). This combination leads to complex behavior in which 13 water molecules are associated with the amide subgroup below the VPT but only 2-3 associate above [8,9].

### 2.2 Theoretical Derivations

The following are derivations modified from the original Flory-Rehner work on crosslinked polymer networks and further development by Hirotsu [10]. Within the mean field theory (MFT) framework, $n_1$ and $n_2$ represent moles of each species: water and polymer chains, respectively. There are $n_2$ chains are made up of $N_m$ monomer units in a lattice of $N_0 = n_1 + N_m n_2$ MFT cells. The size of each species is normalized with the assumption that each species occupies the same size cell ($v_0 = l^3$) referenced to the radius of a water molecule (1.5 Å) [11]. The total volume of the polymer solvent system is $V = N_0 v_0$. 


Figure 2.2. Schematic of polymer hydrogel interactions in a solution (water). Top) Favorable interaction which causes the hydrogel to swell in volume. Bottom) Unfavorable interaction which causes the hydrogel causes collapse.
2.2.1 Osmotic Pressure of Mixing

The concentration for each species, water

\[ \phi_1 = \frac{n_1}{N_0} \quad (2.1) \]

and polymer

\[ \phi_2 = \frac{N_m n_2}{N_0} \quad (2.2) \]

In a crosslinked hydrogel system, the entropy due to species mixing is

\[ \frac{V}{k_B} \Delta S = (-n_1 \ln \phi_1 - n_2 \ln \phi_2) \quad (2.3) \]

and the enthalpy is

\[ \frac{V}{k_B T} \Delta H = n_1 \phi_2 \chi(\phi, T) \quad (2.4) \]

With \( f = \Delta H - T \Delta S \), the free energy of mixing for two species is

\[ \frac{V}{k_B T} f = n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_1 \phi_2 \chi(\phi, T) \quad (2.5) \]

In a hydrogel system, the number of water molecules varies whereas the number of polymer molecules (or chains) remains constant. Thus, the osmotic pressure is the derivative of the free energy with respect to the change in water molecules

\[ \Pi_{\text{mixing}} = \frac{\partial}{\partial n_1} f \quad (2.6) \]

Rewriting \( f \) in terms of \( n_1 \) (\( v_0 \) replaces \( V \)),

\[ \frac{v_0}{k_B T} f = n_1 \ln \frac{n_1}{n_1 + N_m n_2} + n_2 \ln \frac{N_m n_2}{n_1 + N_m n_2} + \frac{n_1 N_m n_2}{n_1 + N_m n_2} \chi(\phi, T) \quad (2.7) \]
The derivative is

$$\frac{v_0}{k_B T} \frac{\partial}{\partial n_1} f = \ln \frac{n_1}{n_1 + N_m n_2} + (N_m - 1) \frac{n_2}{n_1 + N_m n_2} + ...$$

$$\chi(\phi, T) \left( \frac{N_m n_2}{n_1 + N_m n_2} - \frac{n_1 N_m n_2}{(n_1 + N_m n_2)^2} \right) + \frac{n_1}{n_1 + N_m n_2} \frac{\partial}{\partial n_1} \chi(\phi, T) \tag{2.8}$$

Taking the derivative and collecting like terms by replacing with $\phi_1$ and $\phi_2$ gives

$$\frac{v_0}{k_B T} \frac{\partial}{\partial n_1} f = \ln \phi_1 + \phi_2 \left( 1 - \frac{1}{N_m} \right) + \chi(\phi, T) \phi_2 (1 - \phi_1) + \phi_2 n_1 \frac{\partial}{\partial n_1} \chi(\phi, T) \tag{2.9}$$

The relationship between the Flory-Huggins interaction term and empirical derived parameters (mean value) [3, 12, 13] is given by

$$\int_{\phi_2}^1 \chi_{12}(\phi, T) = (1 - \phi_2) g(\phi_2, T) \tag{2.10}$$

Thus, the empirically derived parameters $g(\phi_2, T)$ are [6, 7, 14]

$$g(\phi_2, T) = \sum_{k=0}^{n} g_k \phi_2^k \tag{2.11}$$

where

$$g_n = g_{n1} + g_{n2} T \tag{2.12}$$

Although the physical meaning of this parameter remains to be experimentally elucidated [15], it continues to phenomenologically describe polymer behavior [16]. The series is expanded to the 2nd order term due to exponential decay of the terms following. Thus,

$$g(T, \phi_2) = g_0(T) + g_1(T) \phi_2 + g_2(T) \phi_2^2 \tag{2.13}$$

Now, osmotic pressure due to mixing can be written as

$$\frac{v_0}{k_B T} \Pi_{\text{mixing}} = \ln (\phi_1) + \phi_2 \left( 1 - \frac{1}{N_m} \right) +$$

$$g(T, \phi_2) \phi_2^2 - \phi_2 \phi_1 (g_1(T) \phi_2 + 2g_2(T) \phi_2^2) \tag{2.14}$$
Experimentally, $\phi_2$ is obtained, so the mixing term for osmotic pressure is

$$\frac{v_0}{k_BT} \Pi_{\text{mixing}} = \ln (1 - \phi_2) + \phi_2 \left( 1 - \frac{1}{N_m} \right) + \phi_2^2 \left[ g(T, \phi_2) - (1 - \phi_2)(g_1(T) + 2g_2(T)\phi_2) \right]$$

(2.15)

To simplify following expressions, the following Flory-Huggins parameter relationship for osmotic pressure is used

$$g_{\Pi}(T, \phi_2) = g_0(T) + g_1(T)(2\phi_2 - 1) + g_2(T)(3\phi_2^2 - 2\phi_2)$$

$$= g(T, \phi_2) + g_1(T)(\phi_2 - 1) + 2g_2(T)\phi_2(\phi_2 - 1)$$

(2.16)

Finally, the osmotic pressure due to mixing is

$$\frac{v_0}{k_BT} \Pi_{\text{mixing}} = \ln (1 - \phi_2) + \phi_2 \left( 1 - \frac{1}{N_m} \right) + \phi_2^2 g_{\Pi}(T, \phi_2)$$

(2.17)

### 2.2.2 Osmotic Pressure of Elasticity

For isotropic expansion of a crosslinked hydrogel, the free energy can be calculated using a “phantom network” model [17,18]. Assuming the hydrogel is an imperfect network and the number of polymer chains $N_C = n_2$, the total free energy due to elasticity is difference between the energy due to chain extension ratios and configurational entropy

$$\frac{1}{k_BT} f_{\text{elastic}} = \frac{n_2}{2} (\lambda_x^2 + \lambda_y^2 + \lambda_z^2 - 3) - \frac{1}{3} \ln \frac{V}{V_0}$$

(2.18)

where $\lambda_n$ is the ratio of chain extension in dimension $n$. With the relationship $V/V_0 = \lambda_x \lambda_y \lambda_z$ and assumption $\lambda_x = \lambda_y = \lambda_z$, the free energy of elasticity is

$$\frac{1}{k_BT} f_{\text{elastic}} = \frac{3n_2}{2} \left[ \left( \frac{V}{V_0} \right)^{2/3} - 1 \right] - \ln \left( \frac{V}{V_0} \right)^{1/3}$$

(2.19)
The osmotic pressure due to elastic properties of a hydrogel is given by the isotropic stress

$$\Pi_{\text{elastic}} = \sigma = \frac{d}{dV} f$$  \hspace{1cm} (2.20)

which equates to

$$\frac{1}{k_B T} \Pi_{\text{elastic}} = \frac{n_2}{2} \left[ -\frac{V}{V_0} + 2 \left( \frac{V}{V_0} \right)^{-1/3} \right]$$  \hspace{1cm} (2.21)

The relationship between volume swelling and volume fraction is

$$\frac{V}{V_0} = \frac{\phi_0}{\phi_0}$$  \hspace{1cm} (2.22)

The volume fraction at synthesis is

$$\phi_0 = \frac{N_m n_2 v_0}{V_0}$$  \hspace{1cm} (2.23)

Rewriting $\Pi_{\text{elastic}}$ in terms of $\phi_2$ and $\phi_0$ gives the osmotic pressure due to elasticity in the hydrogel polymer network

$$\frac{v_0}{k_B T} \Pi_{\text{elastic}} = \frac{\phi_0}{2N_m} \left[ 2 \left( \frac{\phi_2}{\phi_0} \right)^{1/3} - \frac{\phi_2}{\phi_0} \right]$$  \hspace{1cm} (2.24)

### 2.2.3 Osmotic Pressure at Equilibrium

The hydrogel system is in equilibrium when the internal osmotic pressure is equal to the external osmotic pressure. Pressure due to ions or electrostatic interactions are assumed negligibly small in comparison to the pressure of mixing and elasticity. For this reason, the total pressure is set to equal zero

$$\Pi_{\text{total}} = \Pi_{\text{mixing}} - \Pi_{\text{elastic}} = 0$$  \hspace{1cm} (2.25)

Now, in terms of $\phi_2$, the total osmotic pressure is

$$\frac{v_0}{k_B T} \Pi_{\text{total}} = \frac{\phi_0}{N_m} \left( \frac{\phi_2}{\phi_0} \right)^{1/3} - \ln (1 - \phi_2) - \phi_2 - \phi_2^2 \Pi_{11}(T, \phi_2)$$  \hspace{1cm} (2.26)
2.3 Mechanical Relations

2.3.1 Osmotic Bulk Modulus

In a crosslinked polymer hydrogel system, the osmotic bulk modulus [10] is related to the total osmotic pressure by

$$K = \phi_2 \left( \frac{\partial \Pi}{\partial \phi_2} \right)_{\Pi=0}$$  \hspace{1cm} (2.27)

The interaction parameter in the bulk modulus expression is

$$g_K(T, \phi_2) = g_0(T) + g_1(T)(3\phi_2 - 1) + g_2(T)(6\phi_2^2 - 3\phi_2)$$

$$= g(T, \phi_2) + g_1(T)(2\phi_2 - 1) + 2g_2(T)\phi_2(5\phi_2 - 1)$$ \hspace{1cm} (2.28)

Finally, the bulk modulus in terms of the volume fraction $\phi_2$,

$$\frac{v_0}{k_B T} K = \phi_2 \left[ \frac{1}{3N_m} \left( \frac{\phi_2}{\phi_0} \right)^{-2/3} - 1 - \frac{1}{1 - \phi_2} \right] - 2\phi_2 g_K(T, \phi_2)$$ \hspace{1cm} (2.29)

2.3.2 Osmotic Shear Modulus

The osmotic shear modulus [19] is, by definition, the derivative of energy due to chain extension ratios

$$\frac{v_0}{k_B T} G = \frac{\phi_0}{2N_m} \left( \frac{\phi_2}{\phi_0} \right)^{1/3}$$ \hspace{1cm} (2.30)

2.4 Summary

In this chapter, an overview of polymer theory, the relevant derivations, and relationships with hydrogel mechanical properties are provided. Recent work explaining the molecular mechanism of the VPT in PNIPAAm is noted. The foundational relationships between volume fraction, interaction parameters and mechanical properties of the hydrogel are described. These serve as a necessary starting place to resolve the qualitative results of the theory towards quantitative predictions.
2.5 References


CHAPTER 3
POLY(N-ISOPROPYLACRYLAMIDE) HYDROGELS

3.1 Chemical Properties

Poly(N-isopropylacrylamide) hydrogels are crosslinked networks of homopolymers synthesized from N-isopropylacrylamide monomers [1]. Similar to linear PNIPAAm chains which transition between mixing in a single phase to demixing into two phases, PNIPAAm hydrogels transition between a water-rich phase and polymer-rich phase. This thermoresponsive behavior primarily results from hydrogen bonding of coordinated water structures with the amide functional group and the coil-to-globule transition [2, 3].

3.2 Hydrogel Synthesis

Polymer synthesis was conducted using well established protocols by others [4]. Briefly, N-isopropylacrylamide (NIPAM) monomer (Sigma, USA) was proportionally mixed with N,N’-Methylenebis(acrylamide) (MBAm) (Sigma, USA) to achieve a target mol/mol crosslink ratio. Crosslinking occurred through free-radical polymerization with ammonium persulfate (APS) (Sigma, USA) as the initiator and tetramethylethlenediamine (TEMED) (Sigma, USA) as the catalyst. Crosslink ratios were nominally 1:200, 1:100, 1:67, and 1:40. Hydrogels were prepared either as free floating discs with a volume of ~500 μL or surface attached films ~500 μm in thickness (water-rich phase).

3.2.1 Hydrogel Films

PNIPAAm hydrogel films were prepared by pipetting a 20μL droplet onto a glass coverslip functionalized with vinyl groups. Another coverslip was placed directly on top of the droplet and rested on glass coverslip spacers 200μL in thickness. After 20 m, the top coverslip was carefully removed.
Figure 3.1. PNIPAAm hydrogel films on glass coverslips approximately 200 $\mu m$ in thickness.

to prevent damage to the hydrogel film. Films were submerged in water and cycled through a 10 m heating, water exchange, and 10 m cooling cycle 3 times to removed unreacted components.

3.2.2 Hydrogel Discs

PNIPAAm hydrogel discs were prepared by adding a 500$\mu L$ solution into a flat-bottom cylindrical tube. After 20 m, the hydrogel was gently agitated to remove from the sides of the tube. Three cycles of solvent exchange were applied (to removed unreacted components) in which the cold solvent (water) was exchanged with warm water (308 K) and allowed to cool for 20 m per cycle.
Figure 3.2. PNIPAAm hydrogel discs after 15 m of cooling in water.
Figure 3.3. PNIPAAm hydrogel discs after 15 m of heating in water.
3.3 Mass Balance Measurements

Measurements were conducted on the hydrogel discs using microbalance and a temperature controller in a temperature range from 300-316K. For temperatures 300-306 K, samples were equilibrated for at least 20 minutes at a temperature point. For temperatures 308-316 K, samples were equilibrated for at least 60 minutes. Samples were weighed by taking them out of solution, dabbing to remove excess water, and placing on a glass coverslip. The coverslip was weighed with any remaining water separately after each sample measurement.

Each run consisted of sequentially increasing the temperature from 300 K to 316 K with equilibration times as noted above. After each run, samples were left in solution for 24 hours until fully swollen in the water-rich phase.

3.4 Summary

This chapter provides an overview of synthesis methods and measurement techniques for PNI-PAAm hydrogels. An overview of the chemical properties are discussed in context of the volume phase transition. The differences in preparation for hydrogel films and discs are described. Finally, details regarding the approach to mass-balance measurements and temperature variation are provided.
3.5 References


Atomic force microscopy is a high-resolution method which uses a probe to scan sub-nanometer features [1]. With this method, a flexible cantilever with a tip is used to scan a sample surface. Measurements are obtained from the change in deflection of the tip-end of the cantilever as determined by changes in the position of the reflection of a laser signal (Figure 4.1). Although AFM is classically known for imaging applications, another predominant use is force spectroscopy [2]. In this mode of operation, a probe is driven into a sample and withdrawn while the deflection of the cantilever is measured as a function of distance traveled by its base (or time). Applications include single-molecule force spectroscopy [3, 4], mechanics of soft materials such as biological cells or biomaterials [5, 6].

Figure 4.1. Schematic of atomic force microscope operation. A laser signal is reflected off of a flexible cantilever. Changes in the detected signal are related to deflection of the cantilever.
4.1 Contact Mechanics

For non-adhesive contact, the Hertz model of an elastic half-space acted on by surface forces is utilized. In the linear elastic region of material deformation, the modulus of elasticity for a material, $E$, is a proportionality coefficient between the tensile stress $\sigma = F/A$ and deformation $\epsilon = \Delta l/l_0$. For small stresses, this relation is $\sigma = E\epsilon$. The displacement in the z-direction, $u_z$, of the surface can be generalized as [7]

$$u_z = \frac{1 - \nu^2}{\pi E} \frac{1}{r} F_z$$ (4.1)

Where the area of deformation is $r = \sqrt{x^2 + y^2}$, $\nu = -d\epsilon_y/d\epsilon_x$ is Poisson’s ratio (negative ratio of transverse to axial strain), and $F_z = \int_0^a p(r)2\pi r dr$. Displacement of points in the contact area for a rigid sphere in contact with an elastic half-space (Figure 4.2) is given by

$$u_z = d - \frac{r^2}{R}$$ (4.2)

Figure 4.2. Schematic of rigid sphere in contact with elastic half-space for Hertz model overlay onto SEM micrograph of AFM probe modified with polystyrene microsphere.
Using a Hertzian pressure distribution $p = p_0 \left(1 - \frac{r^2}{a^2}\right)^{1/2}$ (for a circle shaped area with radius $a$) and reduced elastic moduli $E^* = E/(1 - \nu^2)$, this gives a displacement,

$$u_z = \frac{\pi p_0}{4E^*a} (2a^2 - r^2) \quad (4.3)$$

when $r \leq a$ and a total force $F_z = 2/3(p_0 \pi a^2)$. Solving for the parameters $a$ and $p_0$ for the displacement,

$$\frac{\pi p_0}{4E^*a} (2a^2 - r^2) = d - \frac{r^2}{R} \quad (4.4)$$

and find $a = \frac{\pi p_0 R}{2E^*}$ and $d = \frac{\pi p_0 a}{2E^*}$ such that $a^2 = Rd$ with a maximum pressure

$$p_0 = \frac{E^*}{\pi} \left( \frac{d}{R} \right)^{1/2} \quad (4.5)$$

and a total force

$$F_z = \frac{4}{3} E^* R^{1/2} d^{3/2} \quad (4.6)$$

Now, the depth of indentation can be related to the maximum contact pressure by

$$d = \frac{1}{2} \left( \frac{9F_z}{16RE^*} \right)^{1/3} \quad (4.7)$$

This general solution can be used for practical AFM applications by describing contact as between two spheres with an effective radius of contact,

$$\frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2} \quad (4.8)$$

Both the AFM cantilever and sample material are appreciably elastic at the nanoscale such that

$$\frac{1}{E^*} = \frac{1 - \nu_1^2}{E_1} + \frac{1 - \nu_2^2}{E_2} \quad (4.9)$$
If the material properties for the AFM probe are known (spring constant, elastic modulus, Poisson’s ratio), the Young’s modulus of a material may be determined from the approach force curve (Figure 4.3).

\[ E^* = \frac{3}{4\sqrt{R}} \cdot \frac{F}{\sqrt{d^3}} \]

Figure 4.3. Left, Hertz model assuming contact between two spheres. \( R \) is the radius of the probe, \( d \) is the indentation into the surface, and \( k \) is the spring constant of cantilever. Right, graph of tip travel against force, called force curve.

In the experimental setup employed in this dissertation project, a polystyrene microsphere is attached to a cantilever for colloidal force microscopy. Since the polystyrene microsphere is the probe in contact with the sample, its nominal elastic modulus and poisson ratio are used, \( E_{PS} = 3.4 \text{ GPa} \) and \( \nu = 0.25 \), respectively [8]. For isotropic polymer hydrogels, the poisson ratio can be assumed incompressible as water \( \nu = 0.5 \).

The measured values from an atomic force microscope are (1) the distance traveled by the cantilever, \( \lambda \) (linear variable differential transformer, LVDT) and (2) the detected deflection, \( \delta \). \( \lambda_0 \) is an arbitrary point selected once the probe makes contact with the surface such that \( \Delta \lambda = \lambda - \lambda_0 \)

\[ d = \Delta \lambda - \delta \] \hspace{1cm} (4.10)

\[ F_z = k\delta \] \hspace{1cm} (4.11)

Thus we can obtain the reduced elastic modulus

\[ E^* = \frac{3}{4R^{1/2}} \frac{F_z}{d^{3/2}} \]

\[ = \frac{3}{4R^{1/2}} \frac{k\delta}{\Delta \lambda - \delta^{3/2}} \] \hspace{1cm} (4.13)
and finally, derive the modulus of measured sample material

\[
E_{sample} = \frac{(1 - \nu_{sample}^2)E_{PS}}{E_{PS} - \frac{3(1 - \nu_{PS}^2)}{4R^{1/2}} \frac{k\delta}{\Delta\lambda - \delta^{3/2}}}
\]  

(4.14)

4.2 Colloidal Probes

Measurement of soft materials, particularly biological matter or bio-inspired bio-materials, presents several interesting challenges. Deformation of the material may transition from linearly elastic to hyper-elastic [9], even over the course of a single measurement. If the probe radius is sufficiently small, initial contact with the sample can result in very large pressures, confounding measurements and analysis [10]. In this project, swollen hydrogels displayed moduli < 1kPa. To overcome the challenge of measuring a material with a low moduli while obtaining high resolution data, polystyrene microspheres were attached to AFM cantilevers (Figure 4.4).

![Figure 4.4. Left, top view and right, side view of 90μm polystyrene microsphere attached to AFM cantilever.](image)

The cantilevers were first characterized to determine the spring constant prior to attachment of the microsphere. Following, the sphere was attached by dipping the edge of the probe into an optical glue and placing in direct contact with the microsphere. This was left under UV radiation (320 nm) for 20 m. The probe was imaged with SEM and dimensions collected by measuring...
the width, length, and thickness of the cantilever. These values were use to validate the empirical measurement of the spring constant.

4.3 Force Curve Analysis on Soft Materials

The point at which indentation equals zero is indeterminate in water because repulsive force near the surface cannot be distinguished from the physical contact with the surface [11]. To overcome this, well-characterized AFM probes with a spherical shape were used as described above and the ratio of indentation depth to probe radius was less than 0.1 for all measurements. Each force curve was fit by adjusting the point of zero-indentation until the log/log slope represented a spherical indentation (1.5) and $R^2 > 0.99$.

4.4 Summary

This chapter provides an overview of experimental techniques in this study using an AFM instrument. A brief introduction to AFM imaging and comparison to scanning electron microscopy is given. Theoretical derivations of contact mechanics underlying force spectroscopic experiments are described. Colloidal probes used in this dissertation are characterized specifically for use with soft materials. Soft materials present unique challenges for measurement and a novel analysis method of force curves is reported.
4.5 References


CHAPTER 5

BIOPHYSICAL CHARACTERIZATION OF CELL INTERACTIONS WITH HYDROGELS

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*Shape-changing hydrogel surfaces trigger rapid release of patterned tissue modules*
Olukemi O. Akintewe, Samuel J. DuPont, Kranthi Kumar Elineni, **Michael C. Cross**, Ryan G. Toomey, Nathan D. Gallant
Published 1 January 2015, Copyright © 2015 ScienceDirect
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Acta Biomaterialia, Volume 11

*Measurement of the traction force of biological cells by digital holography*
Xiao Yu, **Michael Cross**, Changgeng Liu, David C. Clark, Donald T. Haynie, Myung K. Kim
Published 2012, Copyright © 2012 OSA Publishing
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Biomedical Optics Express, Volume 3, Number 1
DOI: http://dx.doi.org/10.1364/BOE.3.000153

### 5.1 Abstract

Micro-patterned poly(N-isopropylacrylamide) hydrogels could be useful in regenerative medicine applications because of the ability to control the topographical configuration of cells into tissue modules, cell sheets, and avoid the use of scaffolds in tissue reconstruction. Poly(N-isopropylacrylamide) is a thermoresponsive polymer that undergoes a volume phase transition in a nar-
row temperature range. While previous studies have shown that topography, surface mechanical properties, and mechanical stress at cell-surface junctions play key roles in cell organization and attachment, little is known about mechanisms of cell attachment and detachment from poly(N-isopropylacrylamide). This study characterizes micro-patterned PNIPAAm hydrogels with scanning electron microscopy, atomic force microscopy, and phase-contrast microscopy to determine the effect of defined topographical features on cell release. The central hypothesis of this investigation is that tissue module release occurs due to a mechanical strain-rate coinciding with critical force needed to overcome the dynamic bond strength of cell adhesion molecules. Advances in this area could improve biomaterial design and accelerate the field of regenerative medicine by reducing or eliminating the need for allograft transplants.

5.2 Introduction

In vitro configuration of multicellular constructs into three-dimensional (3D) patterns to mimic in vivo native cellular environments will be necessary for the formation of functional tissues [1, 2]. Dynamic biomaterials, in defined forms of reversible shape-changing structures, serve as a platform for directing cell patterning [3] and can be actuated with temperature [4], light [5], ionic strength [6], pH [7], electromagnetic fields [8, 9], or a combination of these [10–12]. Improved understanding of physical properties and mechanisms of cell-material interactions for dynamic biomaterials will improve design of these materials for use in tissue engineering applications.

Cell-cell and cell-matrix interactions are mediated through proteins collectively known as cell adhesion molecules (CAMs). They include transmembrane proteins, typically integrins [13], cadherins [14], and serum proteins: primarily fibronectin [15], vitronectin [16], and collagen [17]. While the distribution, conformation and fibrillation of serum proteins can play a key role in cell attachment and adhesion, nearly all the molecular mechanisms of cell-matrix interactions are dependent on recognition of the peptide sequence Arg-Gly-Asp (RGD) [18].

Much less is understood about cell-cell and cell-matrix mechanical interactions [19–21]. Topography is suggested to play a key role in cell organization and attachment [22, 23]. Cell attach-
ment has been shown to be sensitive to mechanical properties of a substrate [24]. Mechanical stress at cell-matrix junctions has been found to increase cell-cell adhesion strength [25]. The elongation and polarization of fibroblasts is also dependent on matrix-rigidity [26]. Current models for mechanical interaction include multiparameter tuning [27], tensegrity [28], and adhesion complex [29].

A connection showing mechanical linkage between a transmembrane protein, integrin, and cytoskeletal stiffness was first demonstrated by Ingber [30]. More recently, local sensing of force and geometry have been shown to determine cell morphology which plays a role in regulating gene expression [31]. The cell machinery that provide this local sensing are focal adhesions (FA), protein complexes through which the cytoskeleton of a cell is connected to a substrate (ECM or biomaterial). Studies on FAs suggest that mechanical force is the central mechanism regulating nearly every stage of focal adhesion assembly [32].

Cells migrate and remodel the ECM in a manner that maintains minimal local intercellular shear stress [33]. Concordantly, data reported in this study demonstrates cell release from micropatterned PNIPAAm hydrogels depends on the lateral strain applied to the cells. The hydrogel surface is characterized with scanning electron microscopy and atomic force microscopy. Phase-contrast microscopy is utilized to measure hydrogel swelling and cell viability. With micro-patterned PNIPAAm hydrogels, the volume phase transition induced strain rate \((\epsilon > 0.25 \text{ in } < 10s)\) exceeds the metabolically-dependent rate of integrin turnover in focal-adhesions which has been shown to be about 1-3 minutes [34]. This time scale required for the reorganization of the focal adhesions is far greater than the time over which a rapid increase in force on the integrin-fibronectin bond created by the strain induced by the swelling PNIPAAm.
5.3 Experimental Methods

5.3.1 Reagents

NIH/3T3 cells were purchased from the American Type Culture Collection. Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate buffered saline (DPBS), newborn calf serum (NCS), 0.25% trypsin EDTA (1X), Calcein AM, ethidium homodimer, penicillin and streptomycin were all obtained from Life Technologies (USA).

N-isopropylacrylamide (NIPAM), 2-dimethoxy-2-phenyl acetophenone (DMPA), N, N'-methylene bisacrylamide (MBAm), 3-(trichlorosilyl) propyl methacrylate (TPM), sodium azide (NaN₃), Rho-associated protein kinase (ROCK) inhibitor Y-27632, acetone, and carbon tetrachloride were all purchased from Sigma-Aldrich (USA).

Methacryloxyethyl thiocarbonyl rhodamine B (polyfluor®570) and 3-APMA was purchased from Polysciences (USA). 3,3'-dithiobissulfosuccinimidyl-propionate (DTSSP) was obtained from Thermo Scientific (USA). Silicone elastomer (PDMS) kits (Sylgard ®184) were obtained from Dow Corning (USA).

5.3.2 Preparation of Micro-patterned PNIPAM Hydrogels

Patterns of crosslinked PNIPAAm hydrogel extruded beams (50-100 μm width × 25 μm height × 5 mm length) were fabricated as described previously [35] on # 1.5 22 mm × 22 mm glass coverslips using PDMS molds by employing the micro-molding in capillaries (MIMIC) technique [36]. Briefly, the glass cover slip was surface modified with TPM in carbon tetra chloride (CCl₄). 1-4% MBAm crosslinker (5 mg/mL), 10% DMPA photo initiator (20 mg/mL) and 1% polyfluor®570 (0.5 mg/mL) were added to a 250 mg/mL solution of NIPAAm in acetone. The resulting solution was introduced to the PDMS molds and polymerized with ultraviolet light (350 nm) for 4 minutes. The fabricated surfaces were sequentially rinsed with ethanol and water to remove unreacted components.
5.3.3 Cell Culture

NIH/3T3 mouse embryonic fibroblast cells were cultured in 10% NCS growth medium containing 1% antibiotics (10,000 units/mL penicillin and 10,000 units/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO2. To prepare tissue modules, trypsinized fibroblasts were seeded onto the fabricated responsive hydrogel arrays at a density of 500 - 750 cells/mm² and cultured at 310 K until confluence (24 - 48 h). Studies of release from low cell density (100 cells/mm²) were cultured for 24 hours.

5.3.4 Release of Tissue Modules from Shape-changing Hydrogels

Rapid release of tissue modules was induced by thermally initiated swelling of the hydrogel beams. 2 mL of cold PBS (277-283 K) was introduced 1 mL at a time into the seeded dish containing 2.5 mL of warm medium resulting in a final temperature of approximately 300 K. Cell release was monitored via time-lapse image acquisition on a microscope for at least 70 seconds.

5.3.5 Viability of Cells Released

A cell viability assay was performed on released cells by using a LIVE/DEAD kit following the commercially recommended protocol. Once the cells reached confluence on the hydrogel beams, the tissue modules were released with fresh cold medium and plated onto a new tissue culture polystyrene (TCPS) dish. Following incubation for 24 or 48 hours at 310 K, the cells were stained with 300 µl of 20 µm Calcein AM and 40 µM ethidium homodimer-1 solution. After 30 min, the dish was rinsed twice with warm PBS and replenished with fresh medium prior to imaging.

5.3.6 Phase Contrast Microscopy

Time-lapse analysis (30 frames per second) and micrographs of samples were obtained using an Eclipse Ti-U (Nikon Instruments, Japan) fluorescent microscope equipped with a CCD camera (CoolSNAP HQ2, Photometrics, Tuscon). Cell images were analyzed with NIS-Elements advanced research software Ver. 4.20 (Nikon Instruments) or Image J (NIH, USA). Images were processed to overlay fluorescent channels on the phase-contrast channel for LIVE/DEAD analysis.
5.3.7 Atomic Force Microscopy

An Asylum (USA) MFP3D, atomic force microscope was utilized to obtain nanotopography. The spring constant for μ-masch (USA) AFM cantilevers, NSC35 (Cr-Au-BS), was empirically determined by obtaining the inverse optical lever sensitivity (InVols) on mica and then finding the resonant frequency using the thermal method [37]. The nanotopography of PNIPAM was obtained in tapping mode with a resolution of 1024 × 1024 points at 0.25 Hz scan rate and a cantilever resonant frequency of 282 Khz or 199 kHz.

5.4 Results and Discussion

5.4.1 Theoretical Considerations

An early theoretical framework for mechanical interaction of CAMS was the Bell model [38] in which the bond survival time can be described

\[ t_{off}(f) = t_{off}^* \exp \left( -\frac{f}{f_{\beta}} \right) \]  \hspace{1cm} (5.1)

where \( t_{off} \) is the stressed bond lifetime under, \( t_{off}^* \) is the unstressed bond lifetime, \( f \) is the applied force and \( f_{\beta} = \frac{k_B T}{x_{\beta}} \) is the scaling factor or level of force to drop the barrier by one unit of thermal energy (\( x_{\beta} \) is the location of the activation energy barrier). In a study of the physical role played by CAMs using dynamic force spectroscopy [39], it was found that the property characterizing these bonds was dynamic strength rather than static strength [40].

Catch-bonds, an adhesion state predicted to increase in strength with increase in tension [41], were experimentally verified [42] to first increase and then decrease under conditions of variable mechanical stress. This dynamic strength was shown to have a corollary to Bell’s model in that bond lifetime was dependent on the stress rate

\[ r_f = \frac{\Delta f}{\Delta t} \]  \hspace{1cm} (5.2)
where $\Delta f$ is the change in force and $\Delta t$ is the time duration. The bond is predicted to break at a critical force

$$f_c = f_\beta \ln \frac{r_{f_{off}}}{f_\beta}$$

(5.3)

Thus, the lifetime of a CAM bond is

$$t = \frac{f_c}{r_f}$$

(5.4)

showing that with increased loading speed, bond strength increases slowly while bond survival falls rapidly.

These bonds manifest in clusters of focal adhesions which have been found to exert a constant stress of $5.5 \text{nN} \cdot \mu \text{m}^{-2}$ [43]. Also notable was that these adhesions formed within seconds. Other work has demonstrated further strengthening of the bonds to a saturation point of $200 \text{nN}$ after 4 hours [44]. Comparatively, cell-matrix interactions with soft biomaterials measured using digital holography found the traction force applied by migrating human dermal fibroblasts (Figure 5.1) to be about 40 nN [45].

5.4.2 Characterization of PNIPAAm Surface Topography

Scanning electron micrographs of PNIPAAm hydrogels in air display a qualitatively smooth surface (Figure 5.2). It is worth noting these measurements in air characterize the hydrogel in a near-completely dehydrated state. Measurements in this state provide a lower bound of surface roughness and upper bound of mechanical stiffness.

Further characterization of surface roughness was measured with atomic force microscopy. The root-mean-square (RMS) surface roughness for the hydrogels in air was 5.54 nm in a 5 $\mu \text{m} \times 5$ $\mu \text{m}$ field of view and 6.42 nm in a 20 $\mu \text{m} \times 20$ $\mu \text{m}$ field of view (Figure 5.3). Surface features of nano-scale pores (100-250 nm diameter) contributed most to the roughness. Additionally, stress cracks from the dehydration process were apparent.
Figure 5.1. Examples of cells wrinkling a silicone rubber film. The field of view was $190 \times 176$ $\mu$m$^2$ with $800 \times 742$ pixels. a), e) and i) Bright field images; b), f) and j) Quantitative phase images; c), g) and k) Cross-sections of phase profiles along highlighted lines AB in b), CD in f) and EF in j); d), h) and l) Pseudo-color 3-D rendering of phase images b), f) and j).
Figure 5.2. Scanning electron microscopy of micro-patterned, self-deforming stamp structures. Structures are formed by injecting PDMS molds with PNIPAM solution and crosslinking with UV. Beams are 50-100 μm in width, 20-30 μm in height and separated by a spacing of 1000 μm.

Figure 5.3. Topography maps of PNIPAAm surface at, left, 20 μm × 20 μm and, right, 5 μm × 5 μm. The dark areas (left) are stress cracks which appear after swelling and deswelling of the structure. Circular structures (right) are nano-scale pores on the surface.
5.4.3 Molecular Basis of Release

Cell release from hydrogel surfaces was examined by separately treating seeded samples, after 24 hour incubation, with 2 mM sodium azide (NaN), a compound known to block ATP production [46], 50 μm Y-27632, a selective inhibitor of Rho-associated protein kinases [47], or 2 mM DTSSP, a homobifunctional crosslinker that fixes only integrins bound to the extracellular matrix [44]. Briefly, samples were exposed to NaN for 60 minutes or Y-27632 or DTSSP for 30 minutes prior to initiating tissue module release. To investigate the effect of surface strain on attached cells, the concentration of the network crosslinker (MBAm) in the prepolymer solution was varied from 1 to 4% before MIMIC processing. The one-dimensional width-wise strain in each microbeam was calculated from phase contrast micrographs as follows:

\[ \varepsilon = \frac{\Delta w}{w_{\text{collapsed}}} = \frac{w_{\text{swollen}} - w_{\text{collapsed}}}{w_{\text{collapsed}}} \]  

where \( \varepsilon \) is the Cauchy strain, \( w_{\text{collapsed}} \) is the width of the hydrogel beam in the collapsed state and \( w_{\text{swollen}} \) is the width of the hydrogel beam in the swollen state. Cell detachment was calculated as the percent of cells released from the microbeams within 3 minutes after thermal actuation.

5.4.4 Cell Viability After Release

To examine the fate of cells within released tissue modules, the multicellular stripes were harvested via lateral strain (\( \varepsilon > 0.25 \)) and allowed to re-attach to TCPS15. Cell survival was observed over 48 hours. After 24 hours on TCPS, the organization of the tissue module was generally lost as the morphology became a loose aggregate of spreading cells, as expected for fibroblasts on TCPS (Fig. 5a). After 48 hours, the cell number and area further increased (Fig. 5b). A LIVE/DEAD viability assay indicated that the majority (~94%) of the harvested cells remained viable after release from the shape-changing microbeams (Figure 5.5).

In the polymer-rich phase above the VPT temperature, the micro-patterned PNIPAAm hydrogels are collapsed and maintain a rigid topography upon which cells can be cultured. In the water-rich phase, the hydrogels rapidly swell laterally at the top surface because attachment to the underlying substrate prevents elongation adjacent to the surface. This swelling results in release of
Figure 5.4. Cell release from PNIPAAm hydrogel monoliths is mediated by lateral strain. Untreated cells will not release when the lateral strain $\epsilon < 0.25$. Metabolically inhibited (NaN$_3$) behave similarly indicating ATP production is not a mechanism of release. ROCK-inhibited cells (Y27632), where actin-myosin contractility is prevented and cells treated to covalently bond integrins to the fibronectin target receptor (DTSSP) do not detach, even when $\epsilon > 1$.
Figure 5.5. Fluorescence overlaying phase image of cells released from PNIPAM beams with live stain (calcein, ex/em ~495 nm/∼515 nm) and dead stain (ethidium, ex/em ~495 nm/∼635 nm) at 24 h or 48 h after replating of released cells.
cells while maintaining cell-cell contacts, cell morphology, and tissue organization. Because the topographic changes and resulting release of cells occur rapidly in 30-60 s, extended exposure to low temperatures is minimized and cell detachment induced anoikis [48] can be limited.

Taken together, these results indicate that cells release rapidly as a result of mechanical strain in the underlying surface and also remain viable following release. This mechanical strain depends on topographical features of the micro-patterned PNIPAAm hydrogels. This release appears to be significantly more rapid (seconds to minutes) than previously reported for thin films (minutes to 10’s of minutes). Inhibition of ATP synthesis via sodium azide showed little effect on the release suggesting that metabolism does not contribute to the rapid release mechanism.

5.5 Conclusions

In this study, cells were cultured on and released from extruded micro-patterns of PNIPAAm hydrogel beams. In the collapsed state, cells adhered until confluent and upon temperature modulation to $> 300\,K$, rapidly detached from the surface when the lateral strain $\epsilon > 0.25$. The molecular mechanism was determined by systematically investigating biological functions of the cell and their relation to the strain needed to release cells. Finally, cells were demonstrated to be viable after release.
5.6 References


CHAPTER 6
QUANTITATIVE PREDICTION OF MECHANICAL PROPERTIES FOR
POLY(N-ISOPROPYLACRYLAMIDE) HYDROGELS WITH FLORY REHNER
THEORY

6.1 Abstract

Stimuli-responsive hydrogels such as poly(N-isopropylacrylamide) undergo a large volume phase transition within a small temperature range making them useful for a wide variety of biomedical applications including tissue engineering, drug delivery, and biomedical implants. Current theory can predict non-stimuli-responsive polymer hydrogel swelling in a solution based on an empirically derived interaction parameter. However, for stimuli-responsive hydrogels, theoretical models to predict swelling and mechanical properties of the polymer currently show only qualitative agreement with experiment. In this study, mass-balance measurements and atomic force spectroscopy are used to determine the mass fraction and obtain force curves of poly(N-isopropylacrylamide) hydrogels with crosslinker ratios 1:200, 1:100, 1:67 and 1:40. The volume fraction and Young’s modulus are calculated from these experimental measurements to elucidate their relationship within the Flory-Rehner theoretical framework for quantitative prediction. Results from this study are useful in predicting the mechanical properties of the hydrogel using empirically derived parameters.

6.2 Introduction

Thermoresponsive polymer hydrogels such as poly(N-isopropylacrylamide) (PNIPAAm) undergo a volume phase transition (VPT) when the temperature of the system reaches a critical point [1, 2]. The VPT is a phenomena in which the volume of the hydrogel swells several times its collapsed size, transitioning from a polymer-rich to water-rich phase. This phase transition can be exploited for applications as has previously been reviewed in tissue engineering [3] and drug delivery [4]. Complementary to the focus on applications are unresolved theoretical challenges including non-equilibrium phenomena [5] and phase diagrams [6]. Of particular interest is the quanti-
In contrast to other non-responsive polymers, linear chains of PNIPAAm in solution display a drastic change in phase properties such as solubility [8] and hydrodynamic radius [9] over a relatively narrow temperature range (~304-306 K). Extensive investigation has been conducted towards understanding the underlying mechanism [10,11], reconciling Flory-Rehner theory (FRT) with experimental results [12], and conceptualizing new theoretical frameworks [7]. It continues to remain an important area of investigation for two key reasons related to the previously noted applications:

- Because PNIPAAm displays similar hydration behavior to that observed in physiologically relevant proteins [13], theoretical treatments could be useful in understanding biological systems.

- The elastic modulus of the hydrogel are of similar order of magnitude to human cells [14].

Simple theoretical models make it possible to understand highly complex phenomena while enabling engineering applications with predictive outcomes. For example, Hansen solubility parameters are useful in manufacturing, coating design, identification of compatible polymers, and determining chemical resistance [15]. Though not fully generalizable due to theoretical limitations, the parameters are broadly applicable. In like manner, Flory-Huggins interaction parameters have been used to predict gel swelling [16–18] and mechanical properties [19–21]. With thermoresponsive hydrogels, FRT remains a highly popular analytical starting point for due to its straightforward statistical analysis of crosslinked polymer networks, yet only qualitative agreement with experiment has been found [22]. More specifically, empirically derived Flory-Huggins interaction parameters from cloud point measurements of linear PNIPAAm chains in solution did not quantitatively predict gel swelling across the phase transition.

Following work by Afroze, others sought to build on the quantitative aspects of the work (polymer-rich phase) and extend predictions to mechanical properties of the hydrogel [23]. Puleo applied constrained junction fluctuation modification of FRT to fit experimental values of swelling that also corresponded to shear modulus [24]. Nevertheless, direct predictions of mechanical properties in hydrogels using parameters derived from single PNIPAAm chains has not yet occurred.

In this study, predictions of FRT are compared to experimental results for mass fraction and elastic modulus of PNIPAAm hydrogels. Studies with varied crosslinker ratio and temperature are con-
ducted on hydrogel discs and films. This report seeks to answer the question, “can mechanical properties of PNIPAAm hydrogels be predicted with a general form of Flory-Rehner theory?”

6.3 Experimental Methods

6.3.1 Hydrogel Preparation

Polymer synthesis was conducted using well established protocols by others [22]. Briefly, N-isopropylacrylamide (NIPAm) monomer (Sigma, USA) was proportionally mixed with N,N’-Methylenebis(acrylamide) (MBAm) (Sigma, USA) to achieve a target mol/mol crosslink ratio. Crosslinking occurred through free-radical polymerization with ammonium persulfate (APS) (Sigma, USA) as the initiator and TEMED as the catalyst. Crosslink ratios were nominally 1:200, 1:100, 1:67, and 1:40. Hydrogels were prepared either as free floating discs with a volume of ∼500 𝜇L or surface attached films ∼500 𝜇m in thickness.

6.3.2 Volume Swelling

Measurements were conducted on the hydrogel discs using microbalance and a temperature controller in a temperature range from 300–316 K. For temperatures 300–306 K, samples were equilibrated for at least 20 minutes at a temperature point. For temperatures 308–316 K, samples were equilibrated for at least 60 minutes. Samples were weighed by taking them out of solution, dabbing to remove excess water, and placing on a glass coverslip. The coverslip was weighed with any remaining water separately after each sample measurement.

6.3.3 Colloidal Force Spectroscopy

Mechanical properties of hydrogel films were obtained using an MFP3D (Asylum, USA) atomic force microscope. Samples were mounted in a closed cell, submerged in aqueous solution, and heated from 300-316 K with an MFP3D BioHeater™(Asylum, USA) temperature controller. The cantilever used was a CSC 17 (µmasch, USA) cantilever with a nominal spring constant of 0.15 N/m. The spring constant of the cantilever was determined experimentally by obtaining the inverse optical lever sensitivity (InVols) on mica and then finding the resonant frequency using the thermal method [25]. The value obtained, \( k_{C-SC17} = 0.112N/m \), was within 5% of that calculated from di-
dimensional measurements of the cantilever using scanning electron microscopy (SEM). Polystyrene microspheres were attached to the end of the cantilever with optical glue and the radius determined using SEM micrographs of a top and side view. The aspect ratio from each view was \(1.00 \pm 0.005\) and the radii, \(r_{CS17} = 43.8 \pm 0.4 \mu m\).

6.3.4 Force Curve Analysis

The point at which indentation equals zero is indeterminate in water because repulsive force near the surface cannot be distinguished from the physical contact with the surface [26]. To overcome this, well-characterized AFM probes with a spherical shape were used as described above and the ratio of indentation depth to probe radius was less than 0.1 for all measurements [27]. Each force curve was fit by adjusting the point of zero-indentation until the log/log slope represented a spherical indentation (1.5) and \(R^2 > 0.99\).

6.3.5 Statistical Analysis

Fitting was conducted using non-linear least squares and linear least squares method.

6.4 Results and Discussion

6.4.1 Theoretical Considerations

Interaction potential between network chains in a crosslinked hydrogel network and water molecules is given by

\[
\int_{\phi_2}^{1} \chi(\phi_2 T, N) d\phi = (1 - \phi_2) g(\phi_2, T, N)
\]

(6.1)

Using nomenclature as previously described by Afroze et al [22], the mean interaction is

\[
g(\phi_2, T, N) = \sum_{n=0}^{\infty} g_n \phi_2^n
\]

(6.2)

where

\[
g_n = g_{n0} + g_{n1} T
\]

(6.3)
For simplicity, let
\[ \frac{1}{n} - \chi_n = (n - 1) \left( \bar{g}_{n-2} - \bar{g}_{n-1} \right) \] (6.4)

Thus, three intermediate quantities Z, I, and J are defined:

\[ Z = \sum_{n=2}^{\phi_2} \left( \frac{1}{n} - \chi_n \right) \] (6.5)

\[ I = \phi_2 \frac{\partial}{\partial \phi_2} Z = \sum_{n=2}^{\phi_2} n \phi_2^n \left( \frac{1}{n} - \chi_n \right) \] (6.6)

\[ J = \frac{\phi_0}{N_m} \left( \frac{\phi_2}{\phi_0} \right)^{1/3} \] (6.7)

These relate to the thermodynamic quantities of osmotic pressure, bulk modulus, and shear modulus.

\[ \frac{\upsilon_0}{k_B T} P = Z - J \] (6.8)

\[ \frac{\upsilon_0}{k_B T} K = I - \frac{J}{3} \] (6.9)

\[ \frac{\upsilon_0}{k_B T} G = J \] (6.10)

Further details for these quantities are derived in the supporting information section 6.6, Detailed Derivations.

### 6.4.2 Volume Swelling

Mass fractions \( w_2 \) of free-floating PNIPAAm hydrogel discs display a temperature dependence consistent with findings by others [22, 28] undergoing a discontinuous increase between 308-311 K as a result of hydrogel dehydration and chain conformation within the network. However, this transition occurs at a higher temperature and broader range than that found for linear chains, a finding also observed in cyclic PNIPAAm [29]. Volume fraction \( \phi_2 \), a quantity related to \( w_2 \), is used in FRT and a comparison of mean \( \phi_2 \) as a function of T with a 95% confidence interval is
shown in Figure 1 for the crosslinker ratios studied in this investigation (1:200, 1:100, 1:67, 1:40). Hydrogel behavior in the water rich phase (linear crosslinker ratio dependence) is distinctly different from that in the polymer rich phase (no crosslinker ratio dependence).

A remarkable feature of the VPT in PNIPAAm is that the behavior occurs in linear polymer chains as short as 10 monomer units [30]. Computational models simulating 20-50 monomer units further support this minimum length [11, 31, 32]. For much longer chains, experimental results for polymer chains between 10 KDa and 390 KDa show demixing occurs at a near consistent mass fraction of $0.5 \pm 0.1$ [22, 33]. Essentially, the VPT is independent of the length of the polymer chain (transition temperature may vary depending on the end group [34]) which lends well to generalizable modeling as is encapsulated in FRT. The predominant explanation for the VPT in PNIPAAm is cooperative hydration [35]. At temperatures below the VPT, water molecules form stabilizing structures around the polymer sidechain at the amide group [10, 30]. The VPT occurs around the temperature where the “entropic penalty” for these cooperative structures is higher than the entropic penalty for demixing water and polymer.

In hydrogels of crosslinked polymer networks, the VPT is an approximate description for two correlated transitions: (1) formation to decomposition of cooperative water structures and (2) coil-to-globular transition of the crosslinked polymer network. The resultant discontinuous volume swelling and mechanical properties have not yet been shown as quantitatively predictive within the FRT framework.

### 6.4.3 Elastic Modulus

Another challenge faced in reconciling theory with experiment are the measurements themselves. In aqueous solutions, hydration forces near the point of contact result in distortion of force curves obtained through atomic force spectroscopy [26]. Specifically, precise determination of the zero distance is not possible despite being a crucial parameter in Hertz modeling of contact mechanics. With soft materials, use of sharp probes can penetrate the surface or lead to highly
Figure 6.1. Mean volume fraction ($\phi_2$) as a function of temperature (T) with 95% confidence interval for various crosslinker ratios. Top-left) 1:200 crosslinker ratio shows broad confidence interval in the polymer-rich phase where by contrast, top-right) 1:100, bottom-left) 1:67, and bottom-right), the 95% confidence intervals and equilibrium values of the data are approximately the same.
unreliable pressures just beyond the contact point [27,36].

To understand the relationship between crosslinker ratio, gel swelling, and mechanical properties, both the free-floating hydrogels and thin films (~500 μm) were synthesized from the same stock solution under identical conditions for each crosslinker ratio in this investigation. Extensive data was collected for each temperature point and crosslinker ratio: \( N = 3 \) for hydrogel discs and \( N = 16 \) for hydrogel films. The temperature-dependent order-of-magnitude change in Young’s modulus of these films in the water-rich and polymer-rich phases shown in Figure 6.2 are consistent with previous studies [12, 28, 37–41] and the hydrogel displays elastic deformation without apparent strain hardening. In the water-rich phase, the crosslinker ratio relates with the \( E \) temperature dependence and the modulus increases with temperature. In the polymer-rich phase, the modulus is at least an order-of-magnitude greater than the water-rich phase, does not display crosslinker ratio dependence, and has a relatively broader 95% confidence interval.

In the water-rich phase, the Young’s moduli are dependent on the crosslinker ratio (\( P \ll 0.01 \)). The quantitative relationship with crosslinker ratio exists both for \( \omega_2, \phi_2 \), and elastic modulus in this phase (Figure 6.6, Tables 6.1 and 6.2). As noted previously, there does not appear to be a crosslinker ratio relationship in the polymer-rich phase. A qualitative relationship was observed between \( \phi_2 \) and \( E \) but differed below and above VPT as shown in Figure 6.3.

### 6.4.4 Water-rich Phase

In the limit as \( \phi_2 \to 0 \), osmotic pressure due to chemical mixing potential of the two species (water and crosslinked gel network) will dominate the thermodynamic expressions such that it can be written independent of \( \phi_0 \) or \( N_m \) by using the relationship from when \( P=0 \),

\[
\frac{v_0}{k_B T} E_{\phi_2 \to 0} = \frac{9KG}{3K + G} = 3Z - \frac{Z^2}{I} = \frac{5}{6} \phi_2^2 \left[ g_0(T) - g_1(T) \right]
\]  

(6.11)

This suggests Flory-Huggins parameters can be determined by fitting to the swelling data. This predicts the elastic modulus in the water-rich phase.
Figure 6.2. Young’s modulus ($E$) as a function of $T$ for surface immobilized hydrogel films for different MBAm:NIPAM ratios. At temperatures below 307 K, modulus increases with crosslinker ratio: top-left) 1:200, top-right) 1:100, bottom-left) 1:67, bottom-right) 1:40. A discontinuous modulus increase occurs above 307 K and though intermediate values vary, the moduli appear to converge to 40-45 KPa except for the 1:200 crosslinker ratio which is 20-30 KPa.
6.4.5 Polymer-rich Phase

In the limit as $\phi_2 \to 1$, osmotic pressure due to elastic potential in the crosslinked gel network will dominate thermodynamic expressions such that $I \to \infty$. Now, the relationship between $E$ and $\phi_2$ is

$$\frac{v_0}{k_B T} E_{\phi_2 \to 1} = 3f - \frac{J^2}{I} = 3 \left( \frac{\phi_0^{2/3}}{N_m} \right) \phi_2^{1/3}$$

(6.12)

Within the same Flory-Huggins framework, the composite $\phi_0^{2/3}/N_m$ parameter is determined by fitting to the swelling data. This predicts the elastic modulus in the polymer-rich phase.

The model, thus, remains unmodified leaving the interaction term as a phenomenological parameter in similar fashion to Shibayama et al. and the composite $\phi_0/N$ parameter empirically determined. It remains a generalizable, but piece-wise application of FRT. This is justified because FRT only accounts for average molecular interactions between water and polymer chains but does not capture the correlated transitions of volume in the polymer and dispersal of cooperative water structures.
Figure 6.4. Predicted elastic modulus (red line) from empirical determined parameters in water-rich phase compared to experimental values (blue circles) with 95% confidence intervals (blue shaded regions) for crosslinker ratios 1:200 (top-left), 1:100 (top-right), 1:67 (bottom-left), and 1:40 (bottom-right). Notably, for the 1:40 crosslinker ratio, the theory predicts negative moduli values (not shown) in the polymer-rich phase.
Figure 6.5. Predicted elastic modulus (red line) from empirical determined parameter in polymer-rich phase compared to experimental values (blue circles) with 95% confidence intervals (blue shaded regions) for crosslinker ratios 1:200 (top-left), 1:100 (top-right), 1:67 (bottom-left), and 1:40 (bottom-right).
6.5 Conclusions

In this study, an empirical basis was established to quantitatively apply FRT to thermoresponsive hydrogels. The polymer-rich phase and water-rich phase display significant differences both in experimental results and theoretical treatment. A piece-wise application of the theory is supported by the underlying molecular interactions, namely, decomposition of cooperative water structures and coil-to-globular transition of the polymer.

Volume fraction quantities derived from mass balance measurements were related to Young’s modulus quantities fit with the Hertz model for well-characterized probes using atomic force spectroscopy. The crosslinker ratios were varied to elucidate these relationships within the Flory-Rehner theoretical framework to make quantitative predictions using the empirically derived parameters. These results can be utilized for determining mechanical properties of crosslinked PNIPAAm hydrogels for use in biomedical applications though further investigation is necessary for use in a manufacturing setting.

6.6 Supporting Information

6.6.1 Detailed Derivations

The Flory-Huggins interaction parameter can be written as a series

\[ g(T, \phi, N) = \sum_{n=0} g_n \phi_2^n = g_0 + g_1 \phi_2 + g_2 \phi_2^2 + \cdots \]  \hspace{1cm} (6.13)

with the first derivative

\[ g^{(1)} = \frac{\partial}{\partial \phi_2} g = \sum_{n=1} n g_n \phi_2^{n-1} = g_1 + 2g_2 \phi_2 + 3g_3 \phi_2^2 + \cdots \]  \hspace{1cm} (6.14)

and second derivative

\[ g^{(2)} = \frac{\partial^2}{\partial \phi_2^2} g = \sum_{n=2} n(n-1) g_n \phi_2^{n-2} = 2g_2 + 6g_3 \phi_2 + 12g_4 \phi_2^2 + \cdots \]  \hspace{1cm} (6.15)
The free energy derivation of mixing

\[
\frac{V}{k_B T} f = -\left[ n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_1 \phi_2 g(T, \phi_2, N) \right]
\]  
(6.16)

Where (note, the derivative for \( f \) taken with respect to \( n_1 \) for convenience and rewritten in terms of \( \phi_2 \) by convention), let intermediate interaction term \( D \) be

\[
D = \phi_2^2 g
\]  
(6.17)

\[
D' = 2\phi_2 g + \phi_2^2 g^{(1)}
\]  
(6.18)

\[
D'' = 2g + 2\phi_2 g^{(1)} + \phi_2^2 g^{(2)}
\]  
(6.19)

Rewriting these in summation form

\[
D = \phi_2^2 \sum_{n=0} g_n \phi_2^n
\]  
(6.20)

\[
D' = 2\phi_2 \sum_{n=0} g_n \phi_2^n + \phi_2^2 \sum_{n=1} n g_n \phi_2^{n-1}
\]  
(6.21)

\[
D'' = 2 \sum_{n=0} g_n \phi_2^n + 2\phi_2 \sum_{n=1} n g_n \phi_2^{n-1} + \phi_2^2 \sum_{n=2} n(n-1) g_n \phi_2^{n-2}
\]  
(6.22)

Aligning indices for the three previous equations

\[
D = \sum_{n=2} \phi_2^2 g_{n-2}
\]  
(6.23)
\[ D' = 2\phi_2 \sum_{n=2} g_{n-2}\phi_2^{n-2} + \phi_2^2 \sum_{n=2} (n-1)g_{n-1}\phi_2^{n-2} \]
\[ = \sum_{n=2} \phi_2^2 \left[ \frac{2}{\phi_2} g_{n-2} + (n-1)g_{n-1} \right] \quad (6.24) \]
\[ D'' = 2 \sum_{n=2} g_{n-2}\phi_2^{n-2} + 2\phi_2 \sum_{n=2} (n-1)g_{n-1}\phi_2^{n-2} + \phi_2^2 \sum_{n=2} n(n-1)g_{n}\phi_2^{n-2} \]
\[ = 2 \sum_{n=2} \phi_2^2 \left[ \frac{1}{\phi_2} g_{n-2} + \frac{1}{\phi_2} (n-1)g_{n-1} + \frac{n(n-1)}{2} g_n \right] \quad (6.25) \]

Now, using the intermediate term in the osmotic pressure for mixing expression

\[ \frac{v_0}{k_B T} \Pi_{mixing} = \frac{V}{k_B T} \frac{d}{dn_1} f = \ln (1 - \phi_2) + \phi_2 + D' \quad (6.26) \]

Since \(0 < \phi_2 < 1\) (given the condition \(\phi_1 + \phi_2 = 1\)) the Maclaurin series is used to describe

\[ \ln (1 - \phi_2) = -\sum_{n=1}^{\infty} \frac{\phi_2^n}{n} = -\phi_2 - \sum_{n=2}^{\infty} \frac{\phi_2^n}{n} \quad (6.27) \]

Which leaves a simplified intermediate term, \(Z\) for osmotic pressure due to mixing

\[ Z = \frac{V}{k_B T} \frac{d}{dn_1} f = \frac{v_0}{k_B T} \Pi_{mixing} = D' - \sum_{n=2}^{\infty} \frac{\phi_2^n}{n} \quad (6.28) \]

This can be used to obtain an intermediate term used in the bulk modulus as follows with

\[ Z' = D'' - \sum_{n=3}^{\infty} \frac{n\phi_2^{n-1}}{n} = D'' - \sum_{n=2}^{\infty} \phi_2^n \quad (6.29) \]

and

\[ I = \phi_2 Z' = \sum_{n=2}^{\infty} \phi_2^n \left[ \frac{2}{\phi_2} g_{n-2} + 2(n-1)g_{n-1} + n(n-1)\phi_2 g_n - \phi_2 \right] \quad (6.30) \]
Figure 6.6. A) Elastic modulus measurements as a function of temperature by crosslinker ratio. B) Relationship between mass fraction $w_2$ and crosslinker ratio display temperature independence. C) Relationship between elastic modulus and crosslinker ratio displayed temperature dependence.

<table>
<thead>
<tr>
<th>$T(K)$</th>
<th>$m$</th>
<th>$b$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>0.0112</td>
<td>0.0702</td>
<td>0.914</td>
</tr>
<tr>
<td>302</td>
<td>0.0115</td>
<td>0.0714</td>
<td>0.926</td>
</tr>
<tr>
<td>304</td>
<td>0.0129</td>
<td>0.0795</td>
<td>0.942</td>
</tr>
<tr>
<td>306</td>
<td>0.0134</td>
<td>0.0820</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Given the exponential decay of $\phi_2^2$, only series for the interaction parameter is only taken to the 2nd term:

\[
\frac{1}{2} - \chi_2 = g_0 - g_1
\]  

\[
\frac{1}{3} - \chi_3 = 2(g_1 - g_2)
\]  

\[
\frac{1}{4} - \chi_4 = 3g_2
\]

### 6.6.2 Crosslinker Relationships with $w_2$, $E$, and $g_n$
Table 6.2. Fitting parameters for elastic modulus and crosslinker ratio using model $E = mN_m + b$.

<table>
<thead>
<tr>
<th>$T$(K)</th>
<th>$m$(×10^4)</th>
<th>$b$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>3.36</td>
<td>308</td>
<td>0.999</td>
</tr>
<tr>
<td>302</td>
<td>3.46</td>
<td>356</td>
<td>0.966</td>
</tr>
<tr>
<td>304</td>
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<td>306</td>
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Figure 6.7. Relationship between $g_{\alpha 1}$ parameters and crosslinker ratio.
6.7 References


CHAPTER 7
PROTEIN-SURFACE INTERACTIONS ON STIMULI-RESPONSIVE POLYMERIC BIOMATERIALS

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7.1 Abstract

Responsive surfaces: a review of the dependence of protein adsorption on the reversible volume phase transition in stimuli-responsive polymers. Specifically addressed are a widely studied subset: thermoresponsive polymers. Findings are also generalizable to other materials which undergo a similarly reversible volume phase transition. As of 2015, over 100000 articles have been published on stimuli-responsive polymers and many more on protein-biomaterial interactions. Significantly, fewer than 100 of these have focused specifically on protein interactions with stimuli-responsive polymers. These report a clear trend of increased protein adsorption in the collapsed state compared to the swollen state. This control over protein interactions makes stimuli-responsive polymers highly useful in biomedical applications such as wound repair scaffolds, on-demand drug delivery, and antifouling surfaces. Outstanding questions are whether the protein adsorption is reversible with the volume phase transition and whether there is a time-dependence. A clear understanding of protein interactions with stimuli-responsive polymers will advance theoretical models, experimental results, and biomedical applications.
7.2 Introduction

Protein adsorption at material surfaces manifests both beneficial and adverse outcomes depending on the application, including cell attachment and biofouling, respectively [1–6]. So-called stimuli-responsive polymers have the capacity to mediate protein adsorption through a volume-phase transition (VPT), which offers dynamic control over such physical properties as wettability, roughness, porosity, and stiffness [7–10]. The VPT is characterized by a sharp change in the solubility of the polymer. Stimuli can be broadly classified as either energy- or chemical-dependent as shown in figure 7.1. Examples of energy-based actuation include temperature [11,12], light [13–15], and electromagnetic fields [16]. Chemical-based actuation includes pH [17] and biochemical [18] stimuli.
As of 2015, over 100,000 articles have appeared on the subject of stimuli-responsive polymers, and some notable clinical applications employing this class of materials have emerged. ThermoDox is a temperature-responsive liposome which permeates at hyperthermic temperatures to rapidly release doxorubicin for cancer treatment [19]. As of this writing, it is currently in phase III trials for treatment of hepatocellular carcinoma (www.clinicaltrials.gov, ID: NCT00617981). Budesonide polymer liquid, which is liquid at room temperature and forms a viscous gel at body temperature, was shown to enable localized drug delivery while avoiding systemic exposure [20]. It is currently recruiting for a clinical trial (www.clinicaltrials.gov, ID: NCT02290665). An FDA-approved injectable gel device, Backstop (www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/pmn.cfm, 501K #: K090430), employs a water-soluble thermosensitive polymer which is a viscous liquid at room temperature and becomes a gelatinous plug at body temperature, is used to prevent kidney stone fragments from migrating back into the kidney during extraction [21, 22]. Despite the decades of research on these materials for biomedical applications, translation to clinical therapeutic devices has been limited. The lack of examples of medical devices that employ stimuli-responsive polymeric biomaterials may be because the unique features of the material are difficult to access and control in vivo. Moreover, the durability of such coatings must be addressed.

Despite the promise of using responsive polymers to control protein adsorption and thereby direct mammalian cell or microbial attachment, less than 100 of these articles have specifically investigated VPT-dependent protein adsorption and interactions. Reversible control of protein adsorption is useful in applications such as scaffolds for wound repair [23–25], on-demand delivery methods of therapeutics [26, 27], and antifouling surfaces (i.e. dialysis ports, cardiovascular shunts, or surgical sutures) [28–30]. Ultimately, understanding how and to what extent dynamic properties of stimuli-responsive biomaterials control protein adsorption will be necessary to advance development of these classes of devices [31–33].

A key challenge in the study of protein adsorption phenomena is the comparability of experimental data. Any number of variables such as surface energy, buffer pH, temperature, or time, can substantially affect experimental outcomes to produce incompatible results from study to study [34]. Thus, many of the reports on stimuli-responsive biomaterial interfaces remain ambiguous.
due to this complexity. In particular, while most studies demonstrate that stimuli-responsive polymers show a clear trend of increased protein adsorption in the less soluble state compared to the more soluble state, the issue of reversibility remains an open question.

Relative to non-responsive materials, theoretical treatment of protein adsorption on stimuli-responsive materials is also conceptually challenging. Interparticle interactions in an aqueous electrolyte medium are described in terms of a balance between van der Waals attraction and electrical double-layer repulsion by Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. However, at biologically relevant salt concentrations, this theory fails to adequately predict experimental results [35]. One promising model, a ‘fluctuation’ Hofmeister series, attempts to describe protein stability by accounting for differences in protein interactions with chaotropes and kosmotropes [36]. An overriding issue remains that the model used to describe protein adsorption behavior is determined by the research question asked; consequently, the generalizability of the results may be limited. The study of protein adsorption also requires the use of several theoretical models that can further complicate the comparison of results between studies [37,38].

Methods for constructing stimuli-responsive coatings include surface attached networks and brushes or the adsorption of free-floating structures (as shown in figure 7.2). Similar to non-responsive materials, various surface properties of these coatings are relevant to protein adsorption. For hydrogel monoliths, these include crosslink density and porosity. With polymer brushes, molecular weight and graft density of the brushes are most pertinent. For micelles and microgel particles, the applicable properties are molecular weight and particle diameter.

By far, the most commonly studied stimuli-responsive polymer is poly(N-isopropylacrylamide) (PNIPAAm). In water, PNIPAAm has a temperature-actuated VPT, which occurs near the lower critical solution temperature (LCST) of approximately 32 °C. Below this temperature, PNIPAAm-structures imbibe water and swell, whereas above the temperature the structures expel water and collapse. Furthermore, the VPT temperature can be tuned by copolymerization with other monomers.
Figure 7.2. Schematic of the volume phase transition for stimuli-responsive polymer structures. State change occurs via application of appropriate stimuli and, in general, is reversible. (A) Crosslinked hydrogel monolith displaying volumetric changes with stimuli. Example shown is surface immobilized. (B) Surface attached polymer brushes show conformational changes with stimuli. (C) Free-floating micelles swell or collapse with stimuli.
This review will focus primarily on protein adsorption to PNIPAAm structures, with special emphasis on the relationship between the VPT and adsorbed amount, and whether adsorption can be correlated to property changes associated with the VPT [39, 40]. Despite the focus on this polymer, the findings reviewed herein are generalizable to other stimuli-responsive systems that undergo similarly reversible VPTs [41–43] (this does not apply to single activation or shape-memory materials). While there have been a number of reviews of protein-surface interactions [34, 44–47] and several reviews of stimuli-responsive polymers [48–51], this review critically analyzes protein-stimuli responsive polymer interactions.

Within the context of applications and challenges, this review is divided into three sections. Section 7.3 briefly discusses basic aspects of protein interactions with biomaterials and then explores the phenomena of VPT-dependent protein interaction with PNIPAAm coatings. Section 7.4 delineates the influence of physical properties for grafted brushes including polymer molecular weight and graft density. Additionally, the generally assumed relationship between wettability and protein adsorption is challenged. Particular attention is given to potential molecular mechanisms for VPT-dependent protein interaction. Section 7.5 examines theoretical work, surveys thermoresponsive coatings that are less widely studied, and outlines needed areas of research to advance understanding of protein interactions with stimuli-responsive systems.

### 7.3 Protein interactions with PNIPAAm

Briefly overviewed here are protein interactions with PNIPAAm and the inherent complexities of the phenomena. In general, there is broad consensus that more protein adsorbs above the VPT than below; there is less agreement, however, as to the magnitude of this difference. Most of the studies are carried out between 10 °C to 40 °C, with only single temperature points compared above and below the VPT. Since it has been shown that protein adsorption to non-responsive surfaces is independent of temperature in this range, these reports implicate the importance of the VPT. However, very few studies exist that show adsorption isotherms (i.e. adsorbed amount versus adsorbing
concentration) below and above the VPT, which is standard practice on non-responsive surfaces.

7.3.1 Interfacial protein interactions

Protein adsorption describes accumulation on a surface and typically involves conformational changes that minimize the free energy and stabilize the protein-surface attachment with weak forces. For instance, when an implant is placed in an in vivo environment, proteins from blood non-specifically adsorb to the material. This is critically important because the adsorption of these proteins can trigger the foreign body response of the immune system resulting in non-ideal outcomes for the implant [5, 6, 52–54]. With in vitro experiments, this adsorption can be modeled by submerging a substrate in serum containing medium (or any solution containing proteins) and evaluating cell interactions on the material. Descriptions of protein adsorption focus primarily on the final or equilibrium state. In this case, protein-surface interactions are affected by (1) solution parameters including temperature, pH and ion concentration, (2) interface parameters including chemistry, topography, and stiffness, and (3) for the protein, the amino acid sequence.

Simple adsorption can be modeled via Langmuir isotherms, where proteins are assumed to behave as ideal gas molecules and dynamic equilibrium exists between adsorbed and free floating proteins. Theory, such as the Vroman effect, has been developed to further account for protein mixtures where slower adsorbing but high affinity proteins can displace faster adsorbing small proteins [55]. Biological systems, however, are more complex and involve individual and ensemble phenomena difficult to both model and measure. As shown in figure 3, protein interactions with an interface may include repulsion, varied states of unfolding, reversible adsorption, and protein-protein interaction. Other associated phenomena are cooperative adsorption, overshooting, and relaxation. Computational modeling of these protein-surface interactions that simulate non-equilibrium or near equilibrium conditions have proven to better correspond with experimental results than equilibrium models [56, 57]. Further in-depth analysis of advances in understanding protein adsorption can be found in reviews by others [34, 44–47]. Polymer chemistry provides significant control over tuning properties that regulate protein adsorption. In general, on
non-responsive surfaces, wettability, functional groups, and nanotopography determine adsorption behavior. However, the effect of dynamically modifying these characteristics, as occurs with stimuli-responsive materials, is a nascent area of study.

7.3.2 Protein interaction dependence on volume phase transition (VPT)

Several proteins have been investigated for their interactions with PNIPAAm polymers including bovine serum albumin (BSA) [48, 59–74], human serum albumin (HSA) [75–81], fibrinogen (Fg) [82–84], lysozyme [85–90], immunoglobulin (IgG) [91–93], human gamma globulin (HGG) [94], myoglobin [95–97], assorted serum or plasma proteins [98, 99], and recombinant proteins [60]. While these vary in structure, functionality, and sequence, a general finding is that they adsorb in greater quantities above the temperature at which the VPT occurs.

Kawaguchi et al were among the first to investigate protein adsorption on PNIPAAm microspheres with an observed VPT temperature between 30°C and 35°C [94]. It was found that HGG adsorption changed substantially around 35°C and was not linear with temperature. The measured amount of protein adsorbed at pH 7 was ∼5 mg g⁻¹ (HGG/polymer) above the VPT temperature and ∼2.5 mg g⁻¹ below it. Their hypothesis—that the increase in hydrophobicity enables greater protein adsorption—has been thematic in subsequent work and will be discussed in section 3. Other publications typically report similar results with other proteins; however, there is little agreement in the adsorbed amounts above and below the VPT. Duracher et al investigated protein adsorption of a modified HIV-1 capsid p24 protein onto thermoresponsive positively charged core-shell poly(styrene)-PNIPAAm particles [60]. Minimal adsorption was found at 20°C but significant adsorption, >60 mg g⁻¹ (protein/polymer) at 40°C. The conclusion was that the temperature-dependent hydration state of the particles and resultant change in charge distribution controlled protein adsorption. Gan et al copolymerized core-shell particles of PNIPAAm-poly(ethylene glycol) with monomethyl ether monomethacrylate to reduce adsorption of BSA [59]. In this work, the difference in protein adsorption above and below the VPT temperature on the copolymer particles was negligible (1.2–3.7 mg g⁻¹ at 25°C and 1.3–4.9 mg g⁻¹ at 42°C), while on PNIPAAm only parti-
Figure 7.3. Non-fouling surface resistant to protein adsorption that repels proteins. (B) Proteins adsorbed to surface in various states of denaturation: (1) partial and (2) complete. (C) Cycle of reversible adsorption. (D) Aggregated proteins by protein-protein interaction or entanglement. Human serum albumin model from x-ray crystallography [58].
cles, a two-fold increase was observed (5.1mg g\(^{-1}\) (protein/polymer) at 25 °C and 11.6mg g\(^{-1}\) at 42 °C).

Another common assumption of VPT dependence is the ability to desorb proteins after adsorption above the VPT. Actually, in most studies, some amount of protein remains attached to a PNIPAAm coating even after temperature or rinse cycles. Okubo et al conducted a comprehensive study on poly-styrene-PNIPAAm emulsions with egg albumin, lysozyme, and lactalbumin hydrolysate [85]. Proteins were first adsorbed for 3h then adsorption/desorption monitored through multiple temperature cycles. Although more adsorption was measured at 40 °C than at 25 °C, very little protein desorbed when first adsorbed at 40 °C. Experiments conducted by Shamim et al studied adsorption and desorption of BSA on magnetic nanoparticles coated with PNIPAAm [65]. They found more protein adsorbed at 40 °C (∼170mg g\(^{-1}\) polymer) than that at 30 °C (∼130mg g\(^{-1}\) polymer). Furthermore, upon reducing the temperature to 20 °C, more protein remained on the sample where protein was adsorbed at 40 °C. Interestingly, the absolute amounts desorbed from both were similar (∼110 and 115mg g\(^{-1}\) polymer respectively). Consistent with this finding, Ding et al used magnetic particles coated with PNIPAAm to show that HSA adsorption increased with temperature. Protein was adsorbed for 30min at 35 °C (4.5mg g\(^{-1}\) polymer), 40 °C (6mg g\(^{-1}\) polymer), and 45 °C (8mg g\(^{-1}\) polymer). For all adsorption temperatures, the amount of protein desorbed at 25 °C (2-3mg g\(^{-1}\) polymer), was about the same [75]. Sugiura et al demonstrated that rinse cycling above the VPT temperature desorbed some IgG from a streptavidin-conjugated PNIPAAm microchannel [93]. However, upon temperature cycling, nearly all (>90%) of the protein was desorbed. Together, these studies suggest proteins adsorbed above the VPT temperature will desorb below it—in some cases partial, and others, complete.

7.3.3 Summary

This section provided an overview of interfacial protein interactions with stimuli-responsive systems. Two key ideas emerge for PNIPAAm coatings with respect to the VPT temperature: (1) The amount of protein adsorbed is greater above than below the VPT, and (2) proteins adsorbed
above can be partially or completely desorbed below. However, most studies have examined only a single point each above and below the VPT temperature, as well as a single adsorbing concentration. This oversimplifies the complexity of the adsorption process and consequently, conclusions remain largely qualitative. Only a few studies have reported results of protein adsorption at multiple points above and below this temperature [80, 90, 100]. More investigations like these would provide a clearer picture of the effect of VPT on protein adsorption and reversibility. Some reports have suggested VPT-related changes in hydrophobicity affect protein interactions. Still, an underlying molecular mechanism remains elusive and existing experimental results do not lend well to a generalizable theory for designing materials to dynamically control adsorption.

7.4 Role of physical properties of PNIPAAm brushes on protein adsorption

Work reviewed in this section focuses on investigating the relationship between protein-biomaterial interactions and properties such as wettability, polymer molecular weight, and graft density of the brushes. The majority of reports covering protein interactions with PNIPAAm polymers have focused on polymer brushes on both flat surfaces and spherical particles [69, 99, 101]. Also, the consensus view that VPT-dependent changes in hydrophobicity modulate protein interactions is further considered.

7.4.1 Wettability

While the early reports by Kawaguchi et al discussed above suggested wettability affects protein interactions with PNIPAAm microspheres [94], Huber et al were the first to correlate contact angle measurements (67° at 25 °C and 85° at 50 °C) with protein interactions for a thermo-responsive coating reporting a ~3.5nm layer of adsorbed HSA at 55 °C compared to nearly none at 25 °C [95]. However, the most complete measurements investigating wettability and protein interactions were conducted by Cho et al [62]. In this study, the adhesive forces between a BSA-immobilized AFM tip and PNIPAAm brushes were measured at temperatures ranging from 25 °C to 34 °C. In concert with contact angle measurements, it was reported that the intermolecular force between PNIPAAm and
BSA is temperature dependent. At 25 °C, a force of 0 nN was found indicating no BSA adsorption while at 34 °C, a force of 0.45 nN indicated some adsorption. QCM measurements found small amounts of protein adsorbed at 22 °C (-Δ10 Hz) and greater adsorption at at 37 °C (-Δ54.5 Hz). The discrepancy between AFM (no adsorption at 25 °C) and QCM (small amount of adsorption 22 °C) will be further discussed in section 4. Furthermore, the asserted temperature dependence is not conclusive for two reasons: (1) a control experiment with a non-responsive surface was not conducted and (2) the relationship between the VPT and contact angle is not well established. To this second point, Kidoaki et al found advancing contact angles on PNIPAAm above and below the LCST only ranged 60°-64° regardless of the graft layer thickness [102].

The correlation is further confounded because relatively small changes in contact angle around the VPT seemingly account for significant changes in protein adsorption. Ivanov et al showed a nearly order-of-magnitude increase in adsorption of myoglobin on PNIPAAm brushes from 9 °C (<10mg g⁻¹ polymer) to 35 °C (70-90mg g⁻¹ polymer) whereas the contact angle only increased from 53° to 67°, respectively [96]. Similarly, Yu et al found Fg adsorption on PNIPAAm brushes increased from 50ng cm⁻² at 23 °C to 120ng cm⁻² at 37 °C while, respectively, the contact angle only changed from 57° to 73° [82]. Moreover, De las Heras Alacón et al found adhesion of bacterial cells, a proxy indicating protein adsorption, was sensitive to VPT-dependent changes in hydrophobicity/hydrophilicity of PNIPAAm brushes [103]. A contact angle change (Δθ) was measured ranging from Δ9°-24° in experiments spanning 12 °C to 45 °C at pH 5.6 or 7.4 which correlated with increased cell numbers at 37 °C compared to 10 °C.

The relationship between VPT-dependent contact angles and protein interaction may even be more nuanced as outlined in the following study. Liu et al recently showed that hysteresis in contact angle measurements are associated with surface composition at the molecular level [104]. By varying the graft density of PNIPAAm on a super-hydrophobic surface (contact angles>150°), they found that below a certain density, hysteresis between the advancing and receding contact angles was relatively small. An important finding was that at 25 °C for the most densely grafted PNIPAAm brushes, application of pressure to the droplet reduced the contact angle to 48°. By contrast, surfaces with sparser brush densities retained a contact angle around 150°. At 40 °C, all brush den-
sities had a contact angle greater than 150°. These results indicate that topographical changes in PNIPAAm through the VPT will have an effect on solution interactions, even when grafted to a super-hydrophobic surface.

While contact angle measurements may not fully capture these effects, other studies show a definite change in protein adsorption. Yu et al [79] characterized PNIPAAm brushes with thickness between 4 and 38.1nm but all with a constant graft density of 0.46 chains nm$^{-2}$. The contact angle of the swollen state at 23 °C remained relatively constant at approximately 58° independent of thickness. In this state, the adsorption of HSA was also relatively constant at 8ng cm$^{-2}$ independent of thickness. Contact angles and adsorption at 37 °C behaved differently. In the collapsed state, the contact angle increased linearly from 65.3° to 79.8° for thicknesses between 4 and 38.1nm. Interestingly, the adsorbed amount was approximately constant at 12ng cm$^{-2}$ up to a thickness of 13.4nm and a contact angle of 71.2°. Only at the greatest thickness (38.1nm and a contact angle of 79.8) was a significant increase in the adsorbed amount (33ng cm$^{-2}$) observed.

More recently, Choi et al found that macroscopic surface wettability does not adequately predict protein adsorption or cell adhesion [99]. In this study, PNIPAAm was grafted onto self-assembled monolayer (SAM) surfaces that were functionalized with $CH_3$, $COOH$, (11-mercaptoundecyl)-tetra-(ethylene glycol) (OEG), or OH. Regardless of the underlying surface chemistry, the change in contact angle from 25 °C to 37 °C was less than 10°, and all surfaces fell within a total range between 50° and 70°. Despite this small range, significant differences in protein adsorption were found on nearly all chemistries with grafted PNIPAAm brushes. In addition, brush density was shown to affect adsorption. On denser brushes (0.154 chains nm$^{-2}$) protein adsorption was independent of underlying chemistry with about 40ng cm$^{-2}$ at 37 °C and no more than 20ng cm$^{-2}$ at 25 °C. For less dense brushes (0.069 chains nm$^{-2}$), adsorption varied with underlying chemistry. At 25 °C and 37 °C, respectively, SAMs of $CH_3$ were ~75/120ng cm$^{-2}$, $COOH$ were ~150/200ng cm$^{-2}$, OH were ~40/60ng cm$^{-2}$, and OEG were ~15/25ng cm$^{-2}$. Underscoring the dependence of adsorption on the underlying chemistry for less dense brushes, in contrast, adsorption amounts on ungrafted SAMs (non-responsive surfaces) were similar to those on the less dense brushes but did not show a temperature dependence: $CH_3$ was ~140ng cm$^{-2}$, $COOH$ was ~200ng cm$^{-2}$, OH was ~100ng.
cm$^{-2}$, and OEG was $\sim 30$ng cm$^{-2}$. Thus, protein adsorption correlates with brush density of grafted PNIPAAm above and below the VPT temperature. Furthermore, higher density brushes may reduce effects of the underlying surface chemistry on adsorption. Nevertheless, it remains unclear whether characterization by contact angle is particularly relevant for PNIPAAm coatings.

To better contextualize reports in this section, findings from a systematic study of protein adsorption on non-responsive surfaces with SAMs to control surface chemistry are outlined for reference [105]. In the study, serum proteins were adsorbed onto SAM surfaces with mixtures of terminal functional groups ($\text{CH}_3/\text{OH}$, $\text{CH}_3/\text{COOH}$, and $\text{CH}_3/\text{NH}_2$) in varied ratios to control contact angles. Within the range of contact angles from the preceding studies ($50^\circ$ to $100^\circ$), protein adsorption on these mixed SAM surfaces was constant on $\text{CH}_3/\text{OH}$ ($\sim 200$ng cm$^{-2}$), decreasing with increasing with contact angle on $\text{CH}_3/\text{COOH}$ ($\sim 250$ to $200$ng cm$^{-2}$), and decreasing with increasing with contact angle on $\text{CH}_3/\text{NH}_2$ (from $\sim 275$ to $200$ng cm$^{-2}$). In summary, on non-responsive surfaces, large changes in contact angle are associated with relatively small changes in amounts of protein adsorbed, whereas, on PNIPAAm stimuli-responsive surfaces, small changes in contact angle are associated with relatively large changes in amounts of protein adsorbed. In contrast, Xu and Siedlecki measured a pronounced transition in protein adhesion for contact angles near $60^\circ$ on polyethylene surfaces, and a strong time dependence was observed [106]. However, Prime and Whitesides reported that protein adsorption had a greater sensitivity to chemical groups than to wettability [107]. These results underscores that contact angles alone may not be predictive of adsorption, and the comparison between adhesion measurement with a single protein on an AFM tip and adsorption from concentrated solutions may elucidate unique phenomena. Taken together, these studies suggest that on PNIPAAm either contact angle measurements do not indicate the local hydrophobicity experienced by individual proteins (i.e. difference in scale) or that physical properties other than wettability also mediate protein adsorption.
7.4.2 Molecular weight and graft density

Molecular weight and graft density are physical properties that contribute to surface wettability. These properties also modulate phenomena at the scale of individual protein-surface interactions. Studies investigating the combined effect of PNIPAAm brush density and molecular weight on protein adsorption are limited, however. Here, the few reported studies are compared to outline gaps, conflicts, and limitations.

Plunkett et al showed that the wettability of a PNIPAAm brush layer above the transition temperature is dependent on molecular weight [101]. For brushes of graft density 0.21 chains nm\(^{-2}\) with Mw > 47000, the contact angle increased (\(\Delta 10^\circ\)) while for those with Mw < 19000 the contact angles above and below the VPT temperature were essentially the same. This finding qualitatively agrees with the previously mentioned adsorption results by Yu et al [79] in which the PNIPAAm brush thickness was varied (which is related to molecular weight) while maintaining a graft density of 0.46 chains nm\(^{-2}\).

To date, no study has systematically investigated molecular weight and graft density to independently show a VPT-dependent relationship. It is also possible that within a certain Mw range and graft density, it may not be experimentally tractable. For example, Burkert et al reported that to PNIPAAm brushes with Mw 28000 and chain density 0.12 chains nm\(^{-2}\), no adsorption of HSA was observed at 23 °C, while at 40 °C approximately 50ng cm\(^{-2}\) at equilibrium was measured [78]. However, in the same study, no protein adsorption was found on brushes with Mw 47000 (0.22 chains nm\(^{-2}\)) or 132000 (0.06 chains nm\(^{-2}\)) at either temperature. Similarly, Brouette et al [97] were unable to detect adsorption of myoglobin at either 25°C or 37°C on PNIPAAm brushes with Mw 244300 and density 0.21 chains nm\(^{-2}\). Somewhat consistent with these findings, Morisada et al [67] measured less than 10mg g\(^{-1}\)-polymer of BSA adsorption on PNIPAAm brushes with a Mw 43000 and density of 0.05 chains nm\(^{-2}\). A possible explanation for the discrepancy is that the lower chain density at the higher Mw is associated with some protein adsorption.
Zhao et al found an inverse relationship with molecular weight and protein adsorption in agreement with the above but no brush density was reported [81]. In this study, adsorption of both Fg and HSA decreased 5-fold when the Mw was increased from 21000 to 146000. This decrease in protein adsorption was associated with a decrease in attachment and spreading of L929 mouse fibroblast cells. Xue et al found decreasing adsorption from 55ng g$^{-1}$ (protein/polymer) to 35ng g$^{-1}$ with increased grafting density ranging from 0.08 to 0.21 chains nm$^{-2}$, respectively [69]. But the molecular weight was also varied such that an independent relationship with grafting density cannot be established.

These findings of decreasing protein adsorption with increasing graft density follow trends established in the broader context of non-responsive grafted polymers. Sofia et al measured protein adsorption on poly(ethylene oxide) (PEO) of Mw 3400, 10000, or 20000 grafted to a silicon wafer [108]. Fn was found to adsorb in a layer 12 Å thick on bare silicon and with an increase in graft density of PEO brushes eventually decreased to 0 Å. No protein adsorbed once the graft density was 0.21 chains nm$^{-2}$ for Mw 3400, 0.08 chains nm$^{-2}$ for Mw 10000, and 0.04 chains nm$^{-2}$ for Mw 20000. What can be concluded is that larger brushes occupy more space and thus require lower densities to occlude the surface preventing adsorption. Thus, for PNIPAAm brushes, similar boundary conditions of Mw and brush density may exist for protein adsorption. Nevertheless, limited results on PNIPAAm preclude quantitative relationships for the parameters brush density and molecular weight that could predict protein adsorption.

7.4.3 Summary

In this section, the physical properties of PNIPAAm coatings which modulate protein interactions were discussed. Although the studies covered use contact angles in relation to protein adsorption, it remains unclear how contact angle measurements relate to physical properties that mediate protein adsorption. Notably, for non-stimuli-responsive coatings, the relationship between contact angle and protein adsorption is also not well understood. While one study observed a strong transition effect between 60-65$^\circ$ on protein adhesion for non-stimuli-responsive surfaces [106],
the broader literature reveals an inconsistent dependence of protein adsorption on contact angles \([1, 105, 107, 109, 110]\). Furthermore, others have stated that contact angles under cyclooctane are a better predictor of protein adsorption than contact angles in air \([111]\). They found a trend of decreasing protein adsorption with decreasing contact angle but no consistent relationship with the functionalization of the surface.

Thus, results using contact angles with stimuli-responsive surfaces are even more ambiguous and difficult to interpret due to the dynamic surface properties. The differences in contact angle measurements above and below the VPT temperature are often minimal even as the difference in protein adsorption is significant. While this suggests underlying changes in molecular properties of the surface, results attempting to establish a relationship between protein adsorption and the physical parameters, brush density and molecular weight, are even less clear. Boundary conditions of brush density and molecular weight may exist for protein adsorption but conflicting analysis and limited results only provide a qualitative correlation.

7.5 **Theory, challenges, and future directions**

The discontinuous phase behavior of the VPT in stimuli-responsive biomaterials leads to several challenges in describing protein adsorption, both experimentally and theoretically. With polymer brushes, there are three modes of adsorption: primary to the underlying substrate, secondary to the projected brush surface, and ternary to the brush terminus or backbone \([112]\). A key experimental challenge is to distinguishing between primary and ternary adsorption while a key theoretical challenge is the incomplete description of swollen brushes and hydrogels with self-consistent field theory \([113]\). De novo computationally designed proteins, a modeling approach using non-natural interactions, is a powerful tool to better understand protein adsorption with in vitro and in vivo systems; nevertheless, the approach is limited by the complexity of electrostatic, van der Waals, and hydrophobic interactions \([114]\).
Some experimental aspects of existing studies can be quantitatively compared such as polymer molecular weight, brush density, or amount of protein adsorbed. However, the experimental conditions, including incubation time, protein concentration, and chemistry of the grafting substrate represent confounding variables that preclude effective comparison. There is general consensus that these materials are antifouling or that adsorption is controllable by the VPT. But given the limited literature on protein adsorption, the challenge in comparing studies, and no clear mechanism, more research in this area is needed.

A notably uncharted area of study involves protein adsorption on hydrogel monoliths. For these, modulation of the crosslink density will alter the extent of change in the volume and surface area at the VPT which can affect protein interactions. In addition, pore size of these structures may also play a role [63]. Wu et al were able to demonstrate that lower crosslink densities resulted in larger pore sizes and were thus associated with greater adsorption of BSA (66kDa). They further confirmed this size-dependent interaction by adsorbing insulin (5.8kDa), a smaller molecule, and showing that it would almost completely desorb upon lowering the temperature below the LCST. Several theoretical models related to porosity, though not specifically focused on stimuli-responsive materials, are a useful starting place to describe size-dependent protein adsorption. One proposed framework uses thermodynamic descriptions based on mass action to explain biomolecule adsorption to a surface [115]. In another, a geometric pore-filling model shows good agreement with experimental studies of protein adsorption on mesoporous materials [116]. Lastly, an experimental study showed the complementarity of the Langmuir approach (in the low packing regime) with excluded volume models by obtaining the same theoretical results when compared with experiment for lysozyme adsorption on polystyrene-PNIPAAm core-shell hydrogels [117].

Groundwork continues to be laid for better understanding protein interactions with PNIPAAm through its VPT. On non-responsive polymers, studies investigating protein adsorption are far more numerous and have established clear precedent with substantive progress in advancing understanding of this phenomena [34]. Though the complexity leaves many open questions, some fairly basic ones have been addressed in non-responsive polymers that could be pursued for thermoresponsive polymers. Two of these stand out as particularly helpful in clarifying VPT depen-
Another area of needed research is the time-dependence of protein interactions. As mentioned for work done by Cho et al [62], they noted a discrepancy between AFM and QCM measurement which they suggested was due to the time scales $\leq 1s$ and $4h$, respectively. They speculated that conformational changes in both proteins and PNIPAAm brushes over the longer timescale could explain this. Other studies have shown absolute protein desorption amounts to be similar regardless of temperature above that which the VPT occurs [65, 75]. The adsorption incubation times were different and thus it remains unclear (1) if the adsorption reached saturation, or (2) even if saturation was reached, whether the proteins were undergoing conformational changes. In another example, magnetic latex particles were coated with a PNIPAAm shell to control adsorption of HSA and it was found that the amount desorbed increased with the incubation time for adsorption [76].

More work is needed to better understand the temperature dependence of protein interactions with PNIPAAm coatings and elucidate the underlying molecular mechanisms. Well-established experiments with non-responsive polymers should be conducted on thermo-responsive polymers. Particularly significant gaps in knowledge for these coatings are adsorption modes on brushes, protein interactions with micron to millimeter scale hydrogels, and time-dependence of protein interactions. Time-course studies may also uncover adsorption dependencies not intrinsically temperature related. Another important advance needed is development of experimental techniques to better control for underlying substrate interactions. Measurement of protein interactions at multiple points above and below the VPT temperature will provide quantitative measures but comparable experimental environments are needed (pH, ionic strength, protein used).

7.6 Concluding remarks

This review covers stimuli-responsive biomaterial systems with a focus on understanding protein-biomaterial interactions. PNIPAAm coatings are the most widely studied but the findings regarding VPT-dependent protein interactions are generalizable because (1) the phase change behavior
is similar irrespective of the stimulus and (2) the influence of temperature on protein adsorption over the relevant range is negligible. These biomaterials are highlighted as a versatile platform for regenerative medicine, therapeutics, anti-fouling surfaces, and basic scientific investigation. Several recent studies involving these materials show promise in furthering both control and models of protein interactions with biomaterials. Stimuli can modulate material properties including surface area, functionality, and stiffness. Because of this, control over protein adsorption, desorption, specificity, and conformation is possible. The ability to more closely mimic the dynamics of an in vivo environment is highly applicable for biomedical implants and drug delivery vehicles. In addition, this control is particularly useful for development of theoretical models to describe protein adsorption. Increasingly sophisticated theoretical and computational models of the protein adsorption phenomena are under development but many challenges remain to sufficiently describe experimental results. Basic in vitro systems are still complex and variation of conditions between experiments and labs confound comparison.

Experimental results considered in section 7.3 qualitatively show that proteins do not adsorb well on brushes at points below the VPT temperature but do adsorb above. Only a handful of studies included experiments with multiple points above and below this temperature; thus claims of temperature-dependent protein interactions are inconclusive. Consensus indicates this behavior is due to a change in the polymer surface from hydrophilic to hydrophobic; however, recent work suggests a more nuanced picture in which the mechanism of protein interactions are driven by mode of adsorption which is dependent on the physical structure of the polymer surface.

Physical properties implicated in temperature dependent protein interactions including wettability, polymer molecular weight, brush graft density, and film thickness, were the focus of section 7.4. Wettability measurements via contact angle are ill-defined indicators of hydrophobicity for PNIPAAm coatings and do indicate underlying changes in the molecular structure. This property is, in fact, also a function of molecular weight and brush density of the polymer brushes. A study to show independent relationships between these and protein interactions is needed to advance understanding of the VPT dependence.
Finally, section 7.5 covered theoretical work, limitations, and future directions. The discontinuous phase behavior, modes of protein interactions with brushes, and complexity in aqueous systems present both experimental and theoretical challenges. The literature investigating VPT-dependent protein interactions is limited, and consequently, the study of protein adsorption in the dynamical environment of stimuli-responsive systems remains fairly nascent. Well-established experiments for non-responsive polymers should be conducted for stimuli-responsive polymers. Because the majority of work has covered polymer brushes and microgel particles, there are limited relevant findings for hydrogel monoliths. There is, however, growing interest for this coating due to its potential for bioseparation and bioprinting applications [118–120]. Time-dependence together with VPT-dependence of protein interactions for stimuli-responsive systems also remains unclear. Variations in experimental conditions and results preclude a solid theoretical framework which could predict protein interaction from VPT-dependent physiochemical properties in stimuli-responsive systems.

The studies reviewed comprise PNIPAAm polymers, the most widely studied subset of stimuli-responsive systems. Use of these systems explicitly for the purpose of elucidating VPT-dependent protein interactions is merited. Well characterized in vitro systems that better represent the dynamics of in vivo environments will stimulate new avenues of basic scientific study. Ultimately, a clear understanding of in vivo protein adsorption will enhance the design of implantable stimuli-responsive biomaterials for tissue regeneration, targeted drug therapies, and anti-fouling surfaces.
7.7 References


CHAPTER 8
FUTURE WORK

8.1 Introduction

Biomaterial surfaces support cell adhesion via the adsorption of an interfacial layer of extracellular matrix or serum proteins. These cell-matrix interactions are mediated through proteins collectively known as cell adhesion molecules (CAMs) which include transmembrane proteins such as integrin receptors [1, 2]. The distribution, conformation and fibrillation of serum proteins play a key role in cell attachment and adhesion [3]. What remains unknown is how surface charge density coupled with mechanical properties promote or inhibit binding of serum proteins.

In this chapter, theoretical background, preliminary experimental results and a project proposal are discussed. There are two key areas of theory relevant to measurement methods used: (1) mapping of relative surface charge density and (2) adhesion force spectroscopy. Preliminary experimental results show the effect of surface charge density on the mediation of serum protein adsorption onto micro-patterned PNIPAAm hydrogels. Finally, a project is proposed to relate the surface charge density with the attachment strength of serum proteins implicated in cell attachment.

8.2 Theoretical Considerations

8.2.1 Mapping Surface Charge Density

Surface charge density can be measured using AFM in non-contact mode, a mode generally used for imaging [4, 5]. A driving voltage to the piezo is applied at the resonant frequency of the tip and as the tip scans the surface, interactions between the tip and the surface will dampen the amplitude of the signal from which a height map is obtained. Using the tip-cantilever geometry shown in Figure 8.1, a brief derivation of non-contact AFM mechanics is described [6].
Tip movement can be described with Newton’s equation of motion

\[
F(t) = m^* \frac{d^2 Z_c}{dt^2} + \gamma D \frac{dZ_c}{dt} + k_c Z_c(t)
\]  
(8.1)

where \( m^* = 0.2426 m_c + m_t \) is the effective mass of the cantilever and tip (respectively), \( Z_c = \frac{2}{3L} \frac{dZ_c}{dX} \) is the deflection of the cantilever, \( L \) is the cantilever length, and \( X \) is the horizontal coordinate. We let \( F(t) = F_0 \sin(\omega t) \) and in steady state, solution to this differential equation is

\[
Z_c(t) = Z_0 \sin(\omega t - \phi)
\]  
(8.2)

where

\[
Z_0 = \frac{F_0}{m^* \sqrt{(\omega_0^2 - \omega^2)^2 + (\frac{\omega \omega_0}{Q})^2}}
\]  
(8.3)
and resonant frequency \( \omega_0 = \sqrt{k_c/m^*} \). In vacuum or a gaseous medium, this amplitude is dependent on the angular frequency and with a maximum

\[
Z_0(\omega_{\text{max}}) = \frac{F_0}{\gamma D \sqrt{k_c m^* - \left( \frac{m^* \gamma D}{2} \right)^2}} \tag{8.4}
\]

at

\[
\omega_{\text{max}} = \sqrt{\frac{k_c}{m^* - \left( \frac{\gamma D^2}{2m^*} \right)}} \tag{8.5}
\]

In an incompressible fluid, the resonant frequency (1st harmonic) depends on the following relation [7]

\[
\mu_F = \frac{L \rho_F}{T \rho_C g_1} \tag{8.6}
\]

where \( L \) is the cantilever length, \( T \) is the cantilever thickness, \( \rho_F \) is the density of the fluid, \( \rho_C \) is the density of the cantilever, and factor \( g_1 = \pi/12 \). The resonant frequency is given by

\[
\omega_F^0 = \frac{\omega_0}{\sqrt{1 + \mu_F}} \tag{8.7}
\]

The force in non-contact mode,

\[
F_0 = Z_0(\omega_{\text{max}}) \gamma D \sqrt{k_c m^* - \left( \frac{m^* \gamma D}{2} \right)^2} \tag{8.8}
\]

is a combination of interaction forces: interatomic potential, van der Waals potential, and electrostatic potential. The force on the tip (assumed spherical) can be modeled as an electrostatic boundary-value problem using the method of images [8]

\[
F_0 = -\frac{dU_{sh}}{dz_s} - \frac{dU_{vdW}}{dz_s} - \frac{dU_{el}}{dz_s} \tag{8.9}
\]

where \( U_{sh} \) is the shell-model energy, \( U_{vdW} \) is the van der Waals interaction energy, \( U_{el} \) is the energy due to electrostatic potentials, and \( z_s \) is the distance from the surface to the probe-center. An energy minimum and distance is assumed such that \( \frac{dU_{sh}}{dz_s} = 0 \) and \( \frac{dU_{vdW}}{dz_s} = 0 \). Thus, the force due to a single
charge is related by
\[ F_{ia} = -q_i \frac{\partial \phi_{ind}(\vec{r}_i)}{\partial r_{ia}} \quad (8.10) \]

where \( q_i \) is the induced charge, \( \phi_{ind}(\vec{r}) \) is the image potential at \( r \), the distance from the center of the probe. The electric field inducing the charge is
\[ E = \sum_i \frac{\partial \phi_{ind}(\vec{r}_i)}{\partial r_{ia}} \quad (8.11) \]

The force measured by AFM is related by (where \( Q = \sum q_i \))
\[ F_0 = -QE \quad (8.12) \]

Thus the average surface charge density for an area \( S \) is \( \langle \sigma \rangle = 1/\sqrt{SF_0/E} \)

Others have also described a method of obtaining the relative surface charge density which can be calculated from the difference of tip-sample separations [9]. With well-characterized spherical AFM probes (Figure 8.6) and assuming the sample surface is a flat plane, the force of interaction, \( F \), can be described as a function of the separation distance, \( D \),
\[ F(D) = \frac{4\pi r_{probe} \lambda \sigma_{probe} \sigma_{sample}}{\varepsilon} \exp(-D/\lambda) \quad (8.13) \]

where \( r_{probe} \) is the probe radius, \( \sigma_{probe} \) is the surface charge density of the probe, \( \sigma_{sample} \) is the surface charge density of the sample, \( \varepsilon \) is the dielectric of the medium, and \( \lambda \) is the Debye length. A maximum force, \( F_{tr} \) is set such that the following condition can be imposed for force curves collected at distances \( D_1 \) and \( D_2 \)
\[ F_1 = F_2 = F_{tr} \quad (8.14) \]

This can be written as
\[ \frac{4\pi r_{probe} \lambda \sigma_{probe} \sigma_1}{\varepsilon} \exp(-D_1/\lambda) = \frac{4\pi r_{probe} \lambda \sigma_{probe} \sigma_2}{\varepsilon} \exp(-D_2/\lambda) \quad (8.15) \]
which leaves the final surface charge ratio as

$$\frac{\sigma_1}{\sigma_2} = \exp(-\frac{D_2 - D_1}{\lambda})$$  \hspace{1cm} (8.16)

### 8.2.2 Adhesion Force Spectroscopy

Adhesive contact force can be measured with AFM using the retraction force curve. The Johnson-Kendall-Roberts model of elastic contact [10] is used for analysis. With this model, the force of adhesion $F_A$ can be used to determine the surface energy density $2\gamma = W/A$ between two surfaces [11]. The geometry shown in Figure 4.2 gives a displacement

$$u_z = d - \frac{r^2}{2R}$$  \hspace{1cm} (8.17)

Modeled as contact between two spheres (one rigid and one elastic), we have

$$\frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2}$$  \hspace{1cm} (8.18)

The general solution for pressure distribution in this model is

$$p(r) = p_0 \left(1 - \frac{r^2}{a}\right)^{1/2} + p'_0 \left(1 - \frac{r^2}{a}\right)^{-1/2}$$  \hspace{1cm} (8.19)

The displacement for this model is then

$$u_z = \pi a E^* \left[p_0 + \frac{1}{2} p'_0 \left(1 - \frac{r^2}{2a^2}\right)\right]$$  \hspace{1cm} (8.20)

As before, we have the relation in which we can solve for the parameters $a$, $p_0$, and $p'_0$

$$\pi a E^* \left[p_0 + \frac{1}{2} p'_0 \left(1 - \frac{r^2}{2a^2}\right)\right] = d - \frac{r^2}{2R}$$  \hspace{1cm} (8.21)
The maximum pressure is then

\begin{equation}
\frac{p_0}{\pi R} = \frac{2aE^*}{\pi R} \quad (8.22)
\end{equation}

\begin{equation}
\frac{p'_0}{\pi a} = -\left(\frac{4\gamma E^*}{\pi a}\right)^{1/2} \quad (8.23)
\end{equation}

where \(2\gamma\) is the total surface energy of both surfaces per unit area, and \(1/E^* = \frac{1-\nu_1^2}{E_1} + \frac{1-\nu_2^2}{E_2}\).

To obtain the relation between surface energy density and force, first start with the potential energy in an elastic deformation

\begin{equation}
U_{el} = \frac{1}{2} \int p(x)u_z(x)dx dy \quad (8.24)
\end{equation}

Applied to this system

\begin{equation}
U_{el} = \frac{1}{2} \int_0^a \left[ p_0 \left( 1 - \frac{r^2}{a} \right)^{1/2} + p'_0 \left( 1 - \frac{r^2}{a} \right)^{-1/2} \right] \left[ d - \frac{r^2}{2R} \right] \pi rdr \quad (8.25)
\end{equation}

With substitution and simplification, this integral evaluates to

\begin{equation}
U_{el} = E^* \left( d^2 a - \frac{2}{3} \frac{da^3}{R} + \frac{a^5}{5R^2} \right) \quad (8.26)
\end{equation}

and the total energy for the system is

\begin{equation}
U_{total} = E^* \left( d^2 a - \frac{2}{3} \frac{da^3}{R} + \frac{a^5}{5R^2} \right) - \gamma \pi a^2 \quad (8.27)
\end{equation}

The contact radius at equilibrium can be calculated under the constraint that the energy assumes a minimum value from

\begin{equation}
\frac{\partial U_{total}}{\partial a} = E^* \left( d - \frac{a^2}{2R} \right)^2 - 2\gamma \pi a = 0 \quad (8.28)
\end{equation}

The total energy as a function of the contact radius using \(d = \frac{a^2}{R} \pm \sqrt{\frac{2\gamma \pi a}{E^*}}\) becomes

\begin{equation}
U_{total} = E^* \left[ \frac{8}{15} \frac{a^5}{R^2} + \frac{\gamma \pi a^2}{E^*} \pm \frac{4}{3} \frac{a^3}{R} \sqrt{\frac{2\gamma \pi a}{E^*}} \right] \quad (8.29)
\end{equation}
The force acting is a derivative of the energy with respect to indentation is

\[
F = -\frac{dU_{\text{total}}}{d(d)} = \frac{\partial U_{\text{total}}}{\partial (d)} - \frac{\partial U_{\text{total}}}{\partial a} \frac{da}{d(d)}
\]  
\(8.30\)

Since we assume a contact radius a corresponding to an energy minimum \(\frac{\partial U_{\text{total}}}{\partial a} = 0\)

\[
F = E^* \left[ 2da - \frac{2a^3}{3R} \right]
\]  
\(8.31\)

and force as a function of contact radius is

\[
F = E^* \left[ \frac{4}{3} a^3 - \frac{8\gamma\pi a^3}{E^*} \right]
\]  
\(8.32\)

Thus, the maximum negative force is obtained at the boundary

\[
a = \left( \frac{9}{8} \frac{\gamma\pi R^2}{E^*} \right)^{1/3}
\]  
\(8.33\)

for an adhesive force (Figure 8.2)

\[
F_A = -\frac{3}{2} \gamma\pi R
\]  
\(8.34\)
8.3 Preliminary Experimental Results

8.3.1 Materials and Methods

pNIPAAm hydrogels were synthesized as described in chapter 3. AFM methods were used as described in chapter 4. Laser scanning confocal microscopy (LSCM) was used to capture x-y-z image stacks for 3D rendering of the microbeams. Images were taken with a Leica TCS SP5 confocal laser scanning microscope equipped with 20X/0.7NA and 40X/1.25NA objectives (Leica Microsystems, Germany). An argon laser line, tuned to 543 nm, was applied to excite fluorescent microbeams and an Acousto Optical Beam Splitter was used to filter the emission. Image sections were taken at a constant z-spacing of 0.25 \( \mu \text{m} \) and were captured with photomultiplier detectors using the Leica Application Suite Advanced Fluorescence software version 2.1.0 (Leica Microsystems, Germany).

8.3.2 Surface Characterization

To test the adhesion strength of fibronectin (FN) on PNIPAAm hydrogels, surfaces and probe were adsorbed with the protein in deionized water. Atomic force spectroscopy with unmodified probes (no microsphere) was used to measure the pull-off force, \( F_A \), from the sample. Each measurement consisted of \( N = 256 \) individual scans over a \( 20 \times 20 \mu \text{m} \) area. A systematic series of measurements were conducted under conditions where FN was adsorbed only to the sample, only to the probe, to both the probe and sample, or to neither. This series was duplicated for an untreated probe and a probe treated with plasma to induce a negative charge.

In the untreated sample group, the average pull-off force was highest when FN was adsorbed to PNIPAAm (\( F_A = 17.1nN \)), the probe (\( F_A = 17.2nN \)), or both (\( F_A = 18.4nN \)) as shown in Figure 8.3. A small pull-off force (\( F_A = 0.897nN \)) was observed when no FN was adsorbed.

By contrast, adhesive forces appeared to substantially decrease for probes with induced negative charge (Figure 8.4). Notably, FN still exhibited a strong self-affinity (\( F_A = 16.2nN \)) consistent with the aggregation and fibrillation process [12]. When only adsorbed to the probe or the sample, the pull-off force was minimal, \( F_A = 1.90nN \) and \( F_A = 3.10nN \), respectively. When no FN was
Figure 8.3. Pull-off force measured on samples with adsorbed FN using an untreated probe. FN was either not adsorbed, adsorbed only to the hydrogel sample, both the sample and the AFM probe, or the probe only. Each bar represents a $20 \times 20 \mu m^2$ force map with $N = 256$ force curves. Samples were collected in DI water at 296K.
FN was either not adsorbed, adsorbed only to the hydrogel sample, both the sample and the AFM probe, or the probe only. Each bar represents a $20 \times 20 \mu m^2$ force map with $N = 256$ force curves. Samples were collected in DI water at 296$\degree$K.

adsorbed, the pull-off force was about the same as the untreated probe $F_A = 0.798nN$.

The decrease in surface energy shown for the probes with induced negative charge relative to those that were untreated reflects what others studies have found [13, 14]. Regardless of whether the tips were treated or untreated, both measurement sets showed an increase in adhesion with the addition of FN to either the sample or the probe. This result is explained by the both the increase in surface charge relative to the surface and the additional interaction energy from the adsorbed protein.

This work demonstrates surface charge mediates FN interaction with PNIPAAm hydrogels. Whether it merely increases overall serum protein adsorption or mediates a balance between serum components remains an area of active investigation. This is a crucial distinction because the bal-
Figure 8.5. 3D reconstruction of PNIPAM micro-patterned structure. Rhodamine is embedded in the polymer network and the PLL is adsorbed on the surface. Emission from fluorophores of rhodamine are shown in red and of FITC-labeled PLL, in green. Left, FITC-PLL adsorbed with temperature maintained at 37°C. Right, FITC-PLL adsorbed at room temperature, 25°C. The structures are 100 µm in width. Field of view is 240 x 240 x 55 µm³.

8.3.3 Surface Modification of Micro-patterned PNIPAM Hydrogels

In 8.5, PLL infiltrates the PNIPAAm hydrogel when adsorbed at a temperature below the VPT but only to the surface when adsorption is conducted above the VPT. This finding suggests that PNIPAAm hydrogels are porous in the water-rich phase. A model peptide, FITC-PLL was shown to infiltrate when adsorbed at room temperature and localize on the surface when temperature was maintained above the VPT. Adsorption of PLL was empirically determined to increase cell attachment on the micro-patterned structures. From this data, it is hypothesized that PNIPAAm hydrogels is porous below the LCST such that PLL is adsorbed throughout the gel. Upon returning to the polymer-rich phase by increased temperature, serum protein attachment is mediated by changes in the surface charge.

8.4 Proposed Research

The goal of this project will be to investigate the following:

- Temperature dependence of protein interactions with PNIPAAm hydrogels
- Time dependence of protein interactions with PNIPAAm hydrogels
• Topography dependence of protein adsorption on PNIPAAm hydrogels

• Surface charge dependence of protein adsorption on PNIPAAm hydrogels

8.4.1 Methods

The adhesive strength of serum proteins, fibronectin (FN), vitronectin (VN), and albumin (BSA) will be determined by measuring the pull-off force using atomic force microscopy (AFM). These proteins will be attached to an AFM probe using a method described by Oberhauser [16] or adsorbed onto PNIPAAm hydrogels. AFM probes will be treated with HDMS, plasma treated, or untreated. Measurements will be conducted in solution at several temperature points below and above the VPT.

The adhesion force will be measured for PLL+ and PLL- conditions with serum-containing and serum-free cell culture medium. The relative distribution of the serum proteins will be determined using fluorescent labeled antibodies to each protein and quantifying with laser scanning confocal microscopy (photo multiplier tube). Samples will also be segregated by a time course of 15 m, 1 h, 4 h, and 24 h.

The surface charge density of the hydrogels will be measured with AFM using a gold-palladium coated polystyrene microsphere attached to a tipless cantilever as shown in Figure 8.6. Standards with high surface charge density (silicon dioxide) and low surface charge density (silicon nitride) will be utilized to determine the relationship between force and surface charge density. The nominal charge density will be measured in air. Charge density will be measured in DMEM without serum proteins below and above the VPT using methods described by Heinz [9].

8.4.2 Expected Outcome

Adsorption of fibronectin, vitronectin, and bovine serum albumin will be compared for poly-L-lysine/no poly-L-lysine and serum-containing/serum-free medium. Quantifiable differences in adhesion are expected for these conditions. A model of the adhesion forces and binding energies
Figure 8.6. Assortment of various AFM probes modified with 90 μm polystyrene spheres. (Left) Low spring constant cantilevers (0.05-0.20 N/m) and (right) high spring constant cantilevers (4.5-14.5 N/m).

with which to interpret preliminary data at a molecular level will be developed. It is also expected that protein interactions will vary with adsorption time and temperature. Finally, this investigation is expected to decouple the effect of surface charge and topography from the preceding variables of time and temperature.
8.5 References


CHAPTER 9
CONCLUSION

This dissertation project, a biophysical characterization of PNIPAAm hydrogels, has tested three hypotheses:

- Mechanical properties of PNIPAAm hydrogels are quantitatively predicted based on crosslinker ratio in the water-rich phase.

- Release of cells from micro-patterned PNIPAAm hydrogels occurs when the lateral strain in the surface exceeds $\epsilon > 0.25$.

- The molecular mechanism of rapid cell release from micro-patterned PNIPAAm hydrogels is mediated by the transmembrane protein integrin and its extracellular matrix receptor, fibronectin.

In chapter 6, an empirical basis was established to quantitatively apply FRT to PNIPAAm hydrogels. The polymer-rich phase and water-rich phase display significant differences both in experimental results and theoretical treatment. A piece-wise application of the Flory-Rehner theoretical framework demonstrated quantitative predictions using the empirically-derived parameters.

In chapter 5, cells were demonstrated to rapidly detached from a PNIPAAm hydrogel surface when the lateral strain $\epsilon > 0.25$. The molecular mechanism, breaking of the integrin-fibronectin bond, was determined by systematically investigating biological functions of the cell and their relation to the strain needed to release cells.

The primary methods and analysis utilized in this project include, as covered in chapters 3 and 4:

- Atomic force microscopy

- Scanning electron microscopy

- Phase-contrast microscopy
Mass-balance analysis

Results from this work can be used to determine mechanical properties of crosslinked PNI-PAAm hydrogels for use in biomedical applications. Through additional research, they could also be used in a manufacturing setting as outlined in chapter 8.

Finally, this project contributes both to basic and applied science. It advances theoretical treatment of thermoresponsive biomaterials with quantitative relations of molecular thermodynamic properties to macroscopic bulk measurements and enhances understanding of the cell-substrate adhesion process required for the development of new biomaterials that will be useful for drug-testing platforms, regenerative medicine, and ultimately, bioengineered tissue autografts.
Appendix A List of Publications

Journal Articles

A.1 References


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