Multi-scaled adhesion mechanics of hydrogel contact lenses

A Dissertation

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ABSTRACT

The mechanical and adhesion properties of hydrogel contact lenses play a role in a variety of manufacturing processes, lens handling, user comfort, and lens optical performance. Additionally, the hydrogel mechanical properties, adhesion properties, or both are hypothesized to be factors in a variety of lens-related ocular complications. Thus, there is a current need for accurate and reliable mechanical and adhesion characterization techniques for these biomedical devices. This thesis establishes novel experimental protocols and methods to accurately characterize hydrogel contact lens adhesion and mechanical properties in a variety of contexts and length scales. Throughout all aims/experiments, two commercially available hydrogel lenses are characterized for comparison, Narafilcon A/Acuvue TruEye and Etafilcon A/Acuvue2.

The first aim of this thesis focuses on macroscopic hydrogel lens adhesion characterization achieved via the Planar Adhesion Test (PAT). The PAT is a novel experimental technique that utilizes the lens native geometry to characterize both mechanical and adhesion properties. The lens is compressed and subsequently removed from a planar substrate, during which the applied load $P$, the maximum tensile force or “pull-off” force $P^*$, approach distance $w_0$, and contact radius $a$ are measured. A previously established modified Johnson-Kendall-Roberts (JKR) shell model is employed to calculate the adhesion energy, $\gamma$, between the hydrogel and the substrate, and it is found that $\gamma = 80 \pm 4.6 \text{ mJ/m}^2$ for Acuvue2 lenses and $\gamma = 95 \pm 6.1 \text{ mJ/m}^2$ for TruEye lenses. The effect of lens geometry, or optical diopter $d$, is explored on both the lens mechanical and adhesion behavior.

The second aim of this thesis focuses on characterizing hydrogel lens adhesion against more physiologically relevant samples; donated human corneas. Similar to the PAT, the lens is brought in contact with the cornea and retracted away, recording the “pull-off” force $P^*$ for each
hydrogel material. The “pull-off” force is directly related to the hydrogel-corneal surface adhesion properties, and is found that $P^* = 2.40 \pm 0.20$ mN for Acuvue2 lenses and $P^* = 2.78 \pm 0.19$ mN for TruEye lenses on human corneas.

The third and final aim of this thesis focuses on characterizing single cell adhesion behavior against the two hydrogel materials via Single Cell Force Spectroscopy (SCFS). Individual Human Corneal Epithelial (HCE) cells are immobilized on a specially functionalized Atomic Force Microscope (AFM) cantilever to form a single cell probe. The cell is brought into contact with the hydrogel surface, and after a specified time $\Delta t$, retracted away from the hydrogel. Individual “pull-off” forces $P^*$ are recorded as a function of time in contact to measure the bond-strengthening time, $\tau$, and together $P^*$ and $\tau$ characterize the adhesion properties of these hydrogels at the cellular level. It is found that the $P^*$ forces for HCE cells are significantly larger for Narafilcon A than the $P^*$ forces for Etafilcon A for all hold times $\Delta t \geq 5s$, and that the bond-strengthening time for Etafilcon A is slightly less than Narafilcon A.

Additionally, this work contains an investigation into the mechanochemistry of native type I collagen via small angle light scattering (SALS), which is reviewed as an addendum at the end of this thesis.
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NOMENCLATURE

\( P = \) total load
\( w_0 = \) approach distance/compression depth/indentation depth
\( a = \) contact radius
\( r = \) radial distance from center/apex of lens/shell
\( h, h(r), h(\phi) = \) thickness of lens/shell, non-uniform thickness profile of lens/shell
\( h_0 = \) central thickness value of the shell/lens
\( R = \) radius of undeformed shell/base curvature of contact lens
\( E = \) Young’s modulus
\( \nu = \) Poisson’s ratio
\( \phi_0 = \) angle between axis normal and axis of symmetry of undeformed shell
\( \alpha = \) value of \( \phi_0 \) at the edge of contact region
\( \beta = \) rotation of normal
\( r_0 = \) distance from axis of symmetry before deformation
\( K = E h / (1 - \nu^2), \) in-plane rigidity
\( D = E h^3 / 12(1 - \nu^2), \) bending rigidity
\( w, u = \) deflection parallel and perpendicular to axis of symmetry
\( V, H = \) stress resultants parallel and perpendicular to axis of symmetry
\( P_V, P_H = \) surface loads parallel and perpendicular to axis of symmetry
\( N_\theta, N_0 = \) meridional and circumferential stress resultants
\( M_\phi, M_0 = \) meridional and circumferential moment resultant
\( Q = \) transverse shear resultants
\( \tau = \) bond-strengthening time
\( P^* = \) “pull-off” force
\( a^* = \) “pull-off” contact radius
\( d = \) optical power/diopter of lens
\( \Delta t = \) hold time
\( \gamma = \) adhesion energy
\( k = \) AFM cantilever nominal spring constant
\( \eta = \) AFM cantilever deflection sensitivity
CHAPTER 1: INTRODUCTION

This thesis focuses on hydrogel lens mechanical and adhesion properties. Specifically it produces novel methods and new metrics to characterize lens adhesion and mechanical properties, and investigates multi scaled in vitro hydrogel lens adhesion. Due to the breadth and scope of this thesis, the introduction is split into several sections. First, a brief introduction of hydrogel contact lenses and some of the ocular complications associated with contact lens use are reviewed in Sections 1.1 and 1.2. A review of the corneal epithelium structure and physiology is given in Section 1.3 to provide context to understand hydrogel contact lens interactions with the ocular surface in vivo. Previous techniques to characterize hydrogel lens adhesion and mechanical properties are reviewed in Section 1.4., followed by the problem statement, research objectives, and significance of this body of work.

1.1 Hydrogel Contact Lens Introduction

Contact lenses are safe and effective biomedical devices that are typically utilized as a vision correction alternative to prescription glasses. Soft hydrogel contact lenses were first developed in the early 1970s and are composed of a water swollen network of crosslinked polymer chains. Soft lenses are currently worn by over 140 million people worldwide [1]. Approximately 35 million of those users reside in the United States, contributing to a $6.1 billion global market that is projected to grow to approximately $12.2 billion by 2018 [2]. There are various hydrogel contact lenses available on the market, which differ not only by polymers used to form the hydrogel but also differ by duration of use. One of the most important advances in the contact lens industry was the creation of silicone hydrogel contact lenses, which currently hold a large share of the market, and is projected to further increase its market share [3, 4].
Silicone hydrogel lenses offer higher degrees of oxygen permeability, which greatly reduces issues of lens-induced hypoxia and ultimately enhances user comfort [5].

**1.2 Hydrogel Contact Lens Related Ocular Complications**

Despite significant advances in silicone hydrogel lens technologies, there are still numerous ocular complications associated with and/or induced by soft contact lens wear, some of which are reviewed in the following subsections.

**Microbial keratitis (MK):** Bacterial keratitis, also known as corneal ulcers, is a sight-threatening contact lens complication [6]. MK is a major risk factor among lens users [7, 8], and it is estimated that over 30,000 lens users suffer from MK in the United States [9]. The process of any contact lens related keratitis starts with the adhesion of bacterium to the contact lens surface, and then subsequent bacterium adhesion to the ocular surface. Despite the role of bacterial adhesion to contact lenses as the primary cause for MK, the hydrogel material properties that are critical to determining this adhesion are still not well understood [10].

![Figure 1: Contact lens-induced MK adapted from [8, 11].](image)
Contact lens acute red eye (CLARE): The same micro-organisms which are typically responsible for MK can result in an acute inflammatory response, CLARE [12]. It has been reported that up to 12% of extended wear lens users awaken with foreign-body discomfort accompanied by a redness of the ocular surface [13]. Similar to MK, bacterial adhesion to the contact lens plays a crucial role in the onset of CLARE, yet how the hydrogel lens material properties effect CLARE is still not well understood [10].

Superior epithelial arcuate lesions (SEALs): SEALs are corneal events associated with soft contact lens wear that involves lesions occurring in the area covered by the upper eyelid, usually within 2 to 3 mm of the superior limbus forming an arc between the 10-and 2-o’clock positions [12, 14]. The etiology of SEALs is likely multifactorial and currently unknown, but it is hypothesized that the lens mechanical properties play a role in the formation of SEALs [14].

![Figure 2: Sodium fluorescein staining of a typical superior epithelial arcuate lesion seen with a soft hydrogel contact lens adapted from [14].](image)

Contact lens associated papillary conjunctivitis (CLPC): CLPC is a common reason for discontinuation of contact lens wear, and symptoms typically include redness/enlargement of the palperbral conjunctiva, discomfort, itching, foreign body sensation, excessive lens movement
and blurred vision [15, 16]. The etiology of CLPC is currently poorly understood, however it is likely multifactorial in nature, and it has been hypothesized that this inflammatory condition may be mechanically mediated [12, 17].

Epithelial thinning: Extensive use of contact lens can lead to thinning of the central corneal epithelium, typically characterized by loss of superficial cells of the epithelium and flattening of the remaining wing epithelial cells [18]. The corneal epithelium is crucial for maintaining corneal health and its degradation can introduce the risk of sight-threatening sequelae. The exact mechanisms causing epithelial thinning are unknown, however it is accepted that hypoxia is a significant factor in contact lens induced epithelial thinning [19]. Evidence suggests that hydrogel mechanical properties might factor in the onset of epithelial thinning [20], as stiffer lenses are associated with a higher degree of epithelial thinning [19], but whether this is purely an oxygen diffusion problem or coupled diffusion-mechanical problem has yet to be addressed.

Figure 3: General contact lens induced papillary conjunctivitis adapted from [15].

Corneal erosions: A corneal erosion is a full thickness detachment of the epithelium in a localized region of the cornea that is associated with contact lens wear [21]. The size of these erosions may range from ~100μm in diameter to macro-scale erosions, shown in Figure 4. The
mechanisms that results in the adherent epithelium to be overwhelmed by the action of the lens to produce corneal erosions are still not understood [22], though the incidence of corneal erosions is relatively low [23]. It is hypothesized that contact lens adhesion to the cornea as a possible etiology for formation of erosions [22]. During long durations of relatively little movement (i.e. overnight wear), it is possible an attachment forms between the lens and a section of the epithelium. Once the lens moves, this attachment creates a mechanically induced corneal erosion by pulling a section of epithelium away [12], initiating the erosion process. Like most contact lens related ocular complications, the onset of corneal erosions could be multifactorial.

With proper adherence to the lens manufacturer’s instructions of handling and duration, the incidence rates of these contact-lens related ocular complications are low [12, 24-26]. However, the sheer number of contact lens users justifies these lens related ocular complications as a serious health concern. To date, studies which investigate the onset of these ocular complications have been clinical in nature, in which groups of users are given contact lenses with certain material properties/durations, and are scanned and diagnosed after a certain period of time [27, 28]. These studies provide invaluable statistical data that draw correlations between
lens properties and patient conditions, but they ultimately do not address the fundamental mechanisms that cause these lens related complications. It has been hypothesized that the contact lens mechanical properties play a role in SEALs [14], CLPC [27], and corneal erosions [18, 19, 22, 29]. Similarly, contact lens adhesion properties are believed to play a role in MK [10, 26, 30] and corneal erosions [22]. However, the underlying mechanisms behind these ocular complications are poorly understood [22], making it difficult to prescribe preventative measures and reduce incidence rates among contact lens users. There are a plethora of mechanisms that could and likely do contribute to the onset of these complications, such as oxygen diffusion to the ocular surface [5], and it is likely that the etiology is the result of several factors rather than a single mechanism [5, 12, 17, 22]. To elucidate the role of hydrogel contact lens mechanical and adhesion properties in the etiology of ocular complications, first it is necessary to establish experimental methods and protocols to reliably characterize the lens adhesion and mechanical properties to probe *in vivo* behavior.

### 1.3 Corneal Epithelium Structure and Physiology

To characterize hydrogel lens mechanical and adhesion properties in the context of potentially elucidating lens-related complications, it is first necessary to understand the physiology of the ocular surface. The corneal epithelium covers the front of the cornea, and hence is the tissue of the ocular surface that hydrogel contact lens directly interacts with *in vivo*. The epithelium is comprised of five to six cell layers with a total thickness of approximately 50µm. The outermost/anterior layer is covered with a tear film, which is essential to smooth out optical irregularities of the anterior epithelial surface amongst many other functions [31]. The morphological and physiological features of the epithelial cells change drastically depending upon their location in the epithelium, as shown in Figure 5. The superficial cell layer is
approximately two to three cells thick and takes on a flat polygonal morphology. These cells express microvilli and maintain tight junctional complex between their neighbors, which form a tight barrier to help prevent toxins and microbes from entering the deeper into the cornea [31]. In the middle of the epithelium is comprised of two to three layers of wing cells that are less flat than the superficial cells but have similar tight junctions [31-33]. The bottom layer of the corneal epithelium is the basal layer, which is comprised of a single cell layer of cuboidal cells approximately 20µm thick. Corneal epithelial cells have an average lifespan of seven to ten days, and routinely undergo orderly apoptosis (programmed cell death), desquamation via interaction with the eyelid during blinking, and involution [34]. This leads to a complete turnover of the corneal epithelial layer in about a week as deeper cells replace the desquamating superficial cells in an routine fashion [31]. Thus, the ocular surface exhibits dynamic behavior and is ultimately extremely complex.

![Diagram of a cross-sectional view of the corneal epithelium](image)

**Figure 5:** Diagram of a cross-sectional view of the corneal epithelium, adapted from [35]. Note the distinct morphological features of epithelial cells in different depths of the epithelium, with the most superficial (outermost) cells expressing apical microvilli and a glycocalyx layer.
1.4 Previous Hydrogel Lens Mechanical and Adhesion Characterization

Techniques

Hydrogel lens mechanical and adhesion properties have been characterized by a variety of methods by both academic and industrial researchers. This subsection briefly reviews current standard techniques and discusses their limitations.

1.4.1 Hydrogel Lens Mechanical Characterization

Hydrogels are comprised of a network of polymer chains that are crosslinked to form a complex, swollen network. The mechanical characterization of hydrogel contact lenses has long been of interest to contact lens manufacturers due to the fact that the mechanical properties greatly affect the optical performance of the lens, lens lifetime, fitting onto the cornea, and lens handling. The stiffness of the lens is also related to the comfort and “foreign body” sensation felt by the user [17, 36]. Furthermore, the mechanical properties of hydrogel contact lenses are hypothesized to be involved in the onset of lens related ocular complications, such as mechanical trauma involved in CLPC and corneal erosions [17]. There are several standard experimental methods that have been implemented by the hydrogel lens scientific community for mechanical characterization.

Tensile Testing

The most widespread is conventional tensile-testing to calculate the hydrogel lens elastic modulus, $E$ [36]. In these conventional engineering tension tests, a stress-strain curve is constructed from load-elongation measurements made by commercially available units, such as an Instron tension tester. The lens is typically cut into a rectangular strip and clamped at each end
with enough slack such that there is no tension in the hydrogel strip. The engineering stress, \( \sigma \), is calculated by dividing the load on the lens, \( P \), by the original cross-sectional area of the lens strip, \( A_0 \):

\[
\sigma = \frac{P}{A_0}
\]

(1)

The strain, \( \varepsilon \), is given by the change in gauge length \( \Delta L \) divided by the initial length \( L_0 \):

\[
\varepsilon = \frac{\Delta L}{L_0} = \frac{L-L_0}{L_0}
\]

(2)

where \( L \) is the instantaneous length. Together, the load-extension curve is transformed into a stress-strain curve shown below:

![Figure 6: Typical stress-strain curve, adapted from [36].](image)

The elastic modulus is simply defined as the slope of the initial, linear section of the stress-strain curve:

\[
E = \frac{\sigma}{\varepsilon}
\]

(3)

Typically, the lens is kept hydrated by manual application of contact lens solution throughout the test to minimize the effects of dehydration on the hydrogel lens mechanical characterization.
However, tensile testing of hydrogel lenses has several disadvantages. First, it is very easy to introduce small flaws and tears into the sample when cutting the rectangular strip, which complicates and potentially compromises the data obtained [36]. Second, the intrinsic curvature of the contact lens is ignored when assuming a rectangular geometry. Third, contact lenses have non-uniform thickness profiles that form a lens to correct for the optical imperfections of the user’s cornea, creating a varied thickness across the long-axis of the rectangular strip. Most tensile data analysis does not account for this varied thickness when calculating the stress acting on the lens. Fourth, there is concern of introducing residual stress by clamping the soft hydrogel strips. Regardless of these experimental difficulties associated with conducting tensile testing of hydrogel lenses, there is the question of the relevancy of tensile test characterization in general. It is well known that the mechanical properties of hydrogels are highly dependent on the environmental conditions in which the test was conducted. With this in mind, it is imperative that hydrogel mechanical properties are determined and measured under conditions that are as close to the in situ conditions as possible [37]. Thus, there is concern of the validity, and more importantly, applicability of a lens tensile modulus for a lens that undergoes little to no tension during normal use on-eye or during handling.

**Nano/Microindentation**

The popular alternative mechanical characterization technique to tensile testing is nano or microindentation. Indentation has gained popularity for testing biomaterials, and in particular hydrogel contact lenses due to its non-destructive method and relatively small sample size required for characterization. The hydrogel lens is placed either on a flat substrate or on a custom spherical mold that matches the base radius of curvature of the lens. A custom built or
commercial available load cell either applies a load, \( P \), or displacement, \( w_0 \), via a variety of probes compressing the sample until a maximum load/displacement is reached, at which point the probe retracts from the sample. After the test, the force-displacement data (\( P-w_0 \) curves) are used to mechanically characterize the hydrogel lens. Though seemingly simple, there are a number of experimental difficulties associated with indentation of hydrated, highly compliant materials. Nano or microindentation tests on biomaterials typically utilize a spherical-tipped probe to avoid stress concentrations that can develop when using flat-ended probes [38], and pyramid/conical probes are prone to damaging the soft hydrogel. The lens is typically submerged in solution during indentation, as the hydrogel mechanical properties are greatly affected by dehydration [39]. Most hydrogel contact lenses are extremely compliant and thin, ranging from 80-120\( \mu \)m thickness with indentation moduli as low as \( \sim 50\text{kPa} \) [39]. Due to hydrogels extremely compliant nature, the coupled mechanical response of the hydrogel sample in addition to the substrate must be considered. This “substrate effect” becomes more and more pronounced the thinner the sample is or the higher the indentation depth is. Typically it is accepted that as long as indentation depths are less than 10% of the sample thickness, the mechanical response is simply that of the hydrogel sample, and the substrate contributions can be neglected [40]. Furthermore, major sources of difficulties are indenter size effects [41], issues of thermal drift [42], the presence of adhesion [43, 44], accurate surface detection [45], viscoelastic creep [40, 42], and choosing the appropriate mechanical model when analyzing the indentation data [46-48]. There are many different models that can be used to interpret nano/microindentation data for mechanical characterization. The most widely used model being Hertz contact mechanics [49], which models a sphere of radius \( R \) indenting an elastic half-space. The Hertz model gives the
stress distribution in the contact area, as well as the interrelationship between the measureable quantities such as depth \( w_0 \), contact radius \( a \), and applied force \( P \) given by:

\[
P = \frac{4}{3} E^* R^{1/2} w_0^{3/2}
\]

\[
a^3 = \frac{3PR}{4E^*}
\]

\[
w_0 = \frac{a}{R} = \left( \frac{9P^2}{16E^*^2R} \right)^{1/3}
\]

Where \( E^* \) is the reduced modulus:

\[
\frac{1}{E^*} = \frac{1-v_1^2}{E_1} + \frac{1-v_2^2}{E_2}
\]

The subscripts 1 and 2 denote either the sphere or elastic half-space, respectively. \( E \) is the elastic modulus and \( v \) is the Poisson’s ratio. In the case of indentation of compliant substrates, the rigid spherical indenter is often many orders of magnitude stiffer than the substrate, in which case the reduced modulus takes a simpler form:

\[
E^* = \frac{E_1}{1-v_1^2}
\]

![Figure 7: Hertz contact mechanics for a sphere indenting an elastic half-space.](image)

The Hertz model assumes linear elasticity, even though hydrogel lenses are known to have time-dependent viscoelastic and poroelastic behavior [39, 50, 51]. In recent years,
significant efforts have been made to account for and characterize this complex behavior typically observed in hydrogels [52-54]. However, regardless of the model utilized to analyze and interpret indentation data, it is an inherent limitation of this testing methodology that the results only reflect extremely localized material mechanical properties. It is unclear if the localized mechanical characterization is relevant to typically more macroscopic, global behaviors such as lens manufacturing, user comfort, optical performance, and potentially lens induced ocular complications.

The two widely utilized mechanical characterization techniques, and ultimately the metrics they derive, may or may not be suitable for the task of characterizing mechanical behavior relevant to the on-eye behavior of hydrogel contact lenses. A crux of this body of work is the development of \textit{in situ} mechanical characterization techniques more relevant to these macroscopic lens behaviors.

1.4.2 Hydrogel Lens Adhesion Characterization

The adhesion properties of hydrogel contact lenses are pertinent to many manufacturing processes as well as on-eye behavior. One such manufacturing process is cast molding, in which the cured polymer must be removed from its adhered mold. Another is simply lens handling and storage. Furthermore, the lens adhesion properties are believed to be factors contact lens related ocular complications, such as MK [10] and corneal erosions [12, 22]. The adhesion properties of hydrogel contact lenses have been characterized by a variety of techniques over the years, and are summarized in the following subsections.

Bacterial Adhesion Assays
Susceptibility of hydrogel contact lenses to bacteria attachment and colonization has long been a major concern for lens manufacturers, researchers and practitioners alike. Indeed, the adhesion and subsequent colonization of bacteria onto hydrogel contact lenses is believed to be a vital step in MK [55], in which users place lenses in storage units over night and insert them for a prolonged period of time. Large scale microbial assays are widely used to investigate hydrogel contact lens adhesion properties in the context of bacterial attachment and colonization. Generally, these tests involve various hydrogel materials that are incubated with a specific strain of bacteria (i.e. *Pseudomonas aeruginosa*) in a specific culture medium for a specific time. Afterwards, the lenses are either removed from the medium or a wash is applied to remove unattached bacteria. Next, the cells are quantified by a variety of cell counting methods [56-59], such as Scanning Electron Microscopy (SEM) shown in Figure 8 [60]. The number of cells serves as a metric to gauge that specific hydrogel-bacteria adhesion. Currently, there is no standardized *in vitro* assay to test bacterial adhesion on contact lenses [61], leading to adhesion data differing significantly between various studies and groups of researchers [10]. Furthermore, these large scale microbial adhesion assays only provide an indirect measurement of adhesion; no actual adhesion forces or time dependence of bond-strengthening of individual bacteria and hydrogel surfaces are measured. This makes it extremely difficult to draw conclusions about specific mechanisms behind bacteria adhesion to hydrogel lenses, how their adhesion progresses and matures, and most importantly it does not give researchers and engineers strategies to avoid and reduce bacterial adhesion. Bacterial adhesion to hydrogel contact lenses is a complex and multifunctional process that not only depends on the adhesion properties of the particular type of bacteria cells in question, but also depends on the material and surface properties of the hydrogel lenses used [62]. Despite a plethora of large scale microbial assay studies, the factors which are
critical to determining lens-bacteria adhesion are still not well understood [10], with conflicting results amongst research groups [58, 63-67], suggesting that reported adhesion characterization is extremely sensitive to the distinct experimental protocols and methodologies. Regardless of issues of consistencies, the type of adhesion characterization obtained from large scale microbial assays is useful for contact lens storage, but it sheds little light on the on-eye, *in situ* contact lens adhesion properties.

Figure 8: SEM micrograph depicting bacterial adhesion and biofilm formation on the surface of a hydrogel contact lens, adapted from [60].

**Atomic Force Microscope (AFM) Adhesion Test**

To date, there have been very few studies that quantitatively characterize hydrogel lens adhesion. This dearth of studies is likely due to the fact that standard adhesion measurement tests, such as the celebrated peel test [68], are largely incompatible with soft hydrogel materials that are difficult to grip, dynamically altered by swelling/dehydration, and exhibit extremely low adhesion forces in liquid buffers. To probe the adhesion characteristics of hydrogel contact lenses, researchers have utilized the Atomic Force Microscope. The AFM is a scanning probe modality that offers extremely high resolution in both spatial ($10^{-10}$ m) and force ($10^{-12}$ N)
measurements. The AFM is an extremely versatile machine with the basic components being a cantilever, piezo scanner, position-sensitive photodetector and a feedback controller which are shown in Figure 9. The basic principle behind AFM is to scan the surface of a sample or indent the sample with a sharp tip that is mounted to a cantilever spring. The cantilever is typically silicon or silicon nitride with a tip that has a radius of curvature on the order of nanometers. While scanning or indenting, the tip is brought into proximity of the sample surface, and the force between the tip and the sample causes a deflection in the cantilever. The deflection of the cantilever is measured by using a laser to reflect off of the back end to the photodetector. Thus changes in the measured voltage of the photodetector correlate to deflection of the cantilever [69].

![Figure 9: Schematic diagram of a commercially available AFM combined with an inverted optical microscope.](image)

The AFM can operate in many modes depending on the type of sample information that is of interest, but in the scope of contact lenses, AFM has largely been used for either surface topographical characterization [70-74] or force spectroscopy [75-77]. AFM force spectroscopy is the mode used to characterize local adhesion and mechanical properties of the hydrogel lenses. The number of studies that utilized AFM force spectroscopy to characterize hydrogel lens
adhesion has been limited. Typically, the probe indents the hydrogel surface to a maximum indentation depth or force, and retracts away from the hydrogel until complete separation is reached, creating a force-displacement ($P_{w0}$) curve. The loading curve is used to calculate the elastic modulus via Hertzian contact mechanics typically [49, 78], while the unloading curve is used to measure the force of adhesion required for the probe to “pull-off” of the hydrogel surface, referred to as $P^*$ [75]. A sample AFM $P_{w0}$ curve is shown in Figure 10. $P^*$ is then used as a metric to characterize the adhesion between different hydrogel lens materials [75], or the distance required for complete detachment (“snap-out” distance) has also been used [77].

![AFM P-w0 curve on hydrogel lenses](image)

**Figure 10**: Typical AFM $P_{w0}$ curve on hydrogel lenses, taken from [75]. Three different materials are shown, with a hydrophilic glass substrate serving as a control/baseline. The other f-d curves represent 2-hydroxyethly methacrylate (pHEMA) and 2-hydroxyethyl methacrylate and methacrylic acid p(HEMA+MA). Gray lines are loading curves and black lines are unloading curves.

Due to the inherent microscopic nature of the AFM, the adhesion data obtained during adhesion tests of hydrogel contact lenses represent extremely localized surface properties. Information on this length scale could be important for characterizing and elucidating micro-organism adhesion, such as bacteria [55]. However, it is not clear if these localized properties
scale up to be relevant for macroscopic, entire lens adhesion pertinent to on-eye behavior, in which the lens interacts with the ocular surface over a length scale of centimeters rather than nanometers.

1.5 Problem Statement

Contact lenses are used by approximately 125 million people worldwide, or roughly 2% of the global population [4]. The number of hydrogel contact lens users are expected to grow significantly in the near future [2], especially when considering the increased life expectancy of aging populations in many countries. Since the development of the first soft hydrogel contact lenses in the early 1970s, significant strides have been made in research and development to enhance their optical performance, user comfort, and biocompatibility with the ocular surface [5]. The in vivo behavior of hydrogel lenses and their interaction with the ocular surface is complex; the hydrogel chemical, mechanical, and adhesion properties are all involved in the overall lens-ocular surface behavior. Despite the multi-billion dollar market created by hydrogel lenses and decades of research, lens adhesion and mechanical properties are characterized by:

- Classical engineering techniques (i.e. tensile testing) that are intended for classical engineering materials rather than complex polymeric biomaterials.
- Methodologies that yield some useful information (i.e. nanoindentation, large scale microbial adhesion assays), but it is yet to be shown how relevant this information is pertaining to the entire lens on-eye, in vivo.
- Simply left uncharacterized due to the complexity of the problem (i.e. non-specific attractive intersurface forces).
Accurate characterization of hydrogel lens mechanical and adhesion properties is necessary for lens design, manufacturing, and evaluation of user comfort. Furthermore, hydrogel lens mechanical and adhesion properties are believed to be factors in the etiology for some contact lens related ocular complications [12, 14, 17-19, 22, 23, 26, 79]. To better understand the factors involved in the onset of these complications, their progression, and develop strategies to manage and prevent them, it is first necessary to reliably and accurately measure the suspected material properties.

1.6 Research Objectives

The scope of this work is to quantitatively characterize hydrogel contact lens (CL) mechanical properties, adhesion, and the coupled adhesion-mechanics. To accomplish this goal, first experimental protocols and methodologies must be established to reliably measure hydrogel contact lens mechanical adhesion behavior. Second, novel modeling/calculations must be utilized to quantitatively interpret and characterize the adhesion-mechanics behavior into meaningful metrics. Third, these methodologies must be applied in vitro to help elucidate lens adhesion-mechanics on-eye, in vivo behavior. To this end, the proposed work aims at multi-scaled quantitative adhesion-mechanics characterization of hydrogel lenses with the following specific aims:

i. Hydrogel CL-planar substrate adhesion (macroscopic)

This aim establishes protocols to quantitatively characterize the adhesion-mechanics of entire lenses in their native geometry against a planar substrate. Various lens brands/hydrogels are experimentally tested by measuring the applied force $P$, displacement $w_o$, and contact radius $a$, as well as the “pull-off” force $P^*$. The adhesion energy, $\gamma$, between the contact lens and substrate is calculated by a modified JKR-shell
model [80]. Together, $P^*$ and $\gamma$ serve as quantitative metrics to characterize lens adhesion. The effect of diopter (geometry) of the lens on overall adhesion-mechanics is also investigated.

ii. **Hydrogel CL-Cornea adhesion (macroscopic/microscopic)**

The second aim applies characterization techniques from the first aim towards more physiologically relevant, biological tissues: human corneas. Reliable and repeatable measurements are established and conducted to accurately measure a quantitative adhesion metric, $P^*$, and is compared to the results of Aim 1.

iii. **Hydrogel CL-single cell adhesion in vitro (microscopic)**

This aim characterizes the adhesion between single Human Corneal Fibroblasts (HCF) cells and Human Corneal Epithelial (HCE) cells against hydrogel lenses via Single Cell Force Microscopy (SCFS). Single cell adhesion mechanics are quantified by “pull-off” force $P^*$ and bond strengthening time $\tau$ [55, 81]. The microscopic data is compared to the lens/substrate and lens/cornea systems in aims 1 and 2 to help elucidate scaling effects on the overall adhesion mechanics.

Together, the combination of these specific aims offers a novel quantitative assay to probe hydrogel lens adhesion mechanics. This work utilizes a bottom-up approach based off of rigorous engineering principles to establish useful metrics for industrial/manufacturing considerations, and potentially help develop assays to elucidate the role of hydrogel adhesion mechanics in the etiology of contact lens related ocular complications.
1.7 Significance

This work establishes and measures quantitative metrics characterizing the adhesion-mechanics of hydrogel contact lenses. Furthermore, this work explores the adhesion-mechanics metrics in a biological context aimed at physiological relevance to on-eye behavior. The coupled lens adhesion-mechanics likely play a role in the etiology of many lens related ocular complications, such as corneal erosions [12, 17, 22]. These complications result in user discomfort, disruption of lifestyle, and potentially introduce the risk of sight-threatening sequelae [28]. This work offers novel metrics that characterize physical principles that up to this point have only been surmised to play a role in the etiology of lens related complications. Such data could help elucidate fundamental mechanisms of the onset of these complications, progression, and potentially help provide strategies for their prevention. Furthermore, the adhesion-mechanical characterization in this thesis could be useful in many industrial contexts, such as lens design and large scale manufacturing processes.

1.8 Thesis Organization

The three specific aims of this thesis are broken up into the following three chapters. Chapter 2 outlines the first aim, the Planar Adhesion Test (PAT). Lens mechanical characterization via Parallel Plate Compression (PPC), the backing adhesion theory, mechanical diopter affect, experimental techniques and protocol, and adhesion diopter affect are detailed in this chapter. The adhesion characterized in Chapter 2 is macroscopic and conducted in a non-biological context. Chapter 3 outlines the second aim of the tissue-hydrogel adhesion tests. These tests utilize donated human corneas to develop assays and provide in vitro data that could help shed light on the in vivo performance of the hydrogel lenses. The adhesion characterized in
Chapter 3 bridges the macro-to-microscopic length scales as it addresses macroscopic scale biological tissues comprised of a complex multi-cellular surface. Chapter 4 outlines the third and final aim of this thesis; single cell adhesion via Single Cell Force Spectroscopy (SCFS). The microscopic adhesion behavior of individual cells on the same hydrogel materials is characterized and compared with the adhesion metrics of aim 1 \((\gamma, P^*)\) as well as the cornea adhesion assays of aim 2. At the end of each chapter, conclusions and extensions and suggestions for future work are discussed.

1.9 Division of Labor

It is very important to note that the first specific aim/chapter 2 of was a group effort in collaboration with Jiayi Shi. The JKR-shell model, which is used to calculate the lens-substrate adhesion energy, constitutes a large portion of her thesis work and time spent in the Nano/Micro Biomechanical Characterization Laboratory at Northeastern University. Jiayi also contributed to some of the experiments conducted in the first specific aim of this thesis.
CHAPTER 2: AIM 1 - PLANAR ADHESION TEST (PAT)

Contributors: Kai-Tak Wan, Jiayi Shi, and David Chan

2.1 Overview

This chapter focuses on a comprehensive and detailed summary of the Planar Adhesion Test (PAT) used to characterize the adhesion of hydrogel lenses. Lens adhesion characterization is a multifaceted task, requiring both novel modeling and experimental techniques to produce consistent data not only for adhesion characterization, but for mechanical characterization as well. As outlined in this chapter, accurate mechanical characterization is essential to characterize the lens adhesion. The contents of this chapter spans three published peer reviewed journal articles and one article currently submitted:


This chapter first covers the novel experimental and numerical techniques to accurately measure hydrogel lens elastic modulus, $E$ [82], shown in Figure 11 in section 2.2. The effect of lens optical power/diopter, $d$, on the mechanical behavior of the lenses is also explored [83].
Next, the calculated elastic modulus serves as an input to the modified Johnson-Kendal-Roberts (JKR) shell model [80], which numerically calculates the adhesion energy, $\gamma$, between a planar substrate and an thin elastic shell. The crux of this first specific aim is the experimental protocol, techniques, results, and analysis of data of hydrogel lenses characterized via PAT to measure the “pull-off” force $P^*$ and to calculate $\gamma$. Finally, this chapter closes with exploring the diopter effect on the overall lens adhesion mechanics.

### 2.2 Lens Mechanical Characterization via Parallel Plate Compression

#### 2.2.1 Introduction

The first step in characterizing the adhesion-mechanics of hydrogel lenses is accurate mechanical characterization. As reviewed in Section 1.3.1, it is unclear if the current standard tensile or indentation testing is suitable to characterize the *in-situ* mechanical behavior of hydrogel lenses. Thus, in this section, the macroscopic scale mechanical properties of hydrogel lenses are characterized by novel Parallel Plate Compression (PPC) technique [82], shown in Figure 11. A universal testing machine with nano-Newton and submicron resolutions is used to measure the applied force, $P$, as a function of vertical displacement of the plate/shaft, $w_0$, while a homemade Laser Aided Topography (LAT) system records the *in situ* deformed shell profile and the contact radius $a$. A nonlinear shell theory and an iterative finite difference method are used to account for the large elastic deformation and the interrelationship between the measurable quantities ($P$, $w_0$, $a$). The lens elastic modulus, $E$, can be calculated from $P(w_o)$ alone, which is further confirmed with the predicted lens deformed profile.
2.2.2 Sample Preparation

Two commercially available contact lenses, *Acuvue TruEye* (Narafilcon-A, Vistakon) and *Acuvue2* (Etafilcon-A, Vistakon), serve as the two primary hydrogels for complete multi-scaled adhesion-mechanical characterization throughout this body of work. For PPC, the lenses were characterized at ambient temperature at 22°C. The samples had a base diameter, $2c = 14.2 \pm 0.10$ mm, and vertical height, $h = 3.70 \pm 0.10$ mm. The radius of curvature, $R$, for TruEye and Acuvue2 are 8.3 mm and 8.5 mm, respectively. Lenses with $d=-1$ were chosen due to their near-to-uniform thickness profile, and the effect of variations in lens thickness profile is discussed in Section 2.3.
2.2.3 Lens Thickness Measurement

The lens thickness profile, \( h(r) \), is measured as a function of off-center displacement by Optical Coherence Tomography (OCT). To measure \( h(r) \), central cross sections approximately 2 mm wide of the lenses were cut with a fresh razor blade and laid out flat against a rigid substrate. Great care was taken to ensure that wrinkling and curling of the lens strip did not occur, and any OCT scans that exhibited these artifacts were discarded. Manual application of contact lens solution via pipette was done to minimize the effect of swelling/dehydration on the thickness profile measurements. A clean and fresh glass slide with known thickness was used to calibrate the pixel to micron ratio. Cross-sectional full-thickness images were collected and assembled to form panoramic images of the full length of the lens. The thickness was measured by simple pixel scaling at approximately 250 µm intervals starting from the apex/radial center of the lens. For each lens brand and diopter, three separate lenses were measured and averaged to produce \( h(r) \). Representative OCT scans for TruEye lenses are shown in Figure 12.
2.2.4. Thin Shell Mechanics Introduction

Due to the geometry of the cornea and the desired minimal thickness for oxygen permeability/comfort, contact lenses take the form of shells. Shells as structural elements occupy many roles in mechanical, civil, and bioengineering due to their efficiency of load-carrying behavior, relatively high strength: weight ratio, high stiffness and ability for containment of space [84]. The definition of a thin shell is if the thickness of the shell $h$ and the radius of curvature $R$ meet the criteria that: $\left(\frac{h}{R}\right) \leq \frac{1}{20}$, with hydrogel contact lenses typically having $\frac{1}{100} \leq \left(\frac{h}{R}\right) \leq \frac{1}{50}$. In general, linear shell theories contain the following assumptions [84]:

- Kirchhoff-Love assumptions: That the thickness of the plate does not change during deformation, and that straight lines normal to the undeformed middle surface remain straight and normal to the deformed middle surface.
• The displacements of an arbitrary point of a shell are small in comparison to its thickness (small-displacement assumption).

• The shell is comprised of an isotropic material that exhibits linear elasticity based off of Hooke’s law, $\sigma = -c \varepsilon$, where $\sigma$ is the stress tensor, $\varepsilon$ is the strain tensor, and $c$ is typically called the stiffness tensor.

• The transverse normal stress is negligible compared with other normal stress components and can be ignored.

For the purpose of this work, hydrogel contact lenses are modeled as a thin, linear elastic spherical shell. The earlier models of shells formed by the full revolution of a curve are dated back to the 1960’s. Particularly, Reissner [85] derived the general solutions for shallow spherical shells with small deformation. Furthermore, Updike and Kalnins [86] analyzed an elastic spherical shell compressed between rigid plates by employing a nonlinear shell theory given by Reissner [87]. For modeling hydrogel contact lenses, Updike’s work is expanded upon to account for parallel plate compression of a convex shell.

2.2.5 Theory

The hydrogel contact lenses are modeled as an elastic spherical cap which is being compressed by a flat, rigid surface. The detailed description of the linear elastic shell model used to calculate $E$ lies outside of the scope of this thesis, a brief summary is outlined here, with a comprehensive summary previously reported [82, 83, 88]. A schematic of lens deformation by PPC for the computational model is shown in Figure 13 with the appropriate nomenclature (also listed in the nomenclature section). The top plate remains parallel to the base plane of the shell.
throughout the loading process. When the parallel plate comes into contact with the shell, the shell deforms and conforms to the planar plate geometry, resulting in a planar contact circle.

![Schematic of a lens deformed by parallel plate compression for the computational model. Lens thickness, \( h \), is a function of the meridional angle \( \phi_0 \), and the contact radius in terms of \( \phi_0^* \) is geometrically related to the compression depth. A full list of nomenclature is given in the appendix.](image)

First, the experimentally derived thickness profile \( h(r) \) is converted to \( h(\phi_0) \) by simple geometrical conversion. The hydrogel lens is modeled as a linear elastic shell of spherical cap geometry with elastic modulus, \( E \), Poisson’s ratio, \( \nu \), radius of curvature, \( R \), thickness, \( h(\phi_0) \) with meridional angle, \( \phi_0 \). It is assumed that mechanical strain remains small and linearly elastic despite the large geometrical deformation of the shell. Upon external load, the shell deforms by a mixture of bending and stretching. The large deformation with small strain is constructed following Reissner’s nonlinear shell theory [89]. The axisymmetry of the lens allows the loading configuration of PPC to be governed by a set of equations, where the elastic strain, \( \varepsilon \), bending
curvature, $\kappa$, membrane stress, $N$, and bending moment, $M$, along the meridional $\phi$ and azimuthal $\theta$ directions:

**Strain displacement:**

$$\varepsilon_\phi = \frac{\cos \phi_0}{\cos \phi} \left(1 + \frac{du}{dr} \frac{d\phi_0}{d\phi}ight) - 1 \quad \text{and} \quad \varepsilon_\theta = \frac{u}{r_0}$$

**Bending curvatures:**

$$\kappa_\phi = \frac{d\beta}{d\phi_0} \quad \text{and} \quad \kappa_\theta = \frac{\sin \phi_0 - \sin \phi}{r_0}$$

**Membrane stress:**

$$N_\phi = K \left(\varepsilon_\phi + v\varepsilon_\theta\right) \quad \text{and} \quad N_\theta = K \left(\varepsilon_\theta + v\varepsilon_\phi\right)$$

**Bending moment:**

$$M_\phi = D \left(\kappa_\phi + v\kappa_\theta\right) \quad \text{and} \quad M_\theta = D \left(\kappa_\theta + v\kappa_\phi\right)$$

where $u$ is the deflection component along the normal to the symmetry axis, $\beta$ is the rotation of the normal to the shell surface, $D$ is the bending rigidity $D = Eh^2/12(1-\nu^2)$, $K$ is the in-plane rigidity $K = Eh/(1-\nu^2)$, $V$ is the stress resultant parallel to the axis of symmetry, $H$ is the stress resultant perpendicular to the axis of symmetry, and $r$ is the distance from the shell mid-surface to the symmetry axis. The subscript 0 denotes the undeformed geometry. The flexibility here to include $h(\phi_0)$ in the present context leads to the $\phi_0$ dependencies of the in-plane rigidity $K$ and bending rigidity $D$. Each infinitesimal segment of the shell is described by 3 displacements / rotations: deflection in horizontal direction $u$, vertical direction $v$, and rotation $\beta$, as well as 3 stresses / bending moments: stress in horizontal direction $H$, vertical direction $V$, and meridional moment resultant $M_\phi$. Axisymmetry allows only the right half of a shell to be considered in the numerical method. It is noted that the PPC model is valid when the compression depth is larger than the film thickness, *i.e.* only for large geometrical deformations. Otherwise, the mixed bending-stretching assumption fails and deformation is dominated by an applied load on a half continuum.
In PPC, loading compresses the shell to a central displacement of $w_0$, and increases the contact radius, $a$, and the corresponding the meridional angle at the contact edge, $\phi_0^*$. The deformed shell is divided into two regions: (i) inner region $\beta = \phi_0$ where the shell conforms to and is in intimate contact with the loading plate, and the (ii) outer region of the freestanding annulus. Within the contact, an analytical solution is partially found to provide the three continuity conditions for the inner-outer contact edge. The solution for the outer annulus is then obtained numerically by employing finite difference method to solve the two-point boundary value problem [82]. An iterative numerical approach is adopted to generate the self consistent solution. The finite difference method is employed again to derive the full numerical solution. The deformed shell profiles, along with $P(w_0, a)$ are then obtained.

### 2.2.6 Parallel Plate Compression Experimental Setup

The PPC experimental configuration is shown in Figure 11.b. PPC is conducted on an Agilent T150 UTM with a force and displacement resolution of approximately 30nN and 10nm, respectively. The lens geometry and ultimately the deformed profile of the lens under applied load is captured by a laser beam that increases the optical contrast of the convex/concave lens surfaces, while an orthogonal long focal length microscope captures the image. All experiments are displacement-controlled with the planar surface staying fixed and the base platform/contact lens moving upwards. An aluminum cylindrical plate with radius $r_p=10$ mm was fabricated for PPC. For convenience and consistency, the loading speed was kept at $v=1.00$ mm.min$^{-1}$. The maximum approach distance was confined to the range of $50 \mu m < w_0 < 1200 \mu m$. The lens sat on an aluminum sample holder with a shoulder of radius 7.25 mm fitting to the base radius $c$. A hole was bored through the sample holder axis below the shell to allow free flow of liquid and
thus preventing hydrostatic pressure buildup during loading-unloading. The isotonic solution container sat on a micrometer stage with two horizontal degrees of freedom to align the loading plate with the shell apex, while the entire setup was placed on a vibration isolation table. The buoyancy force and the liquid surface tension acting on the plate/shaft were carefully measured in the ‘pre-travel’ regime of each individual force-displacement curve, and are subtracted from all force measurements. Great care was taken to accurately determine the moment when the probe first came into contact with the sample. It was taken to be the point when the load cell recorded a sudden jump of ~10μN, which was also confirmed by a long-focal digital camera that captured a complete side view of the sample. The camera was positioned horizontal and in the line of sight of the lens. Images were then processed by using software ImageJ, a public domain Java image processing program inspired by National Institute of Health. The deformed profile was extracted by pixel intensity differentiation. Dimensions of the contact area between the lens-plate interface were measured by pixel scaling. For each lens brand/material, five separate lenses were tested at least three times each to ensure repeatable and statistically significant results.

2.2.7 Results

Figure 14.a shows the mechanical response, $P(w_0)$ of TruEye lenses and Figure 15.a shows the mechanical response of Acuvue2 lenses. The averaged experimental data is shown as white circles, with the error bars indicated one standard deviation. The applied load lies in the range of 0-2 mN. The logarithmic plot of $P(w_0)$ in Figure 14.b and Figure 15.b show the characteristic transition [82] from $P \propto w_0$ to $P \propto w_0^{3/2}$ at roughly the lens central thickness for TruEye, $h_{0,\text{TruEye}} = 80\mu m$ and at slightly less than the central thickness for Acuvue 2, $h_{0,\text{Acuvue2}} = 130\mu m$. The vertical lines denotes $w_0 \sim h_0$, where a change in slope is expected. In the initial
loading, the contact radius increases rapidly from zero but remains small compared to the base radius \((a \ll c)\) as shown in Figure 14.c and Figure 15.c, respectively, and \(P(w_0)\) is therefore linear as expected by the classical elastic shell mode. When the central deflection approaches the shell thickness \((w_0 \sim h_0)\), the contact region gradually increases and becomes significant compared to the base radius, \(P(w_0)\) deviates from the linear relationship. As \(w_0\) exceeds 500 μm, the measured load deviates further from the model. The inconsistency is likely due to the violation of shallow shell assumption in the contact region, as \((w_0)_{\text{max}}=800\ \mu\text{m}\) represents roughly 10% of the lens curvature radius and 22% of the shell height. The best fit curve-fits yields \(E = 0.68 \pm 0.04\ \text{MPa}\) and \(E = 0.29 \pm 0.01\ \text{MPa}\) for TruEye and Acuvue2 lenses, respectively.
Figure 14: TruEye mechanical characterization under Parallel Plate Compression, with the best curve fit yielding $E = 0.68 \pm 0.04$ MPa. White dots represent experimental averages (+/- one standard deviation), and gray lines represent theoretical curve fits. A) Applied load as a function of compression depth, with central thickness $h_0$ marked. B) log-log plot of $P(w_0)$. C) Measured contact radius via long focal microscopy as a function of $w_0$. D) Experimentally measured deformed lens profile at a compression depth of $w_0 = 600 \mu$m, with the theoretically predicted lens profile using $E = 0.68$ MPa shown as the black line.

Figure 14.c and Figure 15.c show the contact radius as a function of applied load, $a(P)$. An underestimation of $a$ in the theoretical $a(w_0)$ and $a(P)$ at small $w_0$ is expected mainly due to the difficulty in defining the contact radius. For $w_0 \sim h_0$, the contact radius becomes comparable to the shell thickness. The actual contact with the top plate is larger than the planar section of the deformed lens on the concave side by roughly twice the shell thickness. Since the theoretical model assumes $h_0 \ll a$, a large discrepancy is expected. Figure 14.d and Figure 15.d show the
superimposed deformed profiles for \( w_0 = 600 \, \mu m \), as indicated, and the theoretical curve using the calculated \( E \) for each lens. Within the contact edge, the shell makes full contact with the top plate. The deformed shell resembles a truncated spherical shell with a small deviation at the contact edge for large \( w_0 \). Most elastic energy of the sample is stored at the contact edge where the smallest radius of curvature occurs.

Figure 15: Acuvue2 mechanical characterization under Parallel Plate Compression, with the best curve fit yielding \( E = 0.29 \pm 0.01 \) MPa. White dots represent experimental averages (+/- one standard deviation), and gray lines represent theoretical curve fits. A) Applied load as a function of compression depth, with central thickness \( h_0 \) marked. B) log-log plot of \( P(w_0) \). C) Measured contact radius via long focal microscopy as a function of \( w_0 \). D) Experimentally measured deformed lens profile at a compression depth of \( w_0 = 600 \, \mu m \), with the theoretically predicted lens profile using \( E = 0.29 \) MPa shown as the black line.
2.2.8 Discussion & Conclusion

A novel experimental set up, PPC, was designed and fabricated to capture the mechanical response and the in situ deformed profile. Using classical shell theory, a numerical approach was adopted to account for shells with a large ratio of base curvature to radii curvature and shell deformation. The modulus of the lenses were measured to be $E = 0.29 \pm 0.01$ MPa for Acuvue2/EtafilconA and $0.68 \pm 0.04$ MPa for TruEye/NarafilconA. Current industrial and research standards to mechanical characterize hydrogel lenses are either tensile or nanoindentation, as discussed in Section 1.3.1. A recent study utilizing nanoindentation on TruEye lenses reports a modulus of $0.64 \pm 0.05$ MPa [90], in great agreement with $0.68 \pm 0.04$ MPa reported via PPC. However, it should be noted that this modulus is unique to an indenter with a radius of 50 µm. Using an indenter with a radius of 100 µm increases the calculated elastic modulus near to $0.79 \pm 0.05$ MPa, which is attributed to a phenomena known as the indenter size effect [41]. The PPC testing method does not suffer from size effects [41], surface detection errors [45], complex/altering sample preparation techniques [36], or complexity in choosing an appropriate model [42, 48]. Another benefit of PPC is the lack of significant sample preparation, such as cutting a planar strip from the curved lens that is required in tensile testing which could introduce flaws to the sample by simple human error [36]. Most importantly, the PPC configuration maintains the native lens geometry and utilizes deformations that are on the order of that during on-eye use and handling (~10-1000µm). Considering that accurate hydrogel mechanical characterization requires that the test be done in conditions as close as the in situ conditions as possible [37], PPC likely better represents the mechanical behavior of hydrogel lenses in vivo compared to the current standards of tensile testing and indentation. Furthermore,
PPC provides the framework for further investigation into lens geometry (diopter effect) and adhesion behavior.

### 2.3 Mechanical Diopter Effect

#### 2.3.1 Introduction

This chapter investigates the effect of diopter, or thin shell geometry, of the hydrogel lenses on the overall lens mechanical behavior. The optical power of hydrogel contact lenses, or diopter $d$, was largely not considered in mechanical characterization, primarily due to inherent limitations of standard techniques such as microindentation, which is only concerned with localized mechanical properties. Recently, it was shown that the diopter has a significant role in mechanical behavior of hydrogel contact lenses [83]. This chapter shows that when a contact lens is compressed by a rigid, flat parallel plate (PPC), the constitutive relationships depends not only on the mechanical properties such as elastic modulus, $E$, of the hydrogel materials, but also the lens power, $d$, or thickness variation, $h(\phi_0)$, along the meridional direction $\phi_0$. Hyperopic lenses ($d > 0$) are thicker at the apex along the optical axis and thin out gradually along the meridian, while myopic lenses ($d < 0$) are thinnest at the apex, shown in Figure 16. The mechanical deformation of hydrogel lenses with various $d$ are characterized by the interrelationships between applied force, $P$, vertical displacement of the external load, $w_0$, contact or dimple radius, $a$, and the deformed profile, $w(r)$. The force responses under PPC show that lenses with positive $d$ are apparently stiffer in the initial loading but become more compliant as load increases. Conversely, lenses with negative $d$ are more deformable initially and becomes gradually more resistant to loading. These experimental results are consistent with the theoretical shell model outlined in section 2.2, using the same $E$. In general, the mechanical
behavior of hydrogel contact lenses has significant impacts in defining the degree of user comfort, as well as the adhesion characteristics (discussed in section 2.6).

Figure 16: Sketch of top surface of contact lenses with different powers but the same basal curvature of the inner concave surface. The bottom curve shows $d = 0$ or uniform thickness. (a) Myopic lens ($d < 0$) with thinnest apical thickness and gradual thickening in the meridional direction towards rim. The apical thickness is the same for all $d$. (b) Hyperopic lens ($d > 0$) with thickest apical thickness and gradual thinning in the meridional direction.

2.3.2 Sample Preparation

To explore the effect of geometry/diopter on the mechanical behavior of hydrogel lenses, Acuvue TruEye (Narafilcon-A) hydrogel contact lenses with range of powers, $d = -6, -3, -1, +3,$ and $+6$, and rim diameter $2a = 14.2$mm were characterized. TruEye was chosen since $d = -1$ has already been characterized in Section 2.2 and serves as a baseline. To measure $h(\phi_0)$, a 2mm wide rectangular strip was cut diametrically from each sample, laid flat on a planar glass substrate, and was kept hydrated throughout the preparation as described in section 2.2.3. Optical coherence tomography (OCT) was then used to measure the film thickness as a function of off-center displacement, $h(r)$, which was then converted into $h(\phi_0)$ using a simple geometric conversion. The measured thickness profiles are shown in Figure 17.
2.3.3 Experimental Setup

The mechanical characterization protocol is the same as described in section 2.2.6. Five lenses ($N = 5$) for each power were characterized with 3 repeated loading-unloading cycles. All results reported are from averaged data with one standard deviation.

2.3.4 Results

Figure 18 shows the individual force measurement $P(w_0)$ using PPC and the best theoretical fits with $E = 0.68 \pm 0.04$ MPa, in both linear and log-log scales. Figure 19 shows a comprehensive summary of the $P(w_0)$ curves. Lenses with $d < 0$ exhibit typical J-curve where the slope $(dP/dw_0)$ increases with the compression depth. The behavior can be explained as follows. Since these lenses have the thinnest section at the apex, further compression expands
the contact circle and pushes the contact edge into the gradually thicker section of increasing bending rigidity. Moreover, the apical thickness $h_0 = h(\phi_0=0)$ is identical for all $d < 0$. Lenses with $d = -6$ show the highest gradient of thickness variation, $(dh/d\phi_0)$, and are therefore the most mechanical resistant compared to $d = -3$ and $-1$. Should the data be plotted in a log-log graph (not shown), the slope $(dP/dw_0)$ increases from the initial value of 1 (or linear) to 3/2 that is consistent with our previous model [91]. Conversely, lenses with $d > 0$ have distinctly different behavior, in that, $(dP/dw_0)$ is a maximum at the initial loading and decreases as compression proceeds. Here the bending rigidity decreases as the contact circles grows out from the apex. These lenses have increasing apex thickness as $d$ increases from +3 to +6, and therefore lenses with $d = +6$ shows larger $(dP/dw_0)$ throughout the loading process. The excellent theoretical fitting to the measurement verifies the assumption of the same elastic modulus for all lenses. Another remark is that the theoretical curves for $d = +3$ and +6 are shown for $w_0 > h_0$ where the assumption of mixed bending-stretching as the main mode of deformation is valid.
Figure 18: Individual $P(w_0)$ curves of TruEye lenses under PPC as a function of diopter. Linear (left) and log-log (right) scales, with the central thickness marked by vertical dashed lines. White dots are experimental data, red curves represent the numerical model with $E = 0.68\pm0.04\text{MPa}$.

Figure 19: Summary of the mechanical response of TruEye contact lenses under parallel plate compression (PPC) and the theoretical curve fits. Measurements are shown as the colored data points and theory as the grey curves. Since the model is invalid when the compression depth is below the lens thickness (~100µm), the initial loading stage is not fitted. The elastic modulus is fixed at $E = 0.68\pm0.04\text{ MPa}$ for all dipters.

2.3.5 Discussion

Figure 18 and Figure 19 show distinct mechanical behavior of TruEye lenses of different diopter, despite the same elastic modulus. The theoretical model captures this distinctive mechanical behavior and allows to probe into the shell mechanics. Besides generating the
predicted $P(w_0)$ for lenses with range of $d$, it is also possible to yield the corresponding meridional moment resultant $M_f$ (defined in Eq. 12). To gain perspective as to the distinct mechanical behavior between diopters, the meridional stress couple $M_f(\phi_0)$ of $d = +6$ and $-6$ for PPC as shown in Figure 20. The hyperopic lens with $d = +6$ is shown in the upper graph. The curves denote four consecutive loading with increasing $w_0$, or equivalently, the meridional angle at the contact edge $\phi_0^*$. At initial loading with small compression depth such that $w_0 < h_0$ and $a < h_0$, our theoretical model is inadequate because the loading geometry resembles indentation of a half continuum rather than the assumed mixed bending-stretching of a shell. The red curve represents the deformed shell when $w_0 = h_0$, at which point the model is valid. Within the contact, $M_f$ is a maximum at the apex where $h(\phi_0=0)$ is a maximum and so is the rigidity. $M_f$ is a monotonic decreasing function as the lens thickness decreases, until a discontinuity shows up at the contact edge ($r = a$) at A. Further out in the freestanding annulus, $M_f$ rapidly decreases to zero and swings to the negative regime, before an exponential decay towards the lens rim. An oscillatory behavior of $M_f(\phi_0)$ is expected because of the stress singularity at $\phi_0^*$ as predicted by classical linear elastic fracture mechanics [92]. Increasing external load pushes the contact edge $\phi_0^*$ out to $5^\circ$ (black), $10^\circ$ (blue) and then $15^\circ$ (green), with respective contact edge expanding from B to C then D. An expanding contact radius leads to an increasingly negative minimum $M_f$ being pushed towards the rim. Several characteristics are noted for these curves. Firstly, being an envelope for $M_f(\phi_0)$ for all loads, the curve ABCD maps the monotonic decreasing $h(\phi_0)$ and the diminishing rigidity. Secondly, the four curves converge to $M_f = 0$ at a specific $\phi_0$ as indicated, a distinct feature of the hyperopic lenses.
Figure 20: Theoretical meridional stress couple is plotted as a function of horizontal distance $X$ from the optical axis and meridional angle $\phi_0$ (lower horizontal axis) for a range of compression depth denoted by $\phi_0^*$. The upper graph corresponds to a hyperopic lens with $d = +6$ and the lower being myopic lens with $d = -6$.

The lower graph of Figure 20 shows loading of a myopic lens with $d = -6$ that pushes the contact edge to $\phi_0^* = 5^\circ$ (black), $10^\circ$ (blue) and then $15^\circ$ (green). Contrasting the hyperopic lens,
$M_f$ increases from $\phi_0 = 0$ and reaches a maximum at the contact edge denoted by F, G, H and J, because of the monotonic increasing $h(\phi_0)$ and the rising mechanical resistance. $M_f$ then decreases rapidly and swings towards the negative regime with the magnitude of the minimum almost doubled compared to that of the hyperopic lens. The curve FGHJ serves as the envelope of $M_f$ within the contact circle for all loads. To better compare lenses of $d = \pm 6$, the normalized moment $M_f / M_{\text{max}}$ is adopted, where $M_{\text{max}}$ is the maximum $M_f$ at the apex of the hyperopic lens or the contact edge of the myopic lens. Figure 21 shows a comparison of $M_f(\phi_0)$ between the two extreme powers when the contact circles reach the same dimension or $\phi_0^*$, along with a reference curve for $d = 0$. Since lens with uniform thickness has constant flexural rigidity for all $\phi_0$, $M_f(\phi_0<\phi_0^*)$ is therefore a constant within the contact circle. These differences in stress distributions across the lenses show the important role that the lens geometry plays in the overall lens mechanical behavior under PPC.
Lens comfort as sensed by the user is inherently difficult to quantify. Typically mass user surveys are employed in which hundreds/thousands of users fill out an arbitrary scale of comfort over a specified period of time, and the average response of many users determines the lens comfort. There are many factors that affect lens comfort, such as oxygen permeability, lens wettability, lens mechanical and lens adhesion properties. Here, we show that the lens power can strongly influence the degree of comfort for the user in the context of lens stiffness. An apparent stiffness of the lens can be defined as the gradient \( (dP/dw_0) \) in an arbitrary range of \( 0 < w_0 < 1.5h_0 \). This quantity is predominantly determined by \( d \) rather than the hydrogel material properties. Figure 22 shows \( (dP/dw_0) \) as a function of \( d \). Lenses with uniform thickness \( d = 0 \)
possess the lowest \( \frac{dP}{dw_0} \). Either increases in \( d \) in the hyperopic lenses or decreases in \( d \) in the myopic lenses raise \( \frac{dP}{dw_0} \) and likely make the lens less comfortable for the user. Both our measurements and model show hyperopic lenses of \( d = +6 \) to be by far the most uncomfortable in the sense of mechanical stiffness. When a statistical survey is performed for different contact lens materials or brands to check the degree of comfort in a large user domain, it is recommended that proper grouping due to power is made; otherwise, analysis might lead to an erroneous conclusion such as pinpointing the deficiency of material properties. Furthermore, since lens mechanical properties are believed to be involved in the etiology of several contact lens related ocular complications [12, 14, 17, 19], lens diopter should be taken into consideration in analyzing the incidence rates of ocular complications, and in potentially elucidating mechanisms initiating and progressing these ocular complications during clinical studies.

![Figure 22: Apparent stiffness of lens as a function of power. Measurements are shown as yellow data points, and theoretical model as gray background. Note that the theoretical](image-url)
value at each diopter is computed separately. The curve connects the computational results and does not represent a smooth curve.

### 2.3.5 Conclusion

The mechanical resistance of contact lenses against external load depends on the lens geometry, rather than merely the material properties and chemistry of the constituent hydrogel or the aqueous ophthalmic environment. The metric apparent stiffness of the initial $P-w_0$ curve was formed, and it was found that the apparent stiffness is lowest at uniform thickness lenses, or at zero diopter, but increases when lenses get more hyperopic or myopic. Lenses with $+6$ were shown to be the stiffest. This mechanical diopter affect could be important for determining user comfort, or elucidating contact lens related ocular complications, and should be considered in future investigations.

### 2.4 Lens Adhesion Characterization: JKR-Shell Model

#### 2.4.1 Introduction

The previous sections in Chapter 2 covered novel mechanical characterization of hydrogel contact lenses. The subsequent subsections of chapter two cover novel techniques and numerical models to characterize hydrogel lens adhesion. Chapter 2.4 reviews the Johnson-Kendal-Roberts (JKR) modified shell model [80, 88], and Chapter 2.5 covers the experimental procedures and analysis. Together, Chapter 2 provides an entirely novel framework to characterize hydrogel lens adhesion-mechanics, which could be useful in many industrial/clinical applications. This chapter starts with a basic review of adhesion. In the context of hydrogel contact lenses, non-specific adhesion forces have largely been ignored in lieu of highly specific adhesion interactions of bacteria or cells on specific polymer surfaces [10]. However, non-
specific adhesion forces are present in all hydrogel lens interactions with various surfaces: the ocular surface, tear film, inner eyelid, packing containers, and spin cast molds.

2.4.2 Adhesion Introduction: JKR Theory

When two surfaces attach or detach, it is due to molecular bonds forming and breaking on both surfaces. These bonds can be classified as either strong bonds or weak bonds. Strong bonds include covalent bonding, ionic bonding, and metallic bonding and require specific surface molecular chemistry characteristics or properties to initiate the bond. Weak bonds include hydrogen bonding and Van der Waals forces, the latter being non-specific and always present between atoms/molecules. The adhesion modeling reviewed in this thesis only considers non-specific adhesion because of its omnipresence in contact mechanics. The adhesion energy, or work of adhesion between two surfaces (S1 and S2) is defined as the free energy change, or reversible work done, to separate the two surface from contact to full separation at infinity, and is given in units of energy per unit area (J/m²). The much celebrated Johnson-Kendal-Roberts (JKR) theory improves upon the Hertz contact theory (briefly outlined in section 1.3.1) by accounting for non-specific intersurface attractive forces within the contact area of the elastic sphere indenting the elastic half space [93]. JKR theory models intersurface attraction forces are modeled as an ideal force with an infinite magnitude that acts over zero range, as shown in Figure 23.
Figure 23: Schematic drawing of the disjoining pressure as a function of separation distance of JKR theory [93].

JKR theory showed that given this characteristic infinite magnitude/zero range attractive force, a non-zero contact circle arises even in the absence of an external load, and a “pull-off” force is required to spontaneously pull the adhering sphere out of contact. The surface energy causes an infinite tensile stress to act at the contact edge. The equilibrium relation between radius of contact \( a \) and applied load \( P \) is given as follows:

\[
a^3 = \frac{PR}{K} \left[ 1 + \frac{3\pi\gamma R}{p} + \sqrt{2 \frac{3\pi\gamma R}{p} + \left( \frac{3\pi\gamma R}{p} \right)^2} \right]
\]  

(13)

Where \( \gamma \) is the work of adhesion, with units of J/m², and \( R \) is the radius of the sphere. \( \gamma \) is related to the energy needed per area to create new surface. A sketch outlining the comparison between JKR and Hertz contact is shown in Figure 24. In particular, at the critical “pull-off” point, a non-zero contact radius and a critical “pull-off” force are given as follows:

\[
P^* = -\frac{3}{2} \pi \gamma R
\]  

(14)

\[
a = \left( \frac{\pi \gamma R^2}{2K} \right)^{1/3}
\]  

(15)

Note that the critical “pull-off” force is independent of the Young’s modulus, \( E \).
Figure 24: Comparison and summary of adhesion models. A. Two elastic spheres making contact under a compressive force, \( F \). The deformation profiles and pressure distributions predicted by B. Hertz theory, C. JKR theory, and D. DMT theory. Adapted from [49].

2.4.2 Adhesion Introduction: DMT Theory

Derjaguin-Muller-Toporov (DMT) theory is an alternative model widely used to model adhesive contact and results in a distinctly different mechanical response [94]. DMT theory builds off of Hertzian contact mechanics by assuming the same contact profile of a sphere in contact with an elastic half-space, but with additional attractive interactions outside the area of contact. The DMT interrelationships between applied force, \( P \), and contact radius, \( a \), are described by the following set of equations:

\[
a^3 = \frac{R}{K} (P + 2\gamma \pi R)
\]  

(16)
\[ d = \frac{a^2}{2R} \]  

(17)

Due to the presence of adhesion, the contact radius between the sphere and elastic half-space is nonzero when the applied force is completely removed, similar to JKR adhesion theory. In Eq. 14, to achieve a zero contact radius between the sphere-substrate requires a negative tensile force, the “pull-off” force needed is \( P^* = -2 \gamma \pi R \). A comparison of the deformed profile and pressure distribution of Hertz, JRK and DMT theory is shown in Figure 24.

### 2.4.2 JKR-Shell model

The classical solid sphere JKR adhesion model is widely adopted in virtually all engineering and many cases of biological adhesion [95]. However, the classic JKR theory models an elastic sphere in contact with an elastic planar substrate, and thus is inadequate to account for adhering shells onto planar substrates. In the presence of a strong interfacial adhesion, thin shells deform and conform to their substrate geometry, which has a plethora of biomedical applications such as hydrogel contact lenses, liposomes, microcapsules for drug delivery, or cells. This chapter reviews a modified JKR shell model that considers large geometrical deformation of an elastic spherical shell compressed between two parallel plates [80, 88], which will be utilized to model hydrogel lens adhesion against a planar substrate.

Similar to the PPC model outlined in Section 2.2.5, to account for large deflection of an axisymmetric shell, Reissner’s theory is employed in this model [89]. It is assumed that mechanical strain remains small and linearly elastic despite the large geometrical deformation of the shell. The JKR-shell model directly builds off of the PPC model, in which an isotropic spherical shell is compressed by two parallel plates and adhesion is not considered throughout the deformation process. Adopting the classical JKR framework for modeling intersurface
attractive forces, in the absence of an external load \((P = 0)\), the shell comes into adhesion contact with the rigid planar substrate, producing a non-zero contact radius, \(a\). The total energy of the system, \(U_T\), is the sum of the potential energy of the applied load, \(U_P\), the elastic energy stored in the shell, \(U_E\), and surface energy to create new surfaces, \(U_S\). Under fixed load configuration, \(P\) moves a vertical distance, \(d\delta\), such that \((dU_P)_{\text{P=constant}} = P \cdot d\delta\). The elastic energy is found by computing the area under the force-displacement curve, \(P(\delta)\), as shown in Figure 25, adapted from a numerical shell with radius \(R = 15\,\text{mm}\), \(E = 2.0\,\text{MPa}\), and thickness \(h = 200\,\mu\text{m}\) [80]. The total force acting on the shell, \(P_1\), is the sum of the applied load and the virtual adhesion force. If there is no adhesion forces present, the loading curve transverses along path OA and the contact radius increases to \(a\). Adhesion now keeps \(a\) constant, and elastic relaxation is allowed to reduce the load from \(P_1\) to \(P\) along AB until equilibrium is reached at B. The total elastic energy is, thus, the area enclosed by OABDO. The surface energy is given by \(U_S = -\pi\gamma a^2\), where \(\gamma\) is the adhesion energy or surface energy per unit contact area. The disjoining pressure is taken to be infinite in magnitude but with zero range, so that adhesion becomes a line force at the contact edge and the intersurface attraction vanishes beyond the contact circle \((r>a)\). Shear at the contact interface is ignored.
Figure 25: Load-displacement relation with $E = 2.0$ MPa, $h = 200$ mm, and $R = 15$mm. The elastic energy, $U_E$, is the area under loading curve (in grey). Area $ABDEA$ is the elastic energy associated with elastic recovery due to relaxation. Adapted from [80].

Next, the mechanical adhesion-detachment of the elastic shell against a planar substrate is established by setting up inter-relationships between the measureable quantities applied load $P$, approach distance $\delta$, and contact radius $a$. Figure 26 shows the predicted relation, $a(P)$, for $\gamma$ as stated in comparison to Hertzian contact ($\gamma = 0$). For all $\gamma$, the compressive load enlarges the contact area between the shell and planar substrate. Tension reduces the contact area and stretches the shell in the vertical direction. Note that $a>0$ at $G$ where $P = 0$. Along path FGHJ on the energy balance curve, the external load turns from compression to tension, reaching a maximum tension $P^*$ at $J$. Further increase in tension beyond $P^*$ can no longer satisfy the thermodynamic energy balance. The shell, thus, spontaneously detaches from the substrate at a
non-zero contact radius $a^*$ (gray circles). Weaker adhesion leads to smaller $P^*$ and $a^*$ as shown in Figure 26.

The universal shell adhesion detachment behavior can be derived, similar to Maugis and Barquins’ normalization scheme [96], in which $P$, $\delta$, and $a$ can be non-dimensionalized in terms of the shell material and geometrical parameters, $E$, $h$, $R$, and $\gamma$. All normalized variables are shown in bold. It is shown that the relationship $a(P)$ then collapses into a single universal detachment curve [80, 88]. This detachment curve is independent of adhesion energy, and so are $\delta(a)$ and $P(\delta)$. It was shown that:

\[ P = P \left[ \frac{Eh^2}{\gamma^4 R^4 (1-v^2)} \right]^{1/3} \]  
\[ \delta = \delta \left[ \frac{E^2 h^4}{\gamma^2 R^5 (1-v^2)^2} \right]^{1/3} \]  
\[ a = a \left[ \frac{Eh^2}{\gamma R^4 (1-v^2)} \right]^{1/3} \]

The universal mechanical response is shown in Figure 27. The curves are obtained by averaging many numerical simulations that span a few orders of magnitude of $E$, $h$, $R$, and $\gamma$. Shi et al. [80] showed that $a(P)$, for any $\gamma$ under fixed load, that pull-off occurs at:

\[ P^* = -(13.2 \pm 0.6) \left[ \frac{\gamma^4 R^4 (1-v^2)}{Eh^2} \right]^{1/3} \]
Figure 26: Contact radius as a function of applied load with $E = 2.0$ MPa, $h = 200$ µm, $R = 15$ mm, and a range of adhesion energy as shown. Shell detachment follows path FGHJ. Grey curve and symbols shows the trajectory of fixed load pull-off. Adapted from [80].
Figure 27: Universal shell adhesion behavior in terms of normalized quantities. Error bars denote uncertainties due to limited number of elements used in computation. (a) Normalized contact radius as a function of external load (b) Normalized approach distance as a function of contact radius. (c) External load as a function of approach distance. Pull-off under fixed load occurs at the most negative tension at $P^*$. 
2.5 Lens Adhesion Characterization: Planar Adhesion Test (PAT)

2.5.1 Introduction

This section covers the experimental protocols and techniques behind the Planar Adhesion Test (PAT), as well as the data analysis to calculate the adhesion energy, $\gamma$, and the pull-off force, $P^*$. The content discussed in Chapters 2.1 to 2.4 are all crucial and serve either as inputs or analysis methods utilized in tandem with the experimental protocols and data produced by the PAT. The elastic modulus calculated by PPC in Section 2.2 is needed to as an input into the JKR shell model in Section 2.4, and the diopter mechanical effect of Section 2.3 is necessary to explore the overall diopter effect on lens adhesion-mechanics. This chapter culminates with the development of the PAT to measure $\gamma$ and $P^*$, which serve as novel metrics to quantitatively characterize hydrogel lens adhesion.

2.5.2 Experimental Set Up

The experimental configuration for PPC discussed in Section 2.2.5 must be modified to account for the tensile adhesion force that will act on the lens under the PAT. The hydrogel lenses were held in a custom built fixture that clamped the lenses at the rim by two O-rings with carefully chosen diameters such that they apply pressure perpendicular to the lens surface without introducing any wrinkles (Figure 28). Similar to PPC, all measurements were performed using an Agilent T150 Universal Testing Machine with force and displacement resolutions of 30nN and 10nm, respectively. The clamped lens was pushed against the stationary planar substrate at an actuation speed of 1mm.min$^{-1}$ under ambience conditions of 22°C and ~50% relative humidity. A sheet of rubber was used as the adhesion substrate, with a wetting angle of ~45° as to be not too hydrophobic or hydrophilic. The rubber sheet was glued to the planar
surface of an aluminum flat punch with diameter 1.5cm, and was thoroughly washed with
deionized water between experiments to ensure similar surface conditions. The fixture was
designed with an optical line of site to beneath the lens center/apex to observe the lens-substrate
interface. A boroscope (frame rate = 30fps) was mounted directly underneath the lens with a
light source to capture the contact area between the lens and substrate. The contact radius, $a$, was
measured by using a known length scale on the surface of the rubber sheet and by simple pixel
scaling.

![Figure 28: Detailed schematic and photos of the PAT set up. Left: a slightly modified lens
holder applies uniform and normal pressure via O-rings to gently clamp the lens as a
tensile force $P$ is applied to the lens. Middle: Photograph showing the lens holder, lens,
planar substrate, and camera/light source below the lens, scale bar 1 cm. Right: close up,
long focal micrograph of the lens and substrate.](image)

2.5.3 Sample preparation

The same brands of contact lenses, Acuvue TruEye (Narafilcon-A, $R = 8.5$mm) and
Acuvue2 (Etafilcon-A, $R = 8.3$mm), both with an optical power of $d = -1$ diopter were
characterized via PAT. Lenses with $d = 0$ would be shells with uniform thickness and are ideal
to measure lens adhesion and to validate the JKR shell model. However, they are not made by
the manufacturers, and therefore $d = -1$ was picked due to the near uniform lens thickness profile
(Figure 17 shows for TruEye lenses, $d = -1$ has near uniform thickness for approximately across
the central ~50% of \( h(r) \), which is approximately the maximum contact area between the lens-substrate interface, shown in Figure 33). The elastic modulus of each lens was measured by PPC outlined in section 2.2, with \( E_{\text{TruEye}} = 0.68 \pm 0.04 \text{ MPa} \) and \( E_{\text{Acuvue2}} = 0.29 \pm 0.01 \text{ MPa} \). The measured \( R, E \) and \( h_0 \) are summarized in Table 1. It is well known that hydrogel lenses in general undergo rapid rates of dehydration, and that dehydration has a significant effect on their mechanical properties [77, 97, 98]. Furthermore, the dehydration has an effect on the hydrogel lens adhesion behavior. To ensure repeatability and consistency between lens brands and experiments, great care was taken such that the time between removal of the lens from the solution bath to the point of first contact, “pull-in”, was 4 min ± 10s.

### 2.5.4 Force measurement

The rubber substrate was compressed against the hydrogel lens until a maximum load of 0.3mN was reached, at which point the substrate retracted away from the lens and the unloading sequence began. Tension was applied till the sample lens spontaneously detached from the rubber substrate at the “pull-off” moment. The vertical approach distance, \( w_0 \), and the compressive / tensile force, \( P \), were simultaneously measured during the loading-unloading cycle. The camera mounted below the lens and its fixture captured the in-situ contact area at the lens-substrate interface during loading-unloading. The optical image was analyzed using a custom MATLAB code that correlated the manually measured contact radius, \( a \), to the automatically measured \( P \) and \( w_0 \) from the UTM. Thus, \( a \) was measured as a function of \( P \) and \( w_0 \). After the relation of \( P(w_0, a) \) was obtained, and the critical \( P^*, w_0^* \), and \( a^* \) at “pull-off” were recorded. Force measurements were repeated three times for each lens and five lenses from each of the two brands to ensure repeatable and statistically meaningful results.
2.5.4 Results and Analysis

Figure 29 shows the typical measurements of $P(w_0)$ for both TruEye (yellow) and Acuvue2 (red) lenses. As the planar substrate approached the lens from a distance, no force was measured. The intersurface separation reached a threshold when the long range surface attraction became dominant, at which point “pull-in” occurred. The lens jumped spontaneously into contact with the substrate, creating a non-zero contact circle and a sudden spike of tensile force recorded by the load cell at $w_0 = 0$, shown as A in the figures. The actuator further compressed the lens.
and the external compressive load increases with an increasing contact radius along ABC until $P_{\text{max}} = 0.3\text{mN}$ at C. Figure 32 show the corresponding sequential images ABCDEFGH of the lens-substrate contact area during the compression-tension process. Unloading then took place with the lens driven in the opposite direction with a decreasing $w_0$. The applied load vanished at a nonzero $w_0$ indicating hysteresis in the loading-unloading cycle. Further lowering of the lens shrank the contact area slightly and the tension increased significantly along CDE. At E, a maximum tension, $P^*$, was reached exceeding the initial “jump-in” at A, while $w_0$ remained positive. If the adhesion measurement was performed for a force-controlled configuration, “pull-off” or spontaneous detachment is expected at E. In our displacement controlled measurements, delamination continued while tension diminished along EFG. At F, a nonzero $P$ is necessary to ensure equilibrium though $w_0 = 0$. At G, the lens essentially detached from the rubber substrate, but the meniscus contracted to a water pillar bridging the opposite surfaces and $P$ remained nonzero. Instability finally sets in at H upon a slight decrease in $w_0$, the pillar collapsed and $P$ suddenly vanished at the fixed grips “pull-off”. The area enclosed by $P(w_0)$ represents the total mechanical energy needed to detach the lens from an adhering surface. The averages of the fifteen pull-off events are summarized in Figure 30. PAT measures distinct pull-off forces ($p < 0.01$ for an unpaired student’s T-test) for the two lenses. The pull-off force for TruEye was measured to be $P^* = 4.65 \pm 0.41 \text{mN}$, and for Acuvue2 $P^* = 3.75 \pm 0.29 \text{mN}$. 
Figure 30: Pull-off forces for TruEye and Acuvue2 lenses, $p < 0.01$.

Figure 31: Zoomed in contact area from C in Figure 28, edited for clarity. The lens-substrate contact area is outlined in the inner yellow dotted line and the diameter is labeled as $2a$. The outer red dashed line highlights the optical interference due to the meniscus that surrounds the lens-substrate contact area. Scale bar is 300 µm.

Figure 31 is an expanded image of C from Figure 29, which is at $P_{max}$, showing apparently two concentric “contact” circles. The inner circle of diameter $2a$ indicates the lens-
substrate contact area, while the outer ring is a result of the water meniscus formed at the narrow cusp due to atmospheric moisture or water being expelled from the hydrogel matrix upon mechanical compression. The annular region virtually denotes a cohesive zone at the contact edge. This outer apparent radius is present throughout the unloading of the PAT, although at times is difficult to distinguish from the true contact radius. Images of the lens-substrate interface throughout the entire unloading sequence, A-I, are shown in Figure 32.

Figure 32: A-H: Typical unloading image sequence of an Acuvue2 contact lens under planar substrate compression. A. Pull-in of the lens to the planar substrate at \( w_o = 0 \) \( \mu \text{m} \), B. Loading at \( w_o = 300 \) \( \mu \text{m} \), C. Maximum load at \( w_o = 650 \) \( \mu \text{m} \), D. Unloading at \( w_o = 465 \) \( \mu \text{m} \), E. Pull-off at \( w_o = 210 \) \( \mu \text{m} \), F. Unloading at \( w_o = 0 \) \( \mu \text{m} \), G. Meniscus pillar begins to dominate unloading at \( w_o = -180 \) \( \mu \text{m} \). I. Complete lens delamination from the planar substrate at \( w_o = -250 \) \( \mu \text{m} \).
Figure 33: $a(P)$ detachment curves for TruEye (yellow) and Acuvue2 (red) lenses against a planar surface. For each brand, 10 detachment events over five separate lenses are conducted and analyzed.

Figure 34: $P(w_0)$ detachment curves for TruEye (yellow) and Acuvue2 (red) lenses.
From the force-displacement and contact radius data, the inter-relationships between \( a, P, \) and \( w_0 \) can be formed. The measured \( a(P) \) of the unloading portion of the PAT is shown in Figure 33. Similarly, the \( P(w_0) \) and \( w_0(a) \) detachment curves are shown in Figure 34 and Figure 35. Though there is a fair amount of spread for each lens data set, there are two distinct populations of detachment curves. For the measured \( a(P) \), each brand of hydrogel lens exhibits the typical C-curve of the JKR-shell model discussed in section 2.4.3 (Figure 26). At the onset of unloading (top half of the “C-curve”), there is a drastic change from compressive to tensile load \( P \) with very little change in contact radius. This is due to stiction between the lens and substrate at the interface. Despite having distinct pull-off forces, \( P^* \), the contact radius at pull-off \( a^* \) is approximately the same for the two lenses, \( a^* \approx 2500\mu m \). After \( P^* \) is reached, data is still measured on the lower-half of the C-curve due to our displacement-controlled experiment, allowing us to measure contact radius until complete spontaneous detachment is achieved (\( P \sim 2mN \)). Similarly, the \( P(w_0) \) detachment curve in Figure 34 exhibits the typical J-curve and \( w_0(a) \).
in Figure 34 shows non-linear behavior, which are predicted by the JKR shell model (Figure 26). To calculate the adhesion energy, $\gamma$, of the lenses, we implement the non-dimensionalizing scheme proposed by Shi et al.[80] and given by Eqs. 18-20. All the material properties are known: $R$ and $h(r = 0)$ are measured directly, it is assumed $\nu = 0.5$, and $E$ is measured via PPC, leaving $\gamma$ as the only unknown quantity and lone fitting parameter. By non-dimensionalizing our experimental $a(P)$ data according to Eqs. 18-20, we can obtain $a(P)$ by selecting a $\gamma$ for each lens that collapses the two data sets onto the predicted universal detachment curve (Figure 27).

![Figure 36: Experimental $a$ vs. $P$ data from Figure 32, non-dimensionalized according to Eqs. 18-20, and compared to the universal curve predicted by Shi et al. is shown as the grey line [80]. Acuvue2 (red circles) fits best with $\gamma = 0.080 \pm 4.67 \times 10^{-3}$ J/m², and TruEye (yellow squares) fits best with $\gamma = 0.095 \pm 6.09 \times 10^{-3}$ J/m². The vertical dashed line highlights $P = 0$ and $P^*$ and $a^*$ are highlighted as larger symbols with error bars.](image-url)
The corresponding $a(P)$ plots in Figure 36 show only the unloading or detachment process and is consistent with the JKR-shell model of Figure 27, which is shown as the gray curve. At the onset of unloading with maximum compressive load, $P$ quickly vanishes as highlighted by the vertical dashed line, yet $a$ remains non-zero due to the interfacial adhesion. Fairly small shrinking of the contact area occurred until the tensile load reaches a maximum at $P^*$, indicating “pull-off” under fixed load configuration. Though the two contact lens brands possess different $E$, $\gamma$, $P(w_0)$ and $a(P)$, the normalized $a(P)$ overlapped with one another, especially at $P^*$. The pull-off load for TruEye was measured to be $P^* = 4.65 \pm 0.41$ mN corresponding to $\gamma = 95 \pm 6.09$ mJ/m$^2$, while Acuvue2 yielded $P^* = 3.75 \pm 0.29$ mN and $\gamma = 80 \pm 4.67$ mJ/m$^2$, with $p < 0.01$ for an unpaired Student’s T-test. Our displacement controlled measurement allowed further delamination at decreasing external load to be measured along the lower half of the C-curve of $a(P)$. A comprehensive summary of the material and interfacial properties calculated in Chapter 2 for both Acuvue2 and TruEye lenses are given in Table 1.

**Table 1: Summary of both measured and calculated material properties. Optical coherence tomography is used to measure shell thickness ‡, and PPC to measure elastic modulus §. Adhesion energy is deduced using the shell adhesion model †.**

<table>
<thead>
<tr>
<th></th>
<th>Acuvue2 (Etafilcon A)</th>
<th>TruEye (Narafilcon A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$ (mm)</td>
<td>8.3</td>
<td>8.5</td>
</tr>
<tr>
<td>$h_0$ (µm)‡</td>
<td>130.0</td>
<td>80.0</td>
</tr>
<tr>
<td>$E$ (MPa)§</td>
<td>0.29 ± 0.01</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>$P^*$ (mN)</td>
<td>3.75 ± 0.29</td>
<td>4.65 ± 0.411</td>
</tr>
<tr>
<td>$\gamma$ (J/m$^2$)†</td>
<td>0.08 ± 4.67 x10$^{-3}$</td>
<td>$\gamma = 0.095 ± 6.09$ x10$^{-3}$</td>
</tr>
</tbody>
</table>
2.5.5 Discussion

The PAT method is versatile enough to measure both elastic deformation and adhesion-detachment with a separate planar substrate. The relatively simple experimental set up, repeatability, and ease of analysis via JKR-shell model non-dimensionalization make PAT a valuable tool to characterize hydrogel lens adhesion-mechanics. The TruEye and Acuvue2 lens detachment data \( a(P) \) successfully converted to \( a(P) \) is the first experimental verification of the JKR-shell model proposed by Shi et al. [80]. Despite relatively good agreement of the experimental data collapsing onto a single universal detachment curve, a number of limitations are inevitable. First, the linear elastic model might not be a universally acceptable model for hydrogel shells largely due to the fact that hydrogels exhibit complex visco-poroelastic behavior. Despite this distinction between modeled materials versus actual material properties, the linear elastic shell model allows the preliminary assessment of these materials and captures the complex behavior fairly well.

Secondly, hydrogels are prone to dehydration that results in significant effects on the lens mechanical properties and adhesion [77, 97, 98]. In fact, the rate of dehydration is typically quite high for hydrogel lenses, where significant dehydration can occur on the order \( O \sim 1-10\text{min} \) [97, 98]. Our tests take approximately 1-1.5min from the time of pull-in to complete delamination, suggesting that dehydration could introduce subtle changes in the adhesion-mechanics not captured by the JKR-shell model. Furthermore, when the contact lens is worn \textit{in vivo}, the convex surface is exposed to a mixture of the ambient atmosphere and is constantly rehydrated via blinking and subsequent reapplication of the tear film. Meanwhile, the concave side is in contact with the corneal epithelium in the presence of fluid tear film. It is after all difficult to quantify dehydration and to simulate the actual environment the lens is exposed to \textit{in vivo}. Thus, a
question of the applicability of the PAT derived adhesion mechanics pertaining towards lens
adhesion behavior on-eye, which is addressed in aims 2 and 3 of this thesis.

Third, the presence of meniscus and the finite cohesive annulus around the contact edge
is not accounted for by the JKR-shell model, which is based on zero-range surface force. During
lens-substrate contact, the liquid film forms a meniscus around the contact periphery shown in
Figure 31, the surface tension of which is present in addition to the intrinsic adhesion when the
force is measured. In fact, “pull-in” occurs before the opposing surfaces come into intimate
contact is the result of these long range surface force. Though it is possible to incorporate
cohesive zone and long range attraction into the JKR-shell model, the sophisticated but
comprehensive model will be mathematically involved requiring an intensive numerical
approach. The proposed relatively simple data analysis via a linear elastic JKR-shell model is
sufficient to capture distinct hydrogel detachment events and calculate their adhesion energies.

Another closely related shortcoming is the incapability of the JKR-shell model to account
for shell thickness variation \( h(r) \), \textit{i.e.} contact lenses with nonzero optical power. The current
JKR-shell model utilizes a uniform shell thickness for all calculations. As the contact circle
shrinks under external tension, the mechanical resistance to shell bending at the contact edge
depends significantly on \( h(r) \), which is explored in Chapter 2.6. Again, a comprehensive solid
mechanics model is beyond the scope of the current work.

2.5.6 Conclusion

The adhesion of hydrogel contact lenses have been characterized with a novel
experimental protocol, the PAT, in conjunction with a previously established JKR-shell model.
The adhesion is characterized in two ways: measurement of pull-off force \( P^* \), and measurement
of the adhesion energy $\gamma$ via a JKR-shell model. $\gamma$ is an intrinsic property of the particular lens/substrate materials involved, independent of the lens mechanical and geometrical properties, though the all three influence the overall adhesion-mechanics of lens delamination from a planar substrate. This stands as the first quantitative adhesion characterization technique of hydrogel contact lenses, which could lend useful information to manufacturing processes as well as help understand lens related complications in which the adhesion is believed to play a role.

2.6 Adhesion Diopter Effect

2.6.1 Introduction

This chapter investigates the effect of optical power/diopter, $d$, has on the hydrogel lens adhesion behavior. In a similar fashion, it was shown in Chapter 2.3 that the diopter $d$ can greatly alter the hydrogel lens mechanical behavior, despite having the same mechanical properties ($E$). In Chapter 2.5, the adhesion energy $\gamma$ was calculated via PAT by the inter-relationship between applied force, $P$, approach distance, $w_0$, and the contact radius between the lens-substrate interface $a$. Particularly, TruEye (Narafilcon A) was shown to have an adhesion energy $\gamma = 95$ mJ/m$^2$. However, the JKR shell model utilizes a uniform thickness shell. In this chapter, it is shown that the thickness profile $h(r)$, or optical power $d$, can greatly affect the overall detachment adhesion behavior, which could factor into on-eye behavior in which adhesion plays a role, such as user comfort or ocular complications.

2.6.2 Experimental Setup

To explore the diopter effect on lens adhesion behavior, the same PAT protocol configuration was utilized, as described in Chapters 2.5.2-2.5.4 and shown in Figure 28.
2.6.3 Sample Preparation

TruEye lenses were used to have a comprehensive analysis of lens adhesion-mechanics. The same range and values of $d$ from Chapter 2.3 were chosen, $d = -6, -3, -1, +3, +6$. The thickness profiles, $h(r)$ are shown in Figure 17 as measured by OCT. Though different optical powers, these lenses all have the same elastic modulus and adhesion energy, $E = 0.68$ MPa and $\gamma = 95$ mJ/m². All lens material properties are summarized in Table 1.

2.6.4 Force Measurements

Similar to Chapter 2.5, a rubber substrate with wetting angle of approximately 45° was used for the adhesion tests. To ensure repeatability and consistency between experiments, great care was taken such that the time between removal of the lens from the solution bath to the point of first contact, “pull-in”, was 4 min ± 10s. The lenses were compressed until a maximum load $P_{max} = 0.3$mN was reached, at which point the substrate retracted away from the lens. A boroscope captured the contact area as a function of applied load, and the inter-relationships $a(P, w_0)$ were established. For each diopter, five separate lenses were tested three times each to ensure statistically meaningful results.

2.6.5 Results

The lens thickness profiles $h(r)$ of all diopters are shown in Figure 17. Representative $P(w_0)$ curves for TruEye lenses of various diopters are shown in Figure 37. No force was measured during pre-travel, and as the planar substrate approaches the lens the long range surface attraction forces become dominant, at which point “pull-in” occurs. Each brand exhibits
a distinct “pull-in” negative tensile force as well as initial contact radius after spontaneous contact is made. The different “pull-in” forces and initial contact radii is due to a balance between the inertia of the central lens region and the adhesion forces. Lenses of positive diopter have a larger central thickness and more mass compared to lenses of negative diopter, and thus have a smaller “pull-in” force and initial contact radius compared to lenses of the same \( \gamma \) but lower diopter. All lenses are compressed further after “pull-in”, until \( P_{\text{max}} = 0.3 \text{mN} \) is reached, at which point unloading begins. At zero load, there is still a non-zero contact radius indicating hysteresis across all lens diopters. As \( P < 0 \) and tensile load is applied to the lens, distinct \( P^* \) are measured for each diopter. All lenses with \( d < +6 \) exhibit a smooth detachment curve after “pull-off” due to the presence of a meniscus outside of the contact area. \( d = +6 \) exhibits a sudden “snap-out” moment at approximately \(-200 < w_0 < -100\) due to the extremely large central thickness and the lens inertia is able to overcome the adhesion forces. The summary of all lens detachment curves are shown in Figure 38. All diopters exhibit the typical c-curve shape predicted by the JKR-shell model, but the scale varies dramatically with alterations in \( d \). The range of pull-off forces measured are approximately \( 3 < P^* < 5 \text{ mN} \) spanning contact radii at pull-off of approximately \( 1 < a^* < 2.5 \text{ mm} \). At the onset of unloading (top half of the “C-curve”), all diopters exhibit a drastic change from compressive to tensile load \( P \) with very little change in contact radius for all diopters due to stiction between the lens and substrate at the interface. All diopters exhibit a relatively large degree of spread in detachment data, where diopters drastically different have distinct detachment behavior (\( i.e. +6 \) and -1) while diopters spanning less range exhibit less distinct behavior (\( i.e. -3, -6 \)).
Figure 37: Representative $P(w_0)$ curves for TruEye lenses spanning $d = -6$, -3, -1, +3, +6. For each diopter, five separate lenses are tested three times each.

Figure 38: Comprehensive $a(P)$ curves for TruEye lenses spanning $d = -6$, -3, -1, +3, +6. All are comprised of Narafilcon-A with $E = 0.68$ MPa and $\gamma = 95$ mJ/m².
Figure 39 shows the summary of $P^*$ as a function of diopter, $P^*(d)$ in yellow, superimposed with the central thickness of the lens $h_0(d)$ in red (shown in Figure 17). It is clear that there $P^*$ is inversely proportional to $h_0$, which can be explained by the JKR-shell model. Eq. 15 shows that lenses that are stiffer or thicker will have lower pull-off force values, and lenses with larger adhesion energies or larger base radius curvatures will have higher pull-off forces. Specifically, the JKR-shell model predicts that $P^* \propto h_0^{-2/3}$. A comparison between the uniform thickness $P^*$ predicted by Eq. 15 and the experimentally measured $P^*$ as a function of central lens thickness, $P^*(h_0)$, is shown in Figure 40. All material properties of the TruEye lenses are calculated from Table 1, transforming Eq. 15 into $P^* = 0.0102 \cdot h_0^{-2/3}$. Despite the JKR-shell model’s restriction to uniform thickness shells, Figure 40 shows good agreement of $P^*(h_0)$ between theory and experimental results. This is due to the fact that $h(r)$ is near uniform in the central region of all lenses, and that this near-uniform thickness in $h(r)$ extends beyond the maximum contact radius between the lens-substrate interface, hence $h_0$ is a first-order approximation of $h(r)$ (see Figure 17).
Figure 39: $P^*(d)$ (yellow) superimposed with $h_0(d)$ (red). Central thickness measurements are adopted from the OCT measurements in Figure 16.

Figure 40: $P^*(h_0)$ as predicted by Eq. 15 (gray line) and measured experimentally (yellow dots). All lens material property values were taken from Table 1.
2.6.6 Discussion

In Chapter 2.3 it was shown that the diopter has a substantial effect on the lens mechanical behavior, despite having the exact same mechanical/material properties. Similarly, Figure 37, Figure 38, Figure 39 and Figure 40 show that the diopter greatly affects the lens adhesion behavior, despite having the same adhesion energy across diopters. One of the main limitations of the JKR-shell model is the inability to account for shells of non-uniform thickness profiles. Despite this limitation, the JKR-shell model can predict experimental outcomes using the central thickness $h_0$ alone as a first-order approximation to calculate $P^*$, as shown in Figure 40. Modifications to the JKR-shell model can be made to account for non-uniform shell thickness; however, a rigorous and comprehensive solid mechanics model is beyond the scope of the current work.

The adhesion diopter effect elucidates some of the complexity of macroscopic hydrogel lens adhesion, in which both $\gamma$ and $P^*$ must be considered for applicability to \textit{in vivo} behavior. There have been relatively few studies into measuring adhesion forces on hydrogel contact lenses. The majority of these studies focus on microscopic adhesion force measurements, geared towards specific bacteria strains and their adhesion to the hydrogel surface \cite{55, 81}. Typically, the adhesion is characterized by AFM measured pull-off force, $P^*$, of either a regular cantilever or a specially functionalized cantilever with a bacteria cell attached to it, known as Single Cell Force Spectroscopy (SCFS). Here, in the context of the PAT configuration, we show that the lens geometry, $d$, can greatly alter the macroscopic adhesion behavior of the lens. Specifically, even with the same surface chemistry, different adhesion forces are required for full detachment for different geometrical configurations of the lens. It is clear that the on-eye, \textit{in vivo} configuration is vastly different than the PAT configuration. However, this study sheds light on the role of
shell geometry/d can have on overall lens adhesion-mechanics, and that d should be considered in clinical studies which investigate lens-related ocular complications in which the lens adhesion properties are believed to play a role, such as epithelial thinning [12, 17].

2.7 Aim 1 Concluding remarks and future work

The crux of the first aim of this thesis in Chapter 2 established novel experimental protocols and delivered metrics to characterize hydrogel lens adhesion mechanics. Two distinct data sets from two different hydrogel lens materials exhibit detachment data that collapsed to the “master curve” predicted by the JKR-shell model, which serves as first experimental verification of the work of Shi et al. [80]. Through the PAT, both γ and P* characterize hydrogel lens adhesion. γ is unique to the specific hydrogel and the surface with which the PAT was conducted with, in this case rubber. PAT using such a simple substrate could be useful to establish a baseline of adhesion energies and to compare the adhesion energies of different lens materials, brands, and types. The “pull-off” force P* was shown to be not only a function of lens material, but also lens geometry/optical power. The novel mechanical and adhesion characterization via PAT could be important in many industrial and clinical applications. Mechanical characterization that utilizes the in-situ geometry of the lens is a better standard when correlations are made between lens mechanical properties and ocular complication incidence rates. Furthermore, the diopter effect shows for the first time, clinicians must consider d for incidence rates to elucidate mechanisms behind ocular complications, such as SEALS, CLPC, or epithelial thinning [12, 17]. There are many clinical applications in which accurate adhesion characterization is necessary, such as microbial keratitis (MK) or contact lens acute red eye (CLARE). γ derived via PAT could be useful as a basic material property that influences bacterial adhesion in vivo, which is
currently poorly understood [10, 58]. γ could be measured in tandem with large scale microbial adhesion tests to help understand bacterial adhesion and biofilm formation on hydrogel contact lenses. Furthermore, γ could be useful to study the onset of corneal erosions, offering clinicians a metric of hydrogel lens adhesion as a variable in the onset and progression of the complication [17, 22, 23]. Aim 1 established the framework of the PAT, and Aims 2 and 3 develop in vitro assays to characterize hydrogel adhesion in a more physiologically relevant context: multi/single-cellular adhesion properties of hydrogel contact lenses.

The PAT and the adhesion-mechanical metrics γ and P* serve as a first step and establish the potential of this technique, and there are many facets that could be improved upon to yield more accurate results. To make the PAT experimental configuration more accurate and assert more control over the dynamic hydrogel adhesions state, an environmental chamber could be constructed around the PAT/UTM set up to precisely control relative humidity and temperature, which will likely ensure higher degrees of repeatability between experiments, although as shown here statistical significance is already achieved with 15 tests over five different lenses. Although the simple linearly elastic JKR-shell model is adequate to capture the hydrogel adhesion mechanics, there is concern about the meniscus present on the hydrogel surface lending to long-range attractive forces outside the cohesive zone. Future work could add onto the JKR-shell theory to model these long-range attractive forces through intensive and rigorous numerical modeling. Utilizing a shell adhesion model that accounts for lenses of non-uniform thickness h(r) is necessary to fully characterize the lens adhesion-mechanical diopter effect, however it was shown that the central thickness is a good first-order approximation. Both mechanical and adhesion diopter effects should be expanded to a wider range of diopter, as commercially available contact lenses span d = -12 to d= +8 with 0.25 increments for full characterization.
Finally, the work in this chapter establishes the PAT theoretical and experimental framework and characterized two hydrogel lenses, which could easily be expanded upon to include a wide array of different commercially available contact lenses, as well as industrially and biomedical relevant substrates. For instance, the PAT could be used against planar substrates of lens packaging materials, such as the plastic used for the disposable lens container or the lid stock to optimize material selection and reduce unwanted lens adhesion to the container during large scale manufacturing, shipment, and storage.
CHAPTER 3: AIM 2 – BIOSAMPLE ADHESION TEST

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3.1 Overview

This chapter focuses on characterizing hydrogel lens adhesion against macroscopic biological samples. The first aim of this thesis developed novel methodologies and utilized rigorous numerical models to reliably measure the adhesion energies and “pull-off” forces of lenses against a planar surface. The values $\gamma$ and $P^*$ are specific to the hydrogel material and the substrate surface used to conduct the PAT, in this case rubber. The PAT against simple surfaces could be useful for manufacturing considerations, such as trying to minimize lens adhesion to packaging materials. However, it is unclear if the adhesion characterization by the PAT on such simple surfaces translates to the on-eye lens adhesion behavior in vivo, which could be important to a plethora of lens-related ocular complications [12, 17, 22]. To investigate hydrogel lens adhesion in a more physiologically relevant context, novel protocols and analysis are developed in this chapter to explore lens adhesion against biological samples in vitro. Specifically, human corneas will be utilized for characterization to help elucidate hydrogel lens adhesion behavior in vitro.

3.2 Introduction and previous research

Hydrogel contact lens adhesion is important for many manufacturing considerations, user comfort, and could be a factor in the etiology of several lens-related ocular complications [10, 12, 17, 23, 79]. In particular, it is possible that the lens adhesion could play a role in the onset and progression of epithelial thinning/corneal erosions, in which it is hypothesized that sections
of the corneal epithelium strongly attach to the lens and are disrupted during typical on-eye movement [12, 17, 22]. Despite this concern, cellular adhesion properties with hydrogel lens materials is still largely un-characterized, and the role of adhesion in the etiology of lens related complications is still unknown [22]. Previous research efforts have been made to investigate cell adhesion to lens materials, however it is largely in the context of either lens-bacteria adhesion or Intraocular Lens (IOL) material-cell adhesion. Cunanan et al. developed an in vitro model to assess epithelial cell adhesion to a variety of IOL materials, such as Lidofilcon A [99]. However, Cunanan et al. characterized adhesion indirectly by measuring the number of epithelial cells present on the material surface after a specific incubation period in culture media [99], similar to standard large scale microbial adhesion assays [10]. Lloyd et al. investigated epithelial cell adhesion to hydrogel lenses by similar passive incubation assays, as well as qualitatively measured the damage to confluent endothelial cell cultures after prolonged contact with hydrogel lenses [100]. However, these studies utilize passive means of characterizing adhesion, and thus do not address the physical adhesion forces between the lens materials and multi-cellular tissues.

To the best of our knowledge, only Mateo et al. quantitatively investigated biomaterial adhesion and mechanical properties and their effect on corneal cells/tissues, however it was in the context of IOL materials against rabbit endothelium [101]. Thus, to characterize hydrogel adhesion against multi-cellular biological structures and shed light on the in vivo lens adhesion behavior, two macroscopic in vitro adhesion tests are proposed in chapter 3. First, lens adhesion is characterized against excised human corneas, in which the “pull-off” force $P^*$ can be precisely measured. Second, confluent layers of cultured HCE cells are utilized to quantitatively investigate cell attachment to the hydrogel lens surface and disruption from the culture substrate.
Together, these two adhesion assays elucidate these hydrogel materials behaviors \textit{in vitro}, and can potentially elucidate the material \textit{in vivo} clinical performance.

### 3.3 Experimental protocols

#### Hydrogel Contact Lenses

To characterize hydrogel contact lens adhesion on human corneas, Acuvue TruEye (Narafilcon-A, Vistakon) and Acuvue2 (Etafilcon-A, Vistakon), are characterized for a full comparison between all three aims in this thesis. It was found that the adhesion energy of TruEye and Acuvue2 against rubber substrates were \( \gamma = 95 \text{ mJ/m}^2 \) and \( \gamma = 80 \text{ mJ/m}^2 \), respectively. All adhesion tests were done at ambient temperature of 22ºC and approximately 50% relative humidity. Lenses with \( d = -1 \) were chosen due to their near-to-uniform thickness profile and simplicity. A total of \( N = 10 \) fresh lenses were tested for each brand. Detailed summaries of the hydrogel lens properties are given in Table 1.

#### Human Corneas

A total of ten human corneas were utilized to characterize hydrogel lens adhesion (see Figure 41). The corneas were generously donated from the Lions Eye Bank of Delaware Valley (Philadelphia, PA) and from Dr. Jeffrey Ruberti and Dr. Monica Susilo. The corneas ranged from both male and female donors 59-72 years old. Each cornea was stored in 4ºC and only opened immediately prior to adhesion testing.
Figure 41: Light micrograph of a human corneal stroma. The quality/state of the epithelium is difficult to characterize, as each cornea likely went through slightly different extraction/handling procedures and adhesion testing is done at varied times from corneal extractions (~2 months +/- 7 days after donation). Scale bar 100µm. Inset: Entire human cornea, radius of curvature approximately 0.75cm.

Experimental Set Up

The lens-cornea adhesion test set up is shown in Figure 42. All experiments are conducted on an Agilent T150 UTM Nanobionix in ambient conditions (22°C and ~50% relative humidity). The experimental configuration is extremely similar to the PAT (see Figure 28), with the exception that the planar rubber substrate is essentially replaced with a human cornea sample supported by a stainless steel ball bearing. The hydrogel contact lens (CL) is clamped at the periphery by a custom built 3D printed fixture that applies uniform normal stress via O-rings, and is subsequently attached directly to the UTM load cell. Due to size and consistency variations of the donated cornea samples, a rigid substrate was chosen to support and hold the
cornea in lieu of a custom clamping fixture. Similar to the PAT, the mechanical and adhesion properties of both the corneal tissue as well as the hydrogel lens are functions of their relative state of dehydration. Thus, to ensure consistency and repeatability between experiments, the time from removal of both the cornea tissue and the hydrogel CL to the time of initial contact (“Pull-in”) was kept at 5 min ± 25s. To keep consistency with prior testing via PAT, the tests were conducted with a velocity of $v = 1\text{mm/min}$. A high magnification digital USB microscope (20x-200x) was mounted perpendicular to the lens fixture to continuously monitor and capture the CL-cornea interface and contact area. The cornea was compressed against the CL/load cell until a maximum force of $P_{\text{max}} = 0.3\text{mN}$ was reached, at which point the cornea retracted away from the CL. Each single adhesion test took approximately 11 minutes to conduct, during which both the CL and cornea were dehydrating in the ambient environment. After an individual test, both CL and cornea samples were immediately placed back in their respective solution baths and allowed approximately 5 minutes to help ensure adequate swelling to achieve as similar initial conditions as possible in between individual tests.
Figure 42: Experimental set up for the hydrogel lens adhesion test with human corneas. A) Custom built lens fixture that applies normal pressure via O-rings at the lens periphery to securely clamp the hydrogel contact lens, adapted from [102]. B) Hydrogel lens-cornea adhesion test. The clamping fixture holds the contact lens (CL) and is then attached directly to the Agilent UTM load cell, while the cornea is placed on a solid spherical support ($R = 0.75\text{cm}$, stainless steel).

### 3.4 Results

Typical loading-unloading $P(w_0)$ curves of TruEye and Acuvue2 lenses for the cornea adhesion test are shown in Figure 43. As the cornea approached the CL from a distance, zero force was measured, shown in the pre-travel area in A in Figure 43. As the cornea came within approximately $200\mu\text{m}$ of the hydrogel surface, a small negative force (~$-100\text{nN}$) was measured as the long-range surface attraction between the CL-cornea before “Pull-in”, although this did not happen every test. As the intersurface separation reached a critical threshold, the long range surface attraction became dominant and “pull-in” occurred, leading to initial contact at $w_0 = 0\mu\text{m}$ and a large negative spike of tensile force creating a non-zero contact area at B. The linear
actuator further compresses the cornea against the contact lens along BCDE, increasing the load and contact area non-linearly until $P_{\text{max}} = 0.3 \text{mN}$ is reached at E. Figure 44 shows the corresponding images ABCDEFGHI of the lens-cornea substrate contact area during the entire loading-unloading process. After maximum load is reached, unloading begins immediately as the cornea is retracted away from the hydrogel CL with decreasing $w_0$. The applied load upon unloading vanished at a larger value of $w_0$ than the initial loading segment, indicating hysteresis in the cornea-CL loading cycle. As $w_0$ decreased further, the contact area shrinks slightly as the negative tensile load increases dramatically between EF. At G, a maximum tensile load, $P^*$, is recorded that exceeds the initial “pull-in” at B, while $w_0 > 0$. Similar to the PAT, if the CL-cornea adhesion test was conducted under a load controlled mechanism, spontaneous CL-cornea detachment is expected at point G. However, due to our displacement controlled experimental set up, further CL-cornea delamination is recorded along GHI. Between GH, there is a large loss of tensile load over a relatively small change in $w_0$ as the adhesion forces between the CL-cornea can no longer compete against the bending rigidity of the CL, which begins to dominate and resume a normal CL curvature. At H, a nonzero $P$ is recorded due to adhesion forces. For Acuvue2, at $w_0 = 0 \text{μm}$ the negative tensile load at H is nearly equal to that at “pull-in”, however this is not the case for TruEye lenses. At H, the hydrogel CL essentially detaches from the corneal surface, however the meniscus due to fluids on both the CL and cornea surface contract into a fluid pillar that bridges the two surfaces, and $P$ remains non-zero. At I, the recorded tensile load is solely due to surface tension of this fluid pillar and finally collapses at around $w_0 = -500 \text{μm}$ for Acuvue2 lenses and $w_0 = -750 \text{μm}$ for TruEye lenses. The averages of forty pull-off events for the hydrogel CL materials across ten human corneas are summarized in Figure 45. The hydrogel CL-cornea adhesion test measures distinct “pull-off” forces ($p < 0.01$ for an unpaired
student’s T-test) for the two CL materials. The “pull-off” force for TruEye was measured to be 
\( P^* = 2.78 \pm 0.19 \) mN, and for Acuvue2 \( P^* = 2.40 \pm 0.20 \) mN. The area enclosed by the 
individual \( P(w_0) \) curves is the total mechanical energy needed to detach that particular hydrogel 
CL from that particular corneal surface.

Figure 43: \( P-w_0 \) curves for TruEye/Narafilcon A (yellow) and Acuvue2/Etafilcon A (red) 
against a human cornea. The highlighted red dots correspond to the Acuvue2 image 
sequence in Figure 44.
Figure 44: Low magnification micrographs capturing the CL-cornea contact area interface of an Acuvue2 lens, corresponding to the $P-w_0$ curve. A-E is the loading portion, and E-I is the unloading portion. A) Pre-travel, no contact. B) Initial contact at “Pull-in”, $w_0 = 0\mu$m. C) $w_0 = 850\mu$m. D) $w_0 = 1675\mu$m. E) $P_{\text{max}} = 0.3\text{mN}$, $w_0 = 1970\mu$m. F) $w_0 = 1600\mu$m. G) “Pull-off”, $w_0 = 375\mu$m. H) $w_0 = 0\mu$m. I) $w_0 = -280\mu$m.
3.5 Discussion

The CL-cornea adhesion test produced repeatable and reliable measurements of the adhesion forces between hydrogel lens materials and human corneas. This relatively simple experimental set up is, to the best of our knowledge, the first experimental investigation into the macroscopic adhesion between hydrogel CL materials and corneal tissues. Here, it is shown that though the pull-off forces are different, the relative relationship of $P^*$ between TruEye and Acuvue2 lenses against cornea tissue correlates to the $P^*$ measurements derived from the PAT with a rubber substrate. Indeed, the results from both CL-cornea and PAT tests are remarkably similar (see Figure 45 compared to Figure 30, and Figure 43 compared to Figure 29). A shortcoming of this work is the lack of a rigorous contact area analysis to measure the contact radius as a function of applied load, $a(P)$, for each hydrogel material. This was due in part because of the experimental difficulty associated with measuring the contact diameter of the
cornea-CL interface from a side view angle. The boroscope method implemented for the PAT in Chapter 2 is not applicable in the current cornea-CL experimental set up, primarily because the cornea itself is too opaque to offer a clear line of sight necessary to distinguish the contact area from the meniscus that surrounds the CL-cornea interface. Similar to lens adhesion characterization via PAT in Chapter 2, the CL-cornea adhesion test also suffers from the presence of fluid forming a meniscus at the cornea-lens interface, adding long-range intersurface attraction forces outside of the CL-cornea contact area. Indeed, the surface tension of this meniscus contributes greatly to the total measured force between the CL and cornea. Figure 46 shows an expanded view of the contact area between an Acuvue2 lens compressed against the corneal surface with the particular values of $P = -1.83$ mN and $w_0 = 0\mu$m creating an apparent contact radius of $a \approx 1720\mu$m at the CL-cornea interface. Assuming a simplified situation that the surface tension of the meniscus acts as a line force present along the circumference of the circular contact area, $2\pi a$, and that the surface tension of the lens solution/cornea media mixture is approximately that of water ($\Gamma = 0.072$ N/m), the force due to surface tension at the CL-cornea interface is given by $P_{ST} = 2\pi a \cdot \Gamma \approx 0.78$ mN, or about 43% of the measured force of this particular CL-cornea measurement. Due to fluctuations in the ambient environment, as well as the amount of fluid on both corneal and hydrogel surfaces, the contributions of surface tension between different experiments likely varies significantly and ultimately hinders the adhesion measurement. An environmental chamber could help ensure similar contributions of surface tension forces between experiments, but would not eliminate it all together form the CL-cornea adhesion test.
Furthermore, this study is limited in its inability to calculate the adhesion energy between hydrogel CL materials and individual human corneas. The JKR-shell model [80, 88] is only suitable for a thin shell compressed against a planar, solid substrate. Substantial efforts would be required to modify the numerical model to account of a compliant spherical substrate, which is beyond the scope of this thesis. Regardless of these shortcomings, the results of the CL-cornea adhesion tests suggests that the relative adhesion behavior ($P^*$) of two hydrogel CLs tested under PAT translates over to the physiologically relevant human corneal tissue samples. Despite the use of highly regulated donated human corneas for an adhesion substrate, there are substantial differences between the CL-cornea adhesion test conducted and the CL in vivo environment on-eye. First, though the CL-cornea adhesion test might be a similar loading mechanism to lens insertion/removal from the cornea surface, it is drastically different from the in vivo loading once the lens is on the cornea surface. Typically the only mechanical deformation/loading of the CL
arises from normal and shear forces distributed from the eye lid during blinking [103]; it is very rare during normal use for CLs to undergo large deformations on the scale of hundreds of microns, such as in the conducted cornea-CL experiments. Second, the results presented here represent the adhesion behavior of two complex visco-poroelastic biomaterials [51, 77, 104] undergoing dehydration due to constant exposure to the ambient environment. This is drastically different than in vivo, in which both the cornea and CL are constantly rehydrated via application of the tear film, the thickness and composition of which influences the rate of evaporation/dehydration of the cornea [105], and is reapplied roughly every 2-10 seconds with every blink [106]. Third, the absence of critical components of the in vivo environment, such as the tear film, likely plays important roles in the overall on-eye CL adhesion behavior. Finally, there are likely substantial differences in the epithelium of the donated corneas versus the in vivo epithelium of typical healthy CL users. Indeed, the excised corneas were statically stored in tissue buffer for approximately 2 months before adhesion testing, which no doubt alters the dynamic corneal epithelium that typically sheds epithelial cells continuously from its surface and are replaced by division of basal cells [107], and is completely replaced about every four days [108].

However, despite the seemingly large difference in adhesion testing ex vivo with excised human corneas versus the CL in vivo environment, the CL-cornea test still provides a basic foundation and establishes a frame work in which the relative adhesion behavior of hydrogel CL materials can be easily and reliably tested. The relative differences in adhesion forces ex vivo could be clinically reflective of the hydrogel CL performance in vivo. The information yielded by the CL-cornea test could be useful for clinical studies in which correlations are drawn.
between CL lens material properties and incidence rates, such as corneal erosion, and thus should be accounted for.

### 3.6 AIM 2 Concluding remarks and future work

Aim 2 of this thesis introduced novel experiments to characterize hydrogel CL materials against human corneal tissue, establishing for the first time quantitative measurements of the adhesion forces necessary for CL detachment off of the corneal surface. There are many future outlets to explore that can enhance and improve the CL-cornea test. An accurate and reliable experimental apparatus to measure the contact radius, $a(P)$, in conjunction with changes to the JKR shell model to account for the corneal surface would allow for measurements of the adhesion energy, $\gamma$, between individual corneas and hydrogel CL materials. $\gamma$ is an inherent material property between the two surfaces, and is thus much more valuable than $P^*$ when drawing correlations to CL related ocular complications. Another way to improve the CL-cornea test would be to conduct the experiment in an environmental chamber that controls relative humidity and temperature, which would offer better control over the dynamic dehydration rates of the hydrogel/tissue and ensure higher degrees of repeatability between experiments. Furthermore, the time of the entire test could be decreased such that the time scale is closer to physiological CL-cornea contact times (i.e. increase the testing velocity such that tests take ~20s, roughly the upper time limit between blinks [106]).

Significant improvements could be made to the CL-cornea adhesion test with rigorous characterization of the state of the corneal epithelium both before and after a single adhesion test. This would allow for thorough assessment into the lens adhesion properties and their effect on thinning/erosion of the corneal epithelium. To characterize the state of the corneal epithelium
before adhesion testing, fluorescein staining could be conducted to ensure similar epithelium conditions between experiments [109]. After adhesion testing, the epithelium could potentially be assessed with TEM [18] or SEM [110], and epithelium depositions could be assessed with corneal staining/light microscopy (i.e. acridine orange staining [111]) or SEM as well (see Figure 47 for proof-of-concept). If characterization of donated human corneas proves to be too difficult, highly consistent commercially available Human Corneal Epithelium constructs from SkinEthic (France) could be utilized for hydrogel CL epithelium thinning investigations.

Figure 47: Proof-of-concept for SEM investigations into human corneal epithelial cell adhesion onto hydrogel CL materials. A) An Acuvue2 (EtafilconA) lens is placed convex side down onto a human cornea (69 years old) for 5 minutes. Upon lens removal from the corneal surface, the hydrogel is immediately placed in Karnovsky Fixative (see appendix for full fixation procedure). B) SEM low magnification micrograph of hydrogel surface showing large amounts of corneal deposits along the hydrogel surface. C) Medium magnification micrograph of a cluster of epithelial cells attached to the hydrogel surface. D) High magnification micrograph of a single epithelial cell strongly adhered to the hydrogel surface. All images taken at 3kV accelerating voltage.
CHAPTER 4: AIM 3 – SINGLE CELL ADHESION TEST

Contributors: Kai-Tak Wan and Monica Susilo

4.1 Overview

This chapter focuses on characterizing microscopic, single cell adhesion on hydrogel contact lenses. The first aim of this thesis developed novel metrics to characterize hydrogel lens adhesion against a planar substrate, $P^*$ and $\gamma$. Aim 2 of this thesis developed novel in vitro techniques to investigate hydrogel lens adhesion against biological samples relevant to hydrogel lens behavior in vivo: human corneas. Aim 3, outlined in this chapter, focuses on developing protocols and experimental techniques to characterize the adhesion forces of individual cells against hydrogel contact lenses via Single Cell Force Spectroscopy (SCFS), completing the multi-scaled adhesion mechanics characterization of this thesis. Specifically, two cell types are characterized. First, readily available Human Corneal Fibroblasts (HCF) cells are characterized for method development and serve as a proof-of-concept of the technique. Second, Human Corneal Epithelial (HCE) cells are characterized for potential insights into the in-vivo lens adhesion behavior.

4.2 Introduction and previous research

Hydrogel contact lens adhesion is believed to play a role in some lens-related ocular complications, such as corneal erosions [12, 17, 22]. However, the exact mechanisms contributing to the onset and progression of these complications are yet to be fully understood [22]. These complications are likely multi-factorial in nature, and part of the reason behind the
lack of a solid understanding of their etiology is due to the complexity of the hydrogel interactions with the dynamic ocular surface. The matter is further encumbered by the limited number of tools available to quantitatively investigate hydrogel lens adhesion. One hypothesis of the onset of corneal erosions is that a strong adhesive attachment forms between a section of the epithelium and the hydrogel lens that subsequently detaches the section from the rest of the epithelium during typical lens motion [12]. However, this hypothesis has yet to be investigated. Thus to explore hydrogel lens adhesion in the context of physiologically relevant ocular complications, the adhesion of single cells against two commercially available hydrogels (Etafilcon A & Narafilcon A) is characterized. To reliably measure cellular adhesion forces on the scale of nano-Newton, Single Cell Force Spectroscopy (SCFS) is implemented via AFM (for a brief introduction of the AFM, see Chapter 1.3.2).

SCFS is one of a handful of methods that permits detection and quantification of cellular adhesion forces that are on the scale of $10^{-9}$ to $10^{-12}$ Newtons. In general, AFM-based SCFS is comprised of a cantilever which has been coated with a functional group of interest to achieve strong attachment of the desired cell type (see Figure 48) [112]. After functionalization, a single cell is attached to the cantilever, and the adhesion strength between the cell and the desired surface is quantified by force spectroscopy [113, 114]. First, the cantilever approaches the substrate at a specified velocity until a maximum force is reached, at which point the cantilever piezo maintains this maximum displacement for a defined contact time. Afterwards, the cell is withdrawn and individual bonds between the substrate and the cell rupture until the cell is completely delaminated from the substrate. The loading-unloading curves, or $P-w_0$ curves, capture the “pull-off” forces necessary to achieve complete detachment. The $P-w_0$ curves contain information about both the mechanical properties of the immobilized cell and the interaction
between the cell-substrate surface [115, 116]. SCFS is implemented with an AFM in conjunction with a high powered optical microscope DIC to allow real-time observation of the cellular interactions with the surface.

Figure 48: Single Cell Force Spectroscopy (SCFS) set up. Left: Differential Interference Contrast (DIC) micrograph of SCFS configuration. A single HCF cell is immobilized on a tipless AFM cantilever. Right: diagram showing the set up of a SCFS hydrogel adhesion test.

In general, the AFM has been widely adapted by researchers to investigate hydrogel contact lens mechanical, surface, and adhesion characteristics due to the extremely high spatial and force resolution. Most studies utilizing AFM for hydrogel lenses characterize lens surface roughness [72, 73, 117], as the surface roughness is believed to play a role in bacterial adhesion [56, 118], biofilm formation [119], and affect the optical performance of the lens [120]. There have also been efforts to characterize the localized mechanical properties of hydrogel lenses via AFM nanoindentation [77]. However, there have been substantially fewer investigations into microscopic hydrogel lens adhesion, in which previous studies have focused primarily on either non-specific cantilever [75] or specific bacterial adhesion to the lens surface [55, 81, 121]. For non-specific adhesion forces, the “pull-off” force, $P^*$, was measured and served as the metric to
quantify adhesion of poly(2-hydroxyethyl methacrylate (pHEMA) based hydrogel lenses on a silicon nitride cantilever [75, 77]. Investigations into quantitative specific adhesion are still relatively new in the realm of hydrogel contact lenses. In the case of bacterial adhesion, SCFS was utilized to immobilize bacterial cells of specific strains in order to quantitatively characterize their adhesion. The “pull-off” force, $P*$, is measured as a function of time in contact to deduce the bond-strengthening time, $\tau$. $P*$, the statistical distribution of $P*$, and $\tau$ all serve as metrics to quantify cell adhesion. Furthermore, the probability of bacteria transmission off of the hydrogel surface was calculated via Weibull analysis [55, 81, 121]. This bacterial adhesion characterization is insightful into understanding biofilm formation and microbial transport/infiltration from the lens to the cornea; a step that is crucial for the initiation and progression of ocular complications like Microbial Keratitis (MK) [22, 23, 28, 79, 122, 123]. However, to date there have been no investigations into the adhesion of HCE cells present in the corneal epithelium to hydrogel lens materials, which could shed light on the role of adhesion in the onset and progression of lens-related ocular complications, such as corneal erosions [12, 17, 22].

There are substantial differences between the single immobilized HCE cells in-vitro to the complex organization of HCE cells in the epithelium in-vivo. However, there is currently a dearth of adhesion characterization of HCE cell (or HCF) against hydrogel lens materials. The in vitro SCFS characterization of two hydrogel materials may be clinically reflective of their relative in vivo lens-cornea interactions and overall adhesion behavior of those two hydrogels. To this end, readily available HCF cells are characterized via SCFS as a proof-of-concept against Etafilcon-A and Narafilcon-A substrates, as well as the more physiologically relevant HCE cells.
Comparisons between $\gamma$ and $P^*$ from the PAT in Aim 1, as well as $P^*$ from Aim 2 are made with the SCFS data reviewed here in Chapter 4.

### 4.3 Experimental protocols

Two different cell types were utilized for SCFS adhesion characterization. HCF cells are a readily available robust corneal cell line that was utilized to develop the experimental technique and protocols. Epithelial cells were then tested to achieve more physiological relevance to *in vivo* cell-hydrogel interactions. To conduct SCFS of two different cell types/lines on two different hydrogel materials is a multi-step process. The detailed protocols of each step are outlined in this subsection.

#### 4.3.1 Human Corneal Fibroblast (HCF) cell culture protocol

Primary HCF cells were extracted from a 48 year-old donor cornea using the extraction protocol outlined by Guo *et al.* [124], and stored in -196°C until use for experiments. All cell culture and preparation work was conducted in a sterile biosafety fume hood. Once thawed, the HCF cells were injected into a standard sterilized T-25 culture flask and supplemented with 15mL of sterilized Dulbecco’s Modified Eagle Medium (DMEM) enriched with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic (Antibiotic/antimycotic; mixed 10mg/mL Streptomycin and 25 µl/mL Amphotericin B, Cellgro, Fisher Scientific). All culture flasks are stored in a sterile incubator at 37°C. Every 48 hours, 7.5 mL of DMEM was gently extracted from each culture flask, and 7.5 mL of fresh enriched DMEM was added. To get HCF cells freely suspended in media for SCFS experiments, the HCF cells were passaged via trypsin/EDTA extraction. The old DMEM was completely removed from the culture flask, and a wash with
sterile 1x Phosphate Buffered Saline (PBS) was conducted. 2 mL of trypsin/EDTA was injected into the culture flask and was incubated at 37°C for 5 min., afterwards the flask is gently tipped on its side and tapped to ensure HCF removal from the culture flask substrate (confirmed via DIC microscopy). 10 mL of fresh DMEM was injected into the culture flask, and gently swirled around to ensure cell suspension into the fresh media. The mixture of HCF, DMEM and trypsin/EDTA was then gently pipetted into a sterile 50mL centrifuge tube, and the cells were centrifuged at 2000 rpm for 2 minutes (2 min build up and slow down period). Afterwards, the old DMEM + trypsin/EDTA was exchanged with 17 mL fresh DMEM and vortex to break up the cell pellet/suspend cells into the media. Of the newly suspended HCF/DMEM mixture, 15mL went into a new culture flask and 2mL was placed in a sterile 50mL centrifuge tube, and diluted with 10mL fresh DMEM to be used for SCFS experiments. The suspended HCF cells are stored at 37°C until immediate use in SCFS experiments. Individual HCF cells are observed to survive for up 6 hours in DMEM at room temperature (25°C) for SCFS experiments.

4.3.2 Human Corneal Epithelial (HCE) cell culture protocol

HCE cells (Invitrogen, C-018-5C) were stored in -196°C until intended use. To begin the HCE culture, the cell vial was thawed at 37°C and placed in a sterile biosafety fume hood. The suspended cells were injected into standard sterile T-25 culture flasks and supplemented with 15 mL of 1X Keratinocyte–SFM (Invitrogen, 17005-042), supplemented with L-glutamine, Epidermal Growth Factor 1-53 (EGF 1-53), Bovine Pituitary Extract (PBE) and Antibiotic-antimycotic (1mg/mL) (Invitrogen, 15240-062). The culture flask was placed in a sterile incubator at 37°C, and every 48 hours the media was extracted and fresh media pipetted into the culture flask. To get HCE cells freely suspended in media for SCFS experiments, the HCE cells
were passaged via trypsin/EDTA extraction, similar to the HCF cells in section 4.3.1, with the exception that the HCE cells are incubated with trypsin/EDTA for 10-15 min at 37ºC, and are centrifuged at 2000 rpm for 2min.

4.3.3 Cantilever functionalization protocol

To conduct SCFS, an appropriate cantilever must be functionalized with the appropriate chemicals to ensure a strong bond of that particular cell type to the AFM cantilever. For soft HCE/HCF cells, tipless Silicon Nitride cantilevers (MLCT-010) are utilized with a nominal spring constant of \( k = 0.03 \text{ N/m} \) (probe D), and the approximate width of 20µm is suitable for most HCE/HCF attachment. The AFM cantilevers are functionalized by simple ConA physisorption [112]. Unconjugated Concanavalin A (Sigma, C7275) is mixed in PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)) at 2mg/mL. Cantilevers are first washed with 70% ethanol twice for 5 mins, and rinsed twice with DI water and allowed to air dry. Cantilevers are then incubated in ~100µL droplets of ConA solution for 2 hours at room temperature. Cantilevers are removed from the ConA droplets and stored in PBS at 4ºC until use in the SCFS experiments. Each cantilever can last for approximately 4-15 strong-cell attachment before it must be cleaned/re-functionalized for further tests.

4.3.4 Sample preparation

The same hydrogel materials are used for SCFS to complete the multi-scaled adhesion characterization from Aims 1 and 2. Instead of lenses, flat sheets of both Etafilcon-A and Narafilcon-A (\( h = 750 \mu\text{m} \)) (custom made, Vistakon) were utilized to ensure cell stability on the hydrogel surface; it is difficult to attach cells to an AFM cantilever with a curved lens surface.
Each SCFS uses a fresh hydrogel sample. A fresh razor is rinsed 2x with DI water to cut samples from the bulk planar sheet of approximately 2cm x 2cm. A standard plastic petri dish is used as the AFM sample chamber, and the petri dish is ultrasonically cleaned (Sonicor) in freshly washed glassware and DI water for approximately 10 min. The petri dish is removed from the sonicator, rinsed with 70% ethanol, rinsed with DI water and then allowed to air dry. While the petri dish is drying, the hydrogel sample is rinsed 3x times with fresh DI water, and then placed in the center of the clean petri dish.

The clean petri dish is placed in the AFM petri-dish stage, and approximately 10 mL of the appropriate cell media at room temperature is then gently pipetted into the petri dish as to not disturb the hydrogel (DMEM with FBS and Antibiotic for HCF cells, or Keratinocyte–SFM supplemented with L-glutamine, EGF 1-53, PBE and Antiobiotic-antimycotic for HCE cells). The petri-dish stage is then placed on the magnetic sample holder of the Agilent 5500 AFM, and approximately 1-4 100µL drops of the suspended HCE/HCF cells are pipetted onto the hydrogel surface. 5-10 minutes is allowed for the cells to settle onto the hydrogel surface in the petri dish before the AFM piezo scanner is inserted into the fluid chamber. Great care is taken such that the time the functionalized cantilever is exposed to the ambient environment is minimized to ensure consistent functionalized states between experiments.

### 4.3.5 Adhesion force measurement

All SCFS experiments utilized an Agilent 5500 AFM on an inverted Olympus GX71 DIC microscope. The functionalized MLCT-010 probes were mounted in the head of the AFM scanner, and carefully submerged into the fluid cell. Great care was taken to ensure that all cells had sunk and deposited on the hydrogel/substrate and were not floating to interfere with the
cantilever, and that the cantilever had approximately 1mm separation between the surface of the hydrogel. For these particular probes, an initial deflection of ~ -1.8 to -2.1 V with a set point of ~ -0.8 produced satisfactory surface detection for all SCFS experiments. The main steps of a SCFS experiment are shown in Figure 49. The first step involves attaching the HCE/HCF cell to the functionalized cantilever by “fishing” the cell off of the substrate/hydrogel surface, as shown in Figure 49.a. This is best achieved by positioning approximately 10 microns above the cell, which can be estimated by measuring the cell diameter via DIC microscopy, then making contact with the cell-free substrate near the cell, and precisely retracting away from the substrate to a distance equal to the diameter plus 10 microns. The cantilever is manually positioned such that the cell is at the apex of the tipless cantilever, as shown in Figure 62. An approach velocity faster than 5µm/s produces relatively high amounts of hydrodynamic drag [125], which will often push the cell out from underneath the cantilever before initial contact is made. To combat this, v = 1µm/s is used for “fishing” for a cell.
Figure 49: Sketch of the main steps in a SCFS experiment. A) Precise compression of a functionalized cantilever against a suspended HCF/HCE cell to immobilize the cell onto the cantilever surface. B) Loading of the cell against the hydrogel substrate for a maximum compression depth of 1-2µm. C) Unloading of the cell to measure “Pull-off” force, $P^*$. 

Once initial contact is made, the strength of the attachment can be tested by simply manually moving the cantilever via micro-positioners (very small distances, 1-2 µm) while observing the cantilever under DIC microscopy to see if the cell moves with the cantilever. If the cell does not move with the cantilever, a simple approach-retract $P-w_0$ curve is conducted with a hold time of approximately $t_{\text{hold}} = 60-120$ s. If the cell was still not attached to the cantilever, a new cell was found. After 5 cell attachment failures, a new cantilever was used. Upon successful attachment of a cell to the functionalized cantilever, the cantilever was retracted 50 µm from the surface and the cell-cantilever was allowed to recover for at least 10 min, during which the cell forms firm contact with the functionalized surface [112]. To remove a cell from the cantilever,
the scanner/cantilever was removed from the liquid cell and the surface tension forces were almost always enough to completely detach the cell from the cantilever.

Once a cell has been successfully “fished” off of the hydrogel surface and is firmly attached to the functionalized cantilever, the SCFS test can begin. The cantilever/cell is moved to a fresh region of the hydrogel surface free of any other cells or debris that could interfere with the force measurement. An indentation velocity of 5µm/s is used to reduce the amount of hydrodynamic drag on the cantilever [125]. A maximum of 1V is applied to the piezo scanner, which translates into a maximum compression depth of approximately $0.5 < w_0 < 2 \mu m$ and a force of $P_{\text{max}} \approx 3nN$ of the HCE/HCF cell against theEtafilcon A/Narafilcon A surface. Once the maximum voltage is reached, the immobilized cell is held for a specific hold time of $\Delta t = 0, 5, 10, 15, 20, 30, \text{ or } 45s$. These hold times were chosen because: First, it is estimated that the average person blinks roughly every 5s, with a fairly large range spanning roughly blinking every 2-20s [106], which is the time frame the epithelium-hydrogel would be in contact before disruption from the eye lid. Second, the hold times are robust enough to characterize the time it takes for a strong attachment to form between the cell and hydrogel, also known as the bond strengthening time, $\tau$ [55, 81]. With longer hold times, thermal drift and hysteresis of the AFM components become more prominent. After $\Delta t$ is reached, the cantilever retracts away from the substrate, and during this unloading portion the adhesion forces between the immobilized cell and hydrogel substrate, $P^*$, is measured. The amount of adhesion tests that can be conducted on a single immobilized cell depends heavily on the cell type and the hold times utilized, with the rule of thumb being that cells can undergo many shorter hold time tests and fewer long hold time tests. Each individual cell was tested a maximum of 30 times over four or five different hold times before being discarded and a new single cell probe was made. $P-w_0$ curves that exhibit
artifacts, such as substrate optical interference, are discarded. Similar to the PAT and adhesion tests of Aims 1 and 2, $P^*$ is defined as the maximum tensile force necessary to achieve complete cell detachment from the hydrogel substrate. The SCFS probe was constantly monitored under DIC microscopy to ensure the cell was still attached, centered, and check for cell viability.

At the end of each test, the hydrogel sample and cells are removed from the liquid cell to measure the individual cantilever deflection sensitivity, $\eta$. This is necessary to convert the cantilever deflection, $T$, into the applied force $P$ with the following equation:

$$P = T\eta k \quad (22)$$

Where $k$ is the nominal spring constant of the cantilever in use. $\eta$, with units of (m/V), is measured by indenting the cantilever used for SCFS without an immobilized cell attached against a rigid substrate, and measuring the inverse slope of $P-w_0$ curve. Five $P-w_0$ curves were used for each cantilever for each round of SCFS experiments to determine $\eta$.

The bond-strengthening time, $\tau$, is the time for the adhesion force between the cell-substrate to strengthen. To characterize this strengthening time, the “pull-off” adhesion forces are plotted as a function of hold time and fitted to the following equation:

$$P^*(\Delta t) = P_{0s}^* + (P_{\infty}^* - P_{0s}^*) \left(1 - e^{-\Delta t/\tau}\right) \quad (23)$$

Where $P_{0s}^*$ is the mean “pull-off” force at $\Delta t = 0s$, $P_{\infty}^*$ is the mean “pull-off” force at $\Delta t = 45s$, leaving only $\tau$ as the sole fitting parameter.

### 4.4 Results

For each cell type (HCF or HCE) and each material (Etafilcon-A or Narafilcon-A), seven hold times ($\Delta t = 0, 5, 10, 15, 20, 30, 45s$) were executed for at least 10 separate immobilized cell
probes. The amount of adhesion tests conducted depended heavily on the hold time, $\Delta t$. With larger $\Delta t$, thermal drift of the AFM/cantilever, piezo hysteresis, and optical interference from other non-immobilized cells are all more likely to effect, interfere, and nullify the unloading curve data.

### 4.4.1 HCFC adhesion characterization

A typical loading-unloading $P-w_0$ curve of an immobilized HCFC against a Narafilcon-A hydrogel substrate is shown in Figure 50. The fibroblast probe begins to approach the substrate approximately 7 microns above the surface at point A, during which the cantilever experiences no attractive forces through AB. At B the cell membrane makes contact with the hydrogel substrate without the “pull-in” force, indicating weak long-range intersurface forces between the fibroblast and hydrogel. Loading until a maximum of 1V ($\sim$3nN) occurs at BC, in which both the tipless cantilever and cell itself deform during the compression. The maximum load/compression depth is held at C for the specified $\Delta t$ (in this case, $\Delta t = 45s$). Over the course of $\Delta t$, multiple bonds between the cell membrane components and the hydrogel surface form in the cell-substrate contact area, strengthening the ultimate attachment strength [126]. During $\Delta t$, hysteresis of the cantilever/piezo, thermal drift of the laser/photodetector, and viscoelastic relaxation of the cell itself occur, all of which contribute to the downward shift in load during CD. Once $\Delta t$ is reached, the unloading sequence begins at D. The unloading slope DE is steeper than the loading slope BC, indicating hysteresis of the cell. At point E there is zero force with a non-zero contact area between the cell and hydrogel due to adhesion. A negative tensile load is applied at EF, until the maximum tensile “pull-off” force $P^*$ is reached at point F. It is possible to have simultaneous eruption of multiple cell-hydrogel bonds, which results in a smooth detachment curve, such as shown in EFGH. It is also possible to have sequential eruptions of
bonds which exhibit distinct sudden spikes in force as individual bonds break and unravel, known as “tethers” [127]. During the unloading sequence of DEFG, bonds are simultaneously breaking as well as the cell membrane deforming as the contact area between the fibroblast-hydrogel shrinks with increasing tensile load. After “pull-off” at point F, bonds continue to break as the cell is further withdrawn from the hydrogel until eventually the main body of the cell is detached at G, leaving only a small number of bonds connecting the cell to the substrate [127]. The last remaining attachments/bonds gradually break over the scale of ~2µm and ~ 200pN between GH, until complete detachment is achieved at H and the piezo scanner returns to its initial position. The $P-w_0$ curve does not return to zero load at point A due to the thermal drift of the AFM components and hysteresis of the piezo. $P^*$ is measured as the difference in applied load from F to H, in this case $P^* \approx 950$ pN. The total work done needed to completely detach the fibroblast from the Narafilcon A surface is the shaded area where $P < 0$ enclosing the unloading curve, and is labeled as $U_{ad}$, the adhesion energy (J).
Figure 50: Typical loading (ABC) and unloading (DEFGH) $P$-$w_0$ curve for an HCF cell on Narafilcon A (TruEye) hydrogel substrate. For this particular fibroblast, $\Delta t = 45s$ with $P^* \approx 950$ pN and a “snap out” distance of $\sim 4\mu m$.

Representative unloading $P$-$w_0$ curves of HCF cells on Narafilcon A for each $\Delta t$ are shown in Figure 51, and $P$-$w_0$ curves for Etafilcon A are shown in Figure 52. Even at $\Delta t = 0s$, there is typically a small, non-zero adhesion force present, although it is common for $P^*(\Delta t = 0) = 0$ for both hydrogels. This is due to the fact that even though the hold time $\Delta t = 0s$, the loading-unloading cycle takes approximately 1s to complete. As the hold time increases, generally the “pull-off” force necessary to achieve complete detachment increases as well. However, the “snap-off” distance at which the cell achieves complete detachment from the substrate does not necessarily correlate with the “pull-off” force, which is consistent with other SCFS investigations [112]. The range of the median “pull-off” forces is approximately the same for HCFC on both Narafilcon A and Etafilcon A, spanning $\sim 150$ pN $< P^* < 1000$ pN, although
individual cells have recorded “pull-off” forces as high as ~6 nN. In Figure 51, the increase in $P^*$ is drastic between hold times of $0s < \Delta t < 15s$ and changes very little between $20s < \Delta t < 45s$, indicating that the non-specific fibroblast-Narafilcon A adhesion quickly develops and matures in less than approximately 15s. Similarly, a strong adhesion forms within the first tens of seconds on Etafilcon A in Figure 52, although the transition to larger $P^*$ with increased $\Delta t$ is in general much more gradual than Narafilcon A. Sequential ruptures, parallel ruptures, or both ruptures of the fibroblast-Narafilcon/Etafilcon A adhesion sites/bonds are captured in the $P-w_0$ curves. Additionally, the elastic/viscoelastic deformation of the cell as well as the cell membrane, and the surface chemistry of the cell membrane all contribute to the adhesion forces in $P-w_0$ curves [127], and it is difficult to infer the contributions of these individual factors into the total measured $P^*$. Hundreds of $P-w_0$ curves were recorded for each hydrogel and most exhibited a smooth detachment curve, although sudden detachment events were recorded as well.
Figure 51: Representative unloading $P$-$w_\theta$ curves for HCF cells on Narafilcon A (TruEye lenses) for seven hold times.

Figure 52: Representative unloading $P$-$w_\theta$ curves for HCF cells on Etafilcon A (Acuvue2 lenses) for seven hold times.
The distributions of $P^*$ for all $\Delta t$ on Narafilcon A is shown in the histograms of Figure 53, with a bin size of 0.2 nN. Similarly, the $P^*$ distribution for Etafilcon A is shown in Figure 54. The “pull-off” forces are not normally distributed, instead they tail towards higher detachment forces with a large spread, which is fairly when variances are large and the values cannot be negative, and in particular in SCFS experiments [55, 81, 112, 127-129]. In some cases, the $P^*$ distributions are even bimodal (i.e. $\Delta t = 30s$ for Narafilcon A in Figure 53.F). Because of the non-normal distribution of $P^*$, it is difficult to extract a most probably force value [112]. To characterize the $P^*$ distributions, they were fit to a standard log-normal probability density function in OriginLab data analysis and graphing software, given by:

$$Y = \frac{A}{P^* \sigma \sqrt{2\pi}} \exp \left[ -\ln \left( \frac{P^*}{\mu} \right)^2 / 2\sigma^2 \right]$$

Where $A$ is the amplitude, $\sigma$ is the standard deviation, $\mu$ is the mean, and $Y$ is the relative probability. The individual $P^*$ distributions and the corresponding log-normal fits with $\mu$ and $\sigma$ for Narafilcon A and Etafilcon A are shown in A-G in Figure 53 and Figure 54, respectively. Figure 53.H and Figure 54.H show the summary of the best log-normal fits for all $\Delta t$. The general trend is a widening of the log-normal distribution with increased $\Delta t$ with a simultaneous decrease in the amplitude of the distribution, although the large spread in data makes significant conclusions difficult to draw. The detailed summary of the HCF SCFS statistical data for Narafilcon A and Etafilcon A are given in Table 2 and Table 3, respectively.
Figure 53: A-G: Histograms of the measured “pull-off” force distributions for various hold times of fibroblasts against Narafilcon A, with the $\Delta t$, mean ($\mu$), and standard deviation ($\sigma$) values given in the legend. The blue curve corresponds to the best fit log-normal distribution given in Eq. 24. H: summary of the best-fit log-normal distributions for all seven hold times.
Figure 54: B-G: Histograms of the measured “pull-off” force distributions for various hold times of fibroblasts against Etafilcon A, with the $\Delta t$, mean ($\mu$), and standard deviation ($\sigma$) values given in the legend. Note, no log-normal fit is given for $\Delta t = 0$ s due as distribution of $P^*$ appears to be normally spread. The blue curve corresponds to the best fit log-normal distribution given in Eq. 24. H: summary of the best-fit log-normal distributions for all six hold times ($\Delta t = 0$ s not applicable).
<table>
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<tr>
<th>$\Delta t$ (s)</th>
<th>Mean $P^*$ (nN) ‡</th>
<th>Standard Deviation (nN) ‡</th>
<th>$\mu$ (nN) §</th>
<th>$\sigma$ (nN) §</th>
<th>Median (nN) ‡</th>
<th>Range (nN) ‡</th>
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Table 2: Summary of the SCFS adhesion experiments of HCF cells on Narafilcon A. ‡ indicates calculated assuming a normal distribution, § indicates calculated assuming a log-normal distribution given in Eq. 24.

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<th>Standard Deviation (nN) ‡</th>
<th>$\mu$ (nN) §</th>
<th>$\sigma$ (nN) §</th>
<th>Median (nN) ‡</th>
<th>Range (nN) ‡</th>
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Table 3: Summary of the SCFS adhesion experiments of HCF cells on Etafilcon A. ‡ indicates calculated assuming a normal distribution, § indicates calculated assuming a log-normal distribution given in Eq. 24.
Figure 55: Mean “Pull-off” force (log-normal distribution) as a function of hold time to characterize the bond-strengthening time, $\tau$, for fibroblasts on Etafilcon A (white dots) and Narafilcon A (red dots). The best fit to Eq. 23 is shown by the black and red line for Etafilcon A and Narafilcon A, respectively, and yields $\tau_{\text{eta}} = 6.9s$ ($R^2$ value of 0.858) and $\tau_{\text{nara}} = 4.1s$ ($R^2$ value of 0.520).

The mean “pull-off” force as a function of hold time as calculated by a logarithmic-normal distribution (Eq. 24) is shown in Figure 55. The error bars represent ± one standard deviation. For both hydrogels, it is clear that there is a large spread in the measured $P^*$ for all $\Delta t$, which is fairly common in SCFS experiments [112, 127]. Each group of data is fit with Eq. 23 to extract the bond-strengthening time, $\tau$, for fibroblasts to develop strong attachment to the hydrogel surface. Utilizing the mean $P^*$ values from log-normal distributions given by Eq. 24, the best fits yield a bond strengthening time of $\tau = 6.9s$ for Etafilcon A ($R^2 = 0.858$) and $\tau = 4.1s$ for Narafilcon A ($R^2 = 0.520$). The strength of adhesion forces between fibroblasts and the hydrogel generally increases with longer hold times, although due to the large spread in data there is no statistical significance between measured fibroblast adhesion forces on Etafilcon A versus Narafilcon A. However, the time it takes for a strong adhesion bond to form on Etafilcon
A is slightly longer than compared to Narafilcon A, although the trends themselves are not significantly different between the two hydrogels.

### 4.4.2 HCEC adhesion characterization

Representative unloading $P-w_0$ curves of HCE cells on Narafilcon A for each $\Delta t$ are shown in Figure 56, and $P-w_0$ curves for Etafilcon A are shown in Figure 57. Similar to fibroblasts, at $\Delta t = 0s$ there is typically a non-zero adhesion force required to achieve full epithelial cell-hydrogel detachment, although there are instances of $P^*(\Delta t = 0)$ for both hydrogels. As the hold time increases, the $P^*$ necessary to achieve detachment also increases as the number and strength of adhesive contacts between the epithelial cell and hydrogel increase. The “snap-off” distance is typically between 2-6µm, approximately the same range as fibroblast cells. The mean $P^*$ for epithelial cells on Narafilcon A spans approximately 200 pN < $P^*$ < 700 pN for all hold times, while the mean $P^*$ spans approximately 350 pN < $P^*$ < 1900 pN for Etafilcon A. Both hydrogels show a drastic increase in $P^*$ between $\Delta t = 0s$ and $\Delta t = 5s$, with the mean $P^*$ forces increasing by more than double in the initial five seconds. After this initial quick strengthening, the $P^*$ values increase more gradually. Similar to fibroblast cells, the detachment curves typically exhibit individual/sequential adhesive site/bond ruptures, multiple parallel ruptures, or a combination of both. A majority of the epithelial cell unloading events exhibit the elongated, “smooth” detachment curves, although sudden detachment events were recorded as well.
Figure 56: Representative unloading $P-w_0$ curves for HCE cells on Narafilcon A (TruEye lenses) for seven hold times.

Figure 57: Representative unloading $P-w_0$ curves for HCE cells on Etafilcon A (Acuvue2 lenses) for seven hold times.
The distribution of $P^*$ forces for all $\Delta t$ on Narafilcon A is shown in the histograms of Figure 58, with a bin size of 0.2 nN. Similarly, the $P^*$ force distribution for Etafilcon A are shown in Figure 59. The $P^*$ forces are not normally distributed, and tail towards higher detachment forces with relatively large spreads for all $\Delta t$, similar to fibroblast cells. For Etafilcon A, the distribution if $P^*$ even appears to be bimodal in some cases (i.e. $\Delta t = 15s$ in Figure 59.D)

To characterize the epithelial $P^*$ force distribution, the histograms are fit with the standard log-normal probability density function given in Eq. 24. The individual $P^*$ distributions and the corresponding log-normal fits with the mean, $\mu$, and the standard deviation, $\sigma$, for Narafilcon A and Etafilcon A are shown in A-G in Figure 58 and Figure 59, respectively. Figure 58.H and Figure 59.H show the summary of the best log-normal fits for all $\Delta t$. The general trend is a widening of the $P^*$ distribution with increasing hold time, accompanied by a decreased distribution amplitude. However, the epithelial cell $P^*$ forces are significantly higher on Etafilcon A than for Narafilcon A for all $\Delta t > 5s$ ($p < 0.01$ via Student’s T-test). The detailed summary of the HCE SCFS statistical data for Narafilcon A and Etafilcon A are given in Table 4 and Table 5, respectively.
Figure 58: A-G: Histograms of the measured “pull-off” force distributions for various hold times of epithelial cells against Narafilcon A, with the $\Delta t$, mean ($\mu$), and standard deviation ($\sigma$) values given in the legend. The blue curve corresponds to the best fit log-normal distribution given in Eq. 24. H: summary of the best-fit log-normal distributions for all seven hold times.
Figure 59: A-G: Histograms of the measured “pull-off” force distributions for various hold times of epithelial cells against Etafilcon A, with the $\Delta t$, mean ($\mu$), and standard deviation ($\sigma$) values given in the legend. The blue curve corresponds to the best fit log-normal distribution given in Eq. 24. H: summary of the best-fit log-normal distributions for all seven hold times.
The mean $P^*$ force as a function of hold time assuming a log-normal distribution is shown in Figure 60. The error bars represent ± one standard deviation. For both Etafilcon A and Narafilcon A there is a relatively large amount of spread in the data, and the standard deviations are typically skewed. The mean $P^*$ forces for each hydrogel are fit to Eq. 23 to extract the bond-strengthening time, $\tau$, for epithelial cells to develop strong attachment to the Etafilcon/Narafilcon A surfaces. The best fit for the bond-strengthening equation for $P^*(\Delta t)$ in Figure 60 yields $\tau = 13.9s$ for Narafilcon A and $\tau = 11.7s$ for Etafilcon A, with $R^2 > 0.95$ for both materials. Similar to the fibroblast cells, the pull-off force necessary to achieve complete detachment from the

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<th>$\Delta t$ (s)</th>
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<th>Standard Deviation (nN)</th>
<th>$\mu$ (nN)</th>
<th>$\sigma$ (nN)</th>
<th>Median (nN)</th>
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Table 4: Summary of the SCFS adhesion experiments of HCE cells on Narafilcon A. † indicates calculated assuming a normal distribution, § indicates calculated assuming a log-normal distribution given in Eq. 24.

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<tr>
<th>$\Delta t$ (s)</th>
<th>Mean $P^*$ (nN)</th>
<th>Standard Deviation (nN)</th>
<th>$\mu$ (nN)</th>
<th>$\sigma$ (nN)</th>
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Table 5: Summary of the SCFS adhesion experiments of HCE cells on Etafilcon A. † indicates calculated assuming a normal distribution, § indicates calculated assuming a log-normal distribution given in Eq. 24.
hydrogel surface increases with longer hold times. However, unlike the fibroblast cells, the epithelial cells exhibit much larger pull-off forces for Etafilcon A compared to Narafilcon A for hold times $\Delta t > 5s$, and the $P^*$ values for $\Delta t > 5s$ are significantly distinct between the two hydrogels ($p < 0.01$, student’s t-test).

![Figure 60: Mean “Pull-off” force (log-normal distribution) as a function of hold time to characterize the bond-strengthening time, $\tau$ for fibroblasts on Etafilcon A (white dots) and Narafilcon A (red dots). The best fit to Eq. 23 is shown by the black and red line for Etafilcon A and Narafilcon A, respectively, and yields $\tau_{eta} = 11.7s$ ($R^2$ value of 0.970) and $\tau_{nara} = 13.9s$ ($R^2$ value of 0.955).](image)

4.5 Discussion

SCFS offers a powerful tool to characterize cell-hydrogel adhesion; however, there are many limitations and caveats with interpreting the SCFS data. The cell, the cellular environment, and the experimental AFM parameters all contribute to the measured cell adhesion. In general, the application of SCFS to elucidate cellular behavior is difficult because of the small number of cells that can realistically be studied \textit{in vitro} due to the time intensive experimental preparation.
and procedures. Furthermore, it is often difficult to draw statistically significant conclusions from SCFS mean force values because of large standard deviations, as the data is typically non-parametrically distributed due to differences in individual cells, cell cultures, and heterogeneities in the cell surface and substrate [55, 81, 112, 121, 127]. The HCF/HCE adhesion data exhibits a non-normal distribution with deviations of upwards of 100% the mean $P^*$ value (see Figure 53, Figure 54, Figure 58, and Figure 59), making significant conclusions difficult. Such large standard deviations are common amongst SCFS experiments [55, 81, 112, 127], however, there are likely additional factors which lend to the large spreads in data.

First, the variance in the individual cell’s state has been shown to affect the measured adhesive behavior depending on when they are tested during their culture cycle [130]. To combat this, it is typically recommended that all cells be cultured and treated in the exact same way; cell culture, cell media, phase of the cell cycle, feeding and starving periods, the moment of cell harvesting and transfer to the SCFS experiment, and the application of additional reagents has to be precisely controlled [127]. Due to time and resource limitations and the large number of individual cell probes required for accurate results, HCF cells were cultured at different states and at various numbers of passages over the course of 4 weeks, which likely affected the measured adhesion properties. The SCFS HCE experiments were conducted over the course of 2 weeks, which likely established tighter control of the cell state between experiments. Similarly, due to the inherent difficulties of SCFS, the time from which cells were passaged/harvested to initial attachment to the cantilever ranged from 30min to 12 hours, which could introduce subtle changes in the cell adhesive behavior.

Second, the size difference of individual cells likely affects the measured adhesion forces. All SCFS measurements were done such that the deflection of the cantilever (V), and hence force
was the controlling variable for the maximum compression depth of the cell. In a singular SCFS experiment from a single cell passage, it is common to observe HCF cells with a diameter ranging from approximately 10-25 µm (see Figure 61). Thus, even cells of the same cell line still have variable size, and likely slightly different mechanical and viscoelastic properties [127]. These differences are likely further exaggerated when cells are harvested at different points in their cell cycle. Across all SCFS experiments, a maximum of 1V deflection was applied to form the maximum contact area between the cell and hydrogel. Different cell sizes and mechanical properties will lead to different maximum contact areas being established for the same maximum compression force, which will lead to differences in the measured adhesion forces. Furthermore, different cantilevers will have different deflection sensitivities and slightly different spring stiffness values, which will result in a different maximum applied force to the SCFS probes.

Figure 61: Typical size distribution of HCE cells gently resting on Etafilcon A, approximately 30 min after cell injection into the AFM fluid chamber. Scale bar 50 microns.
Third, tight control over the cellular environment is crucial for accurate and repeatable SCFS results. It has been shown that cells from the same established cell line and culture cycle can react subtly to environmental changes [127, 131], however, the current SCFS experimental set up is limited to account for this in that it lacks a sterile environmental chamber. Furthermore all cells were cultured and maintained at 37 °C, but all SCFS experiments were conducted at room temperature at roughly 25°C. This temperature transition was neither characterized nor regulated. Various studies have shown that the temperature has a dramatic effect on the adhesion properties of cells [132-134], which likely is another factor contributing to the large amount of spread in the SCFS data.

It is important to note that the statistical analysis chosen to characterize the spreads in SCFS data is crucial [135]. Indeed, Table 2 - Table 5 highlights the necessity of utilizing the relevant statistical distribution to interpret SCFS data. Nearly all of the histograms in Figure 53 and Figure 54 exhibit a skewed distribution with tails towards higher adhesion forces, indicating that the data is best characterized with a log-normal distribution that accurately represents the spread in fibroblast/epithelial cell adhesion forces [112]. For SCFS of HCF cells on Etafilcon A and Narafilcon A, there was no statistically significant difference between cell adhesion forces at a given hold time $\Delta t$. There is however a weak difference in the measured bond strengthening time of the two materials. $\tau_{nara} = 4.1s$ for Narafilcon A and $\tau_{eta} = 6.9s$ for Etafilcon A, although the actual mean values utilized to derive these hold times are not distinct from one another.

For HCE cell experiments, Etafilcon A had significantly higher adhesion forces than Narafilcon A for $\Delta t > 5s$ ($p < 0.01$, student’s t-test). However, the bond-strengthening time for Etafilcon A was slightly lower than Narafilcon A ($\tau_{nara} = 13.9s > \tau_{eta} = 11.7s$). Though this is the opposite relationship of bond-strengthening times as was measured with fibroblasts, it likely
accurately reflects the epithelial cell-hydrogel adhesion behavior as a larger number of epithelial cells was tested compared to fibroblasts. Etafilcon A and Narafilcon A were not coated with any functionalizing agent or specific ligand to help mediate adhesion; the adhesion forces measured are likely non-specific in nature and are mediated by weak chemical bonding (i.e. hydrogen bonding, electrostatic, polar, or ionic interactions) between various molecules/structures on the cell membrane (i.e. glycocalyx) and functional groups on the hydrogel surface [136]. Indeed, in general a vast majority of SCFS measured interactions are non-specific with unclear origins [135]. Thus, it is not surprising that different cell types exhibit different adhesion behavior, as HCF cells have stark differences in morphological features [137], cell adhesion molecule recruitment [138], and likely cell surface features compared to HCE cells. Substantial investigation with fluorescent microscopy of the appropriately tagged cell adhesion molecules would be needed to deduce specific adhesion molecular pathways, if any, that contribute to HCF/HCE adhesion to Narafilcon/Etafilcon A.

In Chapter 2 of this thesis, the adhesion energies of Etafilcon A (Acuvue2) and Narafilcon A (TruEye) were calculated to be $\gamma_{\text{eta}} = 80\text{mJ/m}^2$ and $\gamma_{\text{nara}} = 95\text{mJ/m}^2$. With HCF cells, it is difficult to draw relationships between the macroscopic $\gamma$ and single cell behavior due to the lack of significantly different adhesion forces between hydrogels. With HCE cells, hydrogels with higher $\gamma$ as measured from PAT exhibit lower HCE cell adhesion forces. When comparing results from single cell to macroscopic adhesion behavior, it is important to note that higher “pull-off” forces do not necessarily mean higher adhesion energies (see Chapter 2.6). Furthermore, $\gamma$ as measured from PAT in Chapter 2 utilized a rubber substrate that has drastically different surface chemistry than that of the cell membrane features that contribute to the non-specific adhesion in SCFS experiments (i.e. the lipid bilayer of the cell membrane). Indeed, it is
necessary to measure the adhesion energy of individual cell-hydrogel interactions in order to draw meaningful relationships between lens macroscopic substrate and microscopic cellular behavior. Deriving cell-hydrogel adhesion energies entails of significant alterations to the JKR-shell model discussed in Chapter 2 as well as novel experimental methods to measure the cell-hydrogel contact area (see section 4.6). Along similar lines, Etafilcon A lenses exhibited lower $P^*$ forces measured against human corneas in Chapter 3 than Narafilcon A lenses. In both cornea and single cell adhesion measurements, the pull-off force alone is not enough to thoroughly investigate the scaling effects of hydrogel lens adhesion properties. However, the cornea/SCFS results here serve as a first step towards achieving complete multi-scaled adhesion characterization.

![Figure 62: Left: Light microscopic histology of the human corneal epithelium, adapted from [32]. Note the distinct morphological features of the epithelial cells depending on their location in the epithelium, with basal cuboidal cells near the Bowman’s membrane exhibiting different morphological features than the top superficial cells. Right: HCE cell immobilized on a tipless cantilever, scale bar 50 microns.](image)

The interpretation of in vitro SCFS data to elucidate cell-hydrogel behavior in vivo is encumbered by the fact that the cells in vitro are usually significantly different to the physiologically relevant cells in vivo, such as the cultured HCE cells vs. HCE cells comprising the corneal epithelium. It is unclear how the in vitro hydrogel adhesion characterization translates to the on-eye, in vivo behavior of these lens materials. The interaction of hydrogel
contact lenses with the ocular surface in vivo is incredibly complex [139]. The presence of the tear film [140], the tear film renewal upon blinking [141], and the mechanical interaction with the eye lid during blinking are just a few of the factors involved. The tear film itself has distinct lipid, aqueous, and mucous layers and is initially in direct contact with the hydrogel lens [142]. Furthermore, the tear film is responsible for maintaining corneal moisture, protection, transport of nutrients, and is reapplied with every blink [105, 142], and undoubtedly plays a large role in the overall hydrogel lens adhesion behavior in vivo. Another difficulty in interpretation to in vitro SCFS towards in vivo behavior is that there are substantial differences between individual cultured HCE cells immobilized on a cantilever and HCE cells in the corneal epithelium. Figure 62 shows a typical features of the human corneal epithelium via light microscopy histology [32]. The epithelium itself is comprised of approximately five distinct cellular layers, each with unique characteristics. The morphology of the epithelial cells changes drastically throughout the thickness of the epithelium, with the outer most layer comprised of dense superficial cells with a flattened morphology that are strongly connected to one another via tight junctions [32, 33, 143]. The epithelial cells in vivo exhibit microvilli that protrude from the cell membrane and are covered with glycocalyx. The surface of glycocalyx is composed of transmembrane muscins, such as MUC1 [144], MUC4 [145], and MUC16 [146], which generally are important for lubricating the ocular surface and likely play a role in the overall lens adhesion behavior in vivo. Figure 62 shows a DIC micrograph of the morphology of an immobilized HCE cell on a cantilever, with a spherical morphology that is extremely different than the superficial cell layer morphology of the epithelium. Another stark difference is that microvilli and mucin expression are likely not present on the immobilized cell’s surface. Indeed, there is evidence to suggest that
the very act of attaching a cell onto the functionalized cantilever influences the state of the cell [147], and thus could also affect the measured adhesion forces.

Despite these challenges in SCFS data interpretation, the relative differences in $P^*$ and $\tau$ of HCE on Etafilcon A and Narafilcon A could serve as useful metrics that are clinically reflective of the relative lens behavior *in vivo*. In the most straight-forward approach, one would expect hydrogels with larger $P^*$ values to have higher incident rates of lens-related complications in which adhesion is believed to play a role in, such as corneal erosions, than when compared to hydrogels with lower $P^*$ values. Similarly, one would expect lenses with longer $\tau$ to have lower incident rates of lens-related complications when compared to lenses with shorter $\tau$. To the best of our knowledge, there currently has been only one clinical investigation into the comparative *in vivo* performances of Etafilcon A/Acuvue2 versus Narafilcon A/TruEye. During the course of three months across 120 participants, there was only one recorded lens related ocular complication in which a Narafilcon A user developed a superior epithelial arcuate lesion (SEAL), although Etafilcon A users did exhibit statistically higher amounts of limbal redness [148]. Though SCFS is a promising candidate to elucidate the role of cell adhesion in lens related complications, it is currently unclear how well the $P^*$ and $\tau$ metrics are in predicting relative clinical performance of hydrogel contact lenses. We suggest that future SCFS-hydrogel experiments are conducted on hydrogel materials that already have a large amount of clinical performance data for a full comparison.

### 4.6 AIM 3 Concluding remarks and future work

SCFS offers quantitative analysis on the adhesion behavior of individual corneal epithelial cells against hydrogel contact lens materials. The role of hydrogel adhesion properties
in the context of ocular complications is currently unknown, as the \textit{in vivo} environment is quite complex and the onset and progression of these complications is likely dynamic and multifactorial in nature [12, 14, 23]. However, the results here indicate the feasibility of reliably measuring the adhesion properties of many individual cells to investigate if lenses with larger adhesion forces/lower bond-strengthening times are correlated with higher incident rates of lens-related complications. There are many facets to improve upon and future extensions of this work, however. The incorporation of a sterile environmental chamber to the AFM SCFS setup would greatly improve consistency between individual experiments and reduce the subtle effects of environmental conditions on the adhesion force measurements. Increasing the sample size of cells tested would ensure greater statistical significance, although \(~20-30\) cells are adequate. Similarly, increasing the number of hold times would offer higher degrees of accuracy in calculating the bond-strengthening time \(\tau\). SCFS could be applied to a wider range of commercially available hydrogel contact lens materials, such as Senofilcon A or Galyfilcon A, to serve as a benchmark in comparing adhesion properties to clinical performance data and incidence rates.

Rigorous analysis of the hydrogel-cell adhesion energy would be very useful for drawing correlations between lens adhesion properties and lens incident rates. However, this involves accurate measurement of the contact area between the cell-hydrogel as a function of applied force, similar to Chapter 2. The task of measuring the contact radius of a single cell against a soft hydrogel substrate during AFM force measurements is experimentally daunting, as there is currently no way to reliably visualize the cell-hydrogel interface boundary in the current AFM SCFS setup to the best of our knowledge. One possible single-cell force measurement technique that offers the ability of contact area measurement is micropipette aspiration [95], which could
be incorporated in future endeavors to accurately measure the epithelial cell contact area as a function of applied force.
ADDENDUM: Small angle light scattering to detect strain-directed collagen degradation in native tissue


The content of this addendum is largely taken from the final manuscript of “Small-angle light scattering to detect strain-directed collagen degradation in native tissue” in Interface Focus 2011, volume 1 issue 5, pages 767-776.

A1. Abstract

There is mounting evidence that there is a mechanochemical relationship between collagen and collagenolytic enzymes such that increased tensile mechanical strain reduces the enzymatic cutting rate. This mechanochemical relationship has the potential to permit directed remodeling of tissue-engineered constructs in vitro and to shed light on the generation of load-adapted collagen-based connective tissue. In this investigation, we demonstrate that small angle light scattering (SALS) has the sensitivity to dynamically detect the preferential enzymatic degradation of a subset of unloaded collagen fibrils within differentially-loaded native tissue. Detection of the difference in the relative degradation rate of unloaded fibrils versus loaded fibrils was manifested through changes in the spatial distribution of the SALS signal. Specifically, we found a linear increase in the eccentricity of the SALS data that was consistent with preferential retention of the collagen fibrils aligned with the applied tensile strain. We conclude that SALS is simple, inexpensive and may provide a useful optical screening method permitting real-time monitoring of strain-controlled tissue and construct remodeling.
A2. Introduction

In vertebrate animals, there are four classes of extracellular matrix (ECM) molecules whose primary functions are to resist mechanical force; they are: collagen, elastin, proteoglycans (PGs) and hydroxyapatite (HA). In general, collagen and elastin resist tensile force while proteoglycans and hydroxyapatite resist compressive forces. These load-bearing molecules can be found distributed throughout the vertebrate musculoskeletal system extracellular matrix in varying concentrations (e.g. HA 65% dry weight of bone [149], PGs ~3% dry weight of femoral head cartilage [150], collagen 70-80% dry weight of tendon [151], elastin 7% dry weight of ligament [152]). Of the four classes ECM structural molecules, collagen is the most abundant load-bearing material in vertebrates and contributes more than 25% to the total protein mass [153]. There are currently 28 genetically different types of collagen [154], with the primary load-bearing types (types I-III, V, XI) forming complex hierarchical fibril structures that are crucial to their load-carrying capacity. The basic hierarchical structure of type I collagen is outlined in Figure 63. In addition, collagen is considered to be the embryonic template molecule, arriving in the embryo during the early formation of load bearing structures and providing a guiding rudiment (e.g. for mineralization of bone). Thus, understanding the behavior of collagen as a load-responsive material is of paramount importance if we are to gain insight into the development, growth, maintenance and pathology of load-bearing ECM in vertebrate animals.

There is substantial evidence that collagenous tissue, fibrils and monomers gain resistance to some collagenolytic enzymes even when a small mechanical tension (<3 pN per monomer) is applied along their main axis [155-163]. The protective behavior has been termed a “low-force switch” by Camp et al. [157]. The existence of such a switch suggests that collagen would tend to accumulate and be retained in the path of mechanical force, with the collagen fibril
long axis typically aligning with the principal direction of tensile stress (e.g. tendon [164]). Such a property would greatly simplify the initial construction and subsequent remodeling of load-bearing collagenous matrix (both in native tissue and in engineered constructs). However, it remains to be demonstrated that such a mechanism is at work in vivo or in native tissue in vitro using physiologically relevant matrix metalloproteinase enzymes. Part of the difficulty resides in the fact that there are few methods capable of detecting preferential, strain-directed collagen remodeling in tissues.

![Diagram of the structural hierarchy of type I collagen tissues (tendon), with the relationship between type I collagen molecules, fibrils, fibers, fascicles and tendon unites. Adapted from [164].](image)

The Extracellular Matrix Engineering and Research Laboratory at Northeastern University has previously utilized the corneal stroma as a test matrix to probe the concept of strain-protection of collagen against enzyme proteolysis by bacterial collagenase [162, 163]. In an initial paper [163], it was theorized that applied load or strain could preserve a loaded subset of fibrils relative to less loaded set of fibrils in the presence of the collagenolytic enzyme from
*clostridium histolyticum.* The cornea was chosen because it possesses a unique matrix which is well-suited to test the strain-stabilization theory. This is true principally because the cornea comprises arrays of uniform diameter, aligned fibrils packed into lamellae which alternate in direction [165-168]. Further, the lamellae, which are mechanically uncoupled, can be preferentially strained in one direction, leaving a population of less loaded or unloaded “control” lamellae. By the strain-stabilization theory, the fibrils under lower tensile load should be preferentially attacked by the collagenolytic enzyme. In a time-stop series of experiments, one may periodically (and destructively) examine the matrix morphology following the application of a judiciously chosen mechanical force (or strain) with simultaneous exposure to enzyme. In their paper Ruberti and Hallab [163] performed such an investigation and found that application of mechanical force preferentially preserved fibrils in the direction of the applied load. However, the limitation imposed by morphological assessment at fixed time points (tissue processing for Transmission Electron Microscopy (TEM) takes multiple days in practice) effectively prevents real time feedback during the remodeling process. Thus, the dynamics of the enzymatic “remodeling” process were not captured. Quantifying strain-controlled enzyme/collagen degradation dynamics in bulk tissue can provide insight and direction both to tissue engineers seeking to produce organized constructs for load-bearing applications and to basic scientists seeking to understand the development, remodeling homeostasis and pathology of connective tissue.

Small Angle Light Scattering (SALS) offers a relatively simple, inexpensive technique to provide semi-quantitative structural information about dense fibrous connective tissues. Other forms of microscopy such as Scanning Electron Microscopy (SEM) and TEM provide highly detailed information over an extremely small sample area and require intensive sample
preparation that inhibits dynamic imaging. Unlike SEM/TEM, SALS has the ability to give
dynamic information on the macroscopic scale, as typically an incident laser beam samples an
area on the order of hundreds of square microns. SALS is also simpler to use and less expensive
to obtain than second harmonic generation (SHG) imaging which is a plausible alternative to
SALS [169-171]. Due to the inherent nature of light scattering, SALS is only capable of
detecting structures that are on the order of the wavelength of the incident light. Studies on
calfskin [172] and connective tissues [173] have shown that structural information obtained by
SALS is in close agreement with Small Angle X-Ray Scattering (SAXS), which is generally
accepted as an accurate technique map out collagen architecture [174, 175]. This indicates that
SALS is a quicker, simpler alternative to SAXS, but incurs the cost of reduced structural
resolution. Nonetheless, because the operative structural element in native tissues often comprise
bundles of fibrils acting in parallel (lamellae in cornea and fascicles in tendon), SALS
information may accurately reflect the structural competency of the tissue. Previous studies
using SALS on connective tissue [176, 177] treat the collagenous fiber network as a two-
dimensional assembly of single slits, where the effective slit width is an average of the fiber
diameters and inter-fiber spacing. In this simplified model, light is scattered perpendicular to the
fiber axis so that a fiber with an angular orientation of \( \phi \) scatters light intensity to an angular
orientation of \( \phi + \pi/2 \) (Figure 64). We present a simple model to quantitatively capture this
scattering behavior in the results section A4.
Figure 64: A Diagram illustrating light scattering off of collagen fibers. The parallel aligned fibers act as a diffraction grating and scatter incident light perpendicular to the fiber axis. If one fiber population is at an angular orientation of $\phi$, that population contributes scattered light intensity to $\phi+\pi/2$. If this fiber population dominates the total fiber population (i.e. a majority of fibrils lie in one direction), the scattered light will take the shape of an ellipse with the major axis at an angular orientation of $\phi+\pi/2$. If two perpendicular populations exist at angular orientations of $\phi$ and $\phi+\pi/2$, each population contributes scattered light intensity at $\phi+\pi/2$ and $\phi$, respectively. Similarly, if there is a random or pseudo-random distribution of fiber populations, light is scattered pseudo-isotropically.

Collagen fibrils in the cornea are approximately 35 nm in diameter [167, 173], far below the diffraction-limited resolution of SALS. However, the fibrils are bundled together to form fibrous structures several of microns wide called lamellae [165, 178]. In each lamella, collagen fibrils are aligned parallel to each other with inter-fibril spacing much less than the wavelength of light, resulting in the scattered light being nearly in phase for adjacent fibrils. Researchers have used SALS to explore the corneal structure in the past. Most notably, McCally and Farrell used SALS with crossed-polarizers on bovine cornea under uniform pressure [179]. They deduced that the lamellae are not randomly oriented in the cornea, but rather that there exist two predominant fiber populations of unequal number that lie orthogonal to each other at roughly 45° and 135° with respect to the nasal-temporal direction. This agrees reasonably with more recent
SAXS studies conducted on bovine cornea by Hayes et al which found an excess of collagen oriented at slightly more than 90° with respect to the nasal-temporal direction [180]. Thus, our SALS system is well suited to test our strain-stabilization hypothesis in native tissue containing arrays of collagen fibrils.

**A3. Experimental section**

**A3.1. Specimen.** In bovine corneas, monodisperse diameter type I collagen fibrils, packed into lamellae run parallel to each other in one plane, and are approximately orthogonal to adjacent planes (although there is a substantial number of adjacent lamellae which are not strictly orthogonal [166]). The structure provides an opportunity to generate a strained fibril population (experimental) and an unstrained fibril population (control) in the same specimen. Corneas were obtained from 20-45 kilogram cows provided by Research 87 Inc. (Boylston, MA). Corneas were removed from the ocular globes and prepared for loading and exposure to enzyme as described in Zareian et al [162]. Briefly, corneas were excised, debrided of epithelium and endothelium and stored at -80ºC. Multiple freeze-thaw cycles (to -80ºC) were used to devitalize the tissue for further control over the samples. A custom-made cutting die was used to generate repeatable vertically oriented corneal strips (superior-inferior direction) approximately 0.75 +/- 0.1 mm thick X 17.5 +/-2.5 mm long X 6 mm wide. The samples were always kept moist with 37ºC Dulbecco’s Modified Eagle’s Medium (DMEM; Mediatech Inc.) during all excision and mounting into the test chamber.

**A3.2. Mechanical Loading Apparatus.** An environmentally controlled uniaxial bioreactor [162] was used to explore the effect of mechanical strain on the preferential degradation of
fibrillar collagen tissues via SALS. The device comprises a load cell (Honeywell Sensotec, model 31; max, 2.26 kg; resolution 0.9 grams), a uniaxial motor (Zaber Technologies T-LA60; resolution, 16 µm; max speed, 4 mm/s, speed resolution, 0.001 mm/s), and is controlled using a custom LabView program. The system has an accuracy of +/- 0.01 N, and adjacent specimen chamber walls comprise quartz glass to permit incident laser light to illuminate the sample, while an integrated proportional-integral-derivative controller driven temperature control system maintains the chamber at 37ºC +/- 1°C. Each corneal strip was positioned between two cam grips inside the chamber and immersed in DMEM. Cyanoacrylate glue was applied to the sample grip interface and permitted to dry quickly to provide a firm hold of the cornea during testing. The strain, ε, is calculated from the grip displacement l, using the initial specimen gage length, l₀, as the reference: ε=(l−l₀)/l₀.

A3.3. Digestion Protocol. Crude bacterial collagenase (BC) from Clostridium histolyticum (Clostridiopeptidase A; Sigma-Aldrich, no. C0130) with a molecular weight range from 68 to 125 kDa was used. Each mole of collagenase requires four moles of calcium (Ca²⁺) in order to activate the solution. DMEM contains enough calcium to support the bacterial collagenase at the concentrations used in our system. To initiate enzymatic cleavage in an experiment, the DMEM buffer solution was replaced with solution containing BC (DMEM and 0.05 mM BC) for the remainder of the experiment. All BC solutions were made previously and stored in separate containers at -80°C until use for each experiment. At the onset of each experiment, the BC was preheated in a water bath for 30 minutes until a temperature of 37°C was reached, at which point the BC was injected into the chamber. Collagenase activity can vary from batch-to-batch and can
decline with time. However, this is not an issue due to the short duration of our experiments (~1 hr).

**A3.4. Loading Protocol.** All experiments were conducted in strain-control mode of the bioreactor. Cornea strips were first positioned in the bioreactor chamber with DMEM buffer and loaded until a reference load of 0.01N was detected, which we used as our initial specimen gage length $l_0$. To test our hypothesis that strain preferentially preserves loaded fibrils, three types of experiments were conducted:

1) **Loaded, Exposed to BC ($N=10$).** The strain was held at a constant 6% while the load and SALS intensity distributions were recorded. Loaded cornea samples were allowed 20 minutes for the stress to “relax” due to the viscoelastic nature of the corneal tissue. After 20 minutes elapsed, the loaded cornea samples were subject to degradation by BC for 40 minutes to explore the effect of strain on enzymatic cleavage of collagen fibrils/fibers.

2) **Unloaded, Exposed to BC ($N=9$).** The strain was held at a constant 0% for the full 60 minutes while the SALS intensity distributions were recorded. After 20 minutes, the unloaded cornea samples were subject to degradation by BC to explore the effect of enzymatic cleavage on SALS intensity distribution.

3) **Loaded, Unexposed to BC ($N=5$).** The strain was held at a constant 6% for the full 60 minutes, while the load and SALS intensity distribution were recorded. The loaded cornea samples were not subject to degradation by BC to explore the effect of stress-relaxation on SALS intensity distributions.
**A3.5. SALS Dynamic Imaging.** An optical bread board was used to mount a linearly polarized green HeNe laser (Melles Griot; model 05-LGP-173; 0.3mW) with a beam diameter $d=0.8\text{mm}$ and $\lambda=534.3\text{nm}$, with its polarization axis at $135^\circ$ with respect to the $x$-axis (Figure 65). A linearly polarized laser was chosen over an unpolarized laser since in the latter the polarization orientation randomly shifts due to temperature drifts, and collagen is known to exhibit birefringence. Two mirrors on kinematic mounts were used to direct the beam to the sample such that the incident beam was normal to the tissue sample (angle of incidence $=0^\circ$). A biconvex lens (focal length, $f=0.3\text{m}$, Thorlabs) was positioned behind the sample to redirect scattered light from the sample onto a ground glass where it was captured by a long-focal charged-couple-device (CCD) camera (Prosilica, model CV640; black and white; frame rate 120fps; resolution 9.9 x 9.9 $\mu\text{m per pixel}$). At the onset of each experiment, the incident beam was directed to the center of the corneal strip, and scanned in the vertical direction until a nearly isotropic scattering location was found. Once the ideal location was identified, the sampling area remained constant throughout the duration of the experiment.
Figure 65: Experimental set up used to image bovine cornea dynamically under uniaxial load subject to enzymatic cleavage: a) Green HeNe laser linearly polarized to 135°, $\lambda=534.3$ nm, $d=0.8$ mm. b) Adjustable mirror mounts to scan through sample. c) Bovine cornea strip in bioreactor under uniaxial strain (6%). The inset shows both a cornea pre-degradation (smaller inset with c1) and post-degradation (c2). d) Biconvex lens, $f = 30$ cm, to redirect scattered light for imaging and analysis. e) Ground glass to display SALS patterns. f) CCD to capture and analyze SALS data.

Previous methods of analyzing SALS of collagen networks [166, 173] involved collecting the scattered light intensity distribution along the azimuthal direction, $I(\Phi)$ at a constant scattering angle $\theta = \theta_0$. The width of the $I(\Phi, \theta = \theta_0)$ vs. $\Phi$ distribution is proportional to the degree of fiber orientation. Fiber architectures that have fibers loosely oriented in one direction will have a wider $I(\Phi, \theta = \theta_0)$ vs. $\Phi$ peak than fiber architectures that have tightly oriented populations in one direction. However, this analysis is unsuitable for degradation experiments. As fibers are cleaved by BC and removed from the cornea, the scattering angle decreases as there are less and less collagen fibers to scatter the incident light (see Figure 68). Over long enough times ($t \approx 75$ min) in the unloaded experiments the scattering angle reduces to nearly zero, leaving only the intensity distribution of the incident laser beam. The method of
Sacks et al. [176, 177] can give drastically different results depending on the scattering angle $\theta_o$ used to take the intensity distribution over, and hence is not ideal in degradation experiments in which the scattered light distribution is dynamic.

To analyze SALS intensity distributions in the ensemble of loading protocols used, we implement a simple image moment technique [181]. Image moments offer a robust way to quantitatively analyze scattered light distributions as it evolves over time; it requires no manual selection of a scattering angle $\theta_o$ over which to measure intensity distributions. The image moments are defined as:

\[
\bar{y} = \frac{\sum_j l_j y_j}{\sum_j l_j} \tag{1a}
\]

\[
\bar{x} = \frac{\sum_j l_j x_j}{\sum_j l_j} \tag{1b}
\]

\[
\bar{y}^2 = \frac{\sum_j l_j y_j^2}{\sum_j l_j} \tag{1c}
\]

\[
\bar{x}^2 = \frac{\sum_j l_j x_j^2}{\sum_j l_j} \tag{1d}
\]

\[
\bar{xy} = \frac{\sum_j l_j x_j y_j}{\sum_j l_j} \tag{1e}
\]

The image moments can be used to calculate the covariance matrix of each SALS image:

\[
\text{cov}[I(x, y)] = \begin{bmatrix}
\bar{x}^2 - \bar{x}^2 & \bar{xy} - \bar{x}\bar{y} \\
\bar{xy} - \bar{x}\bar{y} & \bar{y}^2 - \bar{y}^2
\end{bmatrix} \tag{2}
\]

It is straightforward then to calculate the eigenvalues of the covariance matrix:

\[
\lambda_t = \frac{x^2 - \bar{x}^2 + y^2 - \bar{y}^2}{2} \pm \sqrt{\frac{4(xy-x\bar{y})^2 + (x^2-x\bar{x}^2+y^2)^2}{2}} \tag{3}
\]

, which represent the major and minor axis of the ellipsoidal SALS intensity distribution. From which we can find the eccentricity:

\[
e = \sqrt{1 - \left(\frac{\lambda_2}{\lambda_1}\right)^2} \tag{4}
\]
We can quantitatively analyze the collagen structure by treating the collagen fiber populations (lamellae) as a two-dimensional assembly of slits, contributing scattered light intensity perpendicular to the orientation of their fiber/fibril axis. The eccentricity then is a direct measurement of the degree of collagen fiber/fibril organization in one direction. Higher values of eccentricity correspond to a larger percentage of the sampled fibers/fibrils that are oriented perpendicular to direction of the major axis of the ellipse. By using the eigenvalues of the covariance matrix to find the eccentricity of each SALS intensity distribution, the subsequent structural changes of collagen fibers/fibrils due to loading and enzymatic cleavage can be analyzed.

A3.6. Transmission Electron Microscopy:

TEM was implemented to provide an alternative method which could be used to verify the SALS results at the ultrastructural level. Specimens from two of the protocols ($N = 2$ from groups 1 and 2) were gently removed after the experiment and collected from the bioreactor chamber. Once safely removed from the bacterial collagenase, the samples were fixed in Karnovsky’s fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1M cacodylate buffer, PH 7.4) and processed for TEM as described in Guo et al [124]. Briefly, all specimens were removed from Karnovsky’s solution and were exposed to 1% osmium tetroxide and then dehydrated in graded ethanol. After the dehydration process, all specimens were embedded in Epon-Araldite and were sectioned perpendicular to applied load in the tensile specimens. At the end, all sections from specimens were stained with uranyl acetate and lead citrate in methanol and mounted on JEOL 1010 electron microscope (JEOL; Tokyo, Japan).
Figure 66: a) Scattering geometry used to model collagen fibrils as infinite cylinders. b) Predicted scattered irradiance off of two cylinder populations, one that is parallel to the x-axis and another parallel to the y-axis. The value $a$ is defined as the ratio of the number of cylinders in the x direction to number of cylinders in the y direction ($a = 1$ when the two populations are equal). The polar plot shows the irradiance for values of $a$, starting at $a = 1$ on the outside, and $a = 0.8, 0.6, 0.4, 0.2$ towards the inside.

A4. Results

A simple model was developed to capture the predicted SALS intensity distributions of collagen fibrils/fibers based on treating collagen fibers/fibrils as a system of two-dimensional diffraction slits. The collagen fibers were treated as uniform cylinders, with the scattering
geometry shown in Figure 66a. Light is incident along the z-axis on a cylinder much smaller than the wavelength and parallel to the x-axis. The field of scattered light at an angle $\theta$ from the z-axis in the y-z plane is independent of $\theta$ if the polarization is parallel to the x-axis and proportional to cosine of $\theta$ if the polarization is parallel to the y-axis. In either case, at a large distance, $z_o$ the field is proportional to the cosine of the angle $\varphi$. For a collection of cylinders all oriented in the same direction along the x-axis, assuming incoherent addition resulting from random positioning of the cylinders, the irradiance will be proportional to the $\cos^2(\varphi)$. Likewise, for cylinders oriented along the y-axis, the irradiance will be proportional to $\sin^2(\varphi)$. If the density of cylinders in the x direction decreases, being proportional to $a$, where $a=1$ when the density of cylinders in the y direction is equal to that in the x direction, then the irradiance will be proportional to $a\cos^2(\varphi) + \sin^2(\varphi)$. The polar plots in figure 3b show this function for a range of values of $a$, in which as $a$ decreases, the intensity distribution becomes more anisotropic with the longer axis parallel to the x-direction.
Figure 67: Typical load vs. time behavior of corneal tissues in the ensemble of loading protocols used in this study. Squares represent group 1, the triangles represent group 2, and the circles represent group 3. In the loaded samples from groups 1 and 3, the cornea is quickly strained to 6% and the load is recorded. Due to the viscoelastic nature of the cornea, stress-relaxation is observed. After approximately 20 minutes the sample is “relaxed” and bacterial collagenase (BC) at a concentration of 0.05mM is added, indicated by the dashed line. As the enzyme cleaves collagen fibers, the load decreases until it reaches a minimum (~0.02 +/- 0.01N). b) An individual SALS intensity distribution from a sample from group 1 at $t = 20$ min thresholded for clarity. c) SALS intensity distribution at $t = 30$ min. d) $t = 40$ min. e) $t = 50$ min. f) $t = 60$ min.

The typical mechanical response of a cornea under all three types of loading/enzyme experiments run in this study is shown in Figure 67. In group 2, the unloaded samples exposed to BC are strained until a force of 0.01N is reached, and kept constant at that strain (~0%) for the entire experiment. In groups 1 and 3, the loaded samples exhibit significant stress-relaxation and reach equilibrium at approximately 20 minutes after initial loading. At $t = 20$ min, the BC is...
added to group 1 and there is a significant reduction in load carrying capability in the cornea due to enzymatic cleavage. However, by the end of the experiment ($t=60$ min), the strained corneas in group 1 still have an appreciable load (~0.02-0.03N), indicating that at least some fibrils/fibers still remain. A series of filtered, thresholded SALS intensity distributions of a loaded cornea exposed to BC are shown at specific time intervals in Figure 67b-f. All together, Figure 67 shows that a majority of the load carrying capacity of the cornea, and most likely the cornea fibers/fibrils, are cleaved in the first 15 minutes after exposure to 0.05mM concentration of BC. During this exposure to BC, the SALS intensity distribution becomes more and more anisotropic, taking the shape of an ellipse with the major axis perpendicular to the direction of strain.

Typical raw images of SALS intensity distributions for both the unloaded, degraded cornea from group 2 are directly compared with the loaded degraded cornea from group 1 in Figure 68. After 40 minutes exposure to BC, both the loaded and unloaded cornea samples undergo a decrease in scattering angle. However, in the case of the unloaded cornea (Figure 68a, b) the decrease in scattering angle is fairly uniform, indicating that fibers in all directions are degrading at roughly the same rate. For the loaded cornea (Figure 68c, d), the decrease in scattering angle along the direction of strain is noticeably greater, indicating a preferential removal of fibers unaligned with the direction of strain.
Figure 68: Typical SALS patterns from cornea strips subject to enzymatic cleavage from both unstrained control tests (group 2) and strained tests (group 1). Both cornea samples were allowed 20 minutes to relax before BC was added to initiate cleavage. a) 0% strain and 0 min exposure to BC. b) 0% strain and 40 min exposure to BC. c) 6% strain and 0 min exposure to BC (strain direction indicated by white arrows). d) 6% strain and 40 min exposure to BC.

It should be noted that the eccentricity is a direct measurement of the degree of fiber orientation, however, it is the change in eccentricity that allows us to use SALS to dynamically test our hypothesis that strain prevents collagen fibril/fiber degradation by BC. The lamellar architecture of the bovine cornea is not constant across the center-superior-inferior strip [180], and can possibly vary slightly from cornea to cornea. It is also likely that sample preparation and loading into the bioreactor slightly affected the lamellae architecture of the cornea. It is not uncommon then to get fairly distinguished eccentricity values from different corneas, even under...
the same loading/strain protocol and approximate sampling locations. We thus use statistical analysis to ensure that the change in SALS intensity distribution is significant by implementing a paired, two-tailed Student’s *t* test to evaluate the relative change in eccentricity during the degradation of the corneal stromal tissue.

![Figure 69](image)

**Figure 69:** The relative change in eccentricity vs. time for the ensemble of experiments used in this study (above), with the P-values from a paired Student’s *t*-test to test for statistical significance in group 1 (below). Squares represent group 1 (*N* = 10), the black line is a linear fit to group 1 (*R*^2^ = 0.951), the triangles represent group 2 (*N* = 9), and the circles represent group 3 (*N* = 5). The change in eccentricity represents a shift in the distribution of scattered light, which in turn corresponds to a structural change of the collagen fiber/fibril architecture. In both control groups 2 and 3, there is no statistically significant increase in SALS eccentricity (P-values not shown). In group 1 there is a significant increase in eccentricity from 35 minutes onwards characterized by a linear fit with a slope of 8x10^-4 min^-1.

The SALS distributions in the form of the relative change in eccentricity, Δ*e*, for the ensemble of loading configurations are shown in Figure 69. Δ*e* is equal to the difference in the initial value of *e* at *t* = 20 min (the time when BC is added) and the value of *e* at the specific time of the measurement. We can see that the eccentricity of the loaded samples unexposed to BC...
from group 3 show no significant changes in SALS intensity distributions ($p > 0.05$ throughout the duration of the experiment). Though obvious, this shows that the SALS intensity distribution is constant when: 1) enough time has elapsed and the cornea is sufficiently ‘relaxed’, 2) the collagen fiber/fibrils do not undergo any changes in the structural arrangement after approximate equilibrium is reached.

Similarly, we see that the SALS distribution from the unloaded samples exposed to BC from group 2 show no significant changes over time ($p > 0.25$ throughout the duration of the experiment). It should be noted that once BC is added to the cornea, the scattering angle decreases (Figure 68a, b) indicating fiber/fibril structural changes (enzymatic cleavage).

For the loaded samples exposed to BC in group 1, there is a significant change in the SALS eccentricity ($p < 0.02$) from 35 minutes onward (15 minutes after BC addition). Over the course of 40 minutes of exposure to BC at a constant strain the eccentricity increases linearly as the overall scattering angle decreases, although not uniformly (Figure 68c, d).
Figure 70: Low and high magnification TEM micrographs of cross sections from corneal samples exposed to BC. Each cross section is perpendicular to the direction of the applied tensile load. 

a) Low magnification taken from a sample subject to 6% tensile strain and 40 minutes exposure to BC from group 1. Black arrows indicate areas where there appears to be remnants of fibrils transverse to the direction of load. Bar is 2 µm. 

b) Low magnification taken from sample subject to 40 minutes exposure to BC from group 2. White arrows indicate remnants of fibrils both aligned and transverse to the direction of load. Bar is 2 µm. 

c) High magnification of sample from group 1. Black arrows indicate what appear to be boundaries between fibrils aligned to the direction of load and remnants of fibrils transverse to the direction of load. Bar is 1 µm. 

d) High magnification of sample from group 2. White arrows highlight areas of fibril degradation both aligned and transverse to the direction of load. Bar is 1 µm.
Typical high-magnification TEM micrographs of corneal tissue cross sections (perpendicular to the loading direction) from both groups 1 and 2 are shown in Figure 70. Figure 70a,b are lower magnification micrographs of the loaded and unloaded samples following exposure to BC. The loaded sample exhibits a considerably altered architecture from that of a typical cornea, which comprises alternating arrays of aligned fibrils. Close examination of Figure 70a shows that the fibril populations which survive the BC exposure were predominantly aligned with tensile load. Areas can be seen in the figure which appear to be remnants of fibril arrays which were transverse to the applied load (black arrows). In Figure 70b, there are significantly less fibrils and more open spaces compared to the loaded sample for the same time of exposure to the enzyme. Moreover, populations of fibrils that were both aligned with and transversely to the tensile load exhibit degradation (white arrows). Figure 70c, d show higher magnification micrographs of loaded and unloaded samples, respectively. In the loaded case, there are fairly distinct boundaries between subsets of fibrils aligned with and transversely to the load (black arrows), with the transverse (unloaded) fibrils preferentially degraded. In the unloaded case in Figure 70d, there is general fibril degradation with no discernable orientation preference.

A5. Discussion

The uniform decrease in scattering angle exhibited by unloaded samples (Figure 68a, b) indicates that the degradation rate (loss of fibril scatterers) is consistent across fibrils/fibers for all orientations in the absence of a directional load. The SALS data is supported by high-magnification TEM micrographs (Figure 70b, d), in which fibril populations orthogonal to each other and under similar load (~0 N) both undergo substantial degradation. Our null hypothesis specified that applying a unidirectional load to the tissue should not alter this uniformly
decreasing pattern in the SALS signal, indicating no effect of load on the rate of degradation of the tissue. The first problem encountered in testing the hypothesis is the fact that mere application of the uniaxial load, in the absence of the enzyme, changed the SALS pattern to suggest a more vertical fibril alignment (i.e. with the load; see Figure 68c). Because of this not unexpected complication [182, 183], it was necessary to compare the eccentricity of the initial SALS pattern to all subsequent patterns at different time points within the same experiment. Figure 68d shows that following degradation under mechanical strain there appears to be increasing preferential alignment emerging from the initial eccentric pattern in the same sample. Figure 69 confirms this observation statistically and also provides quantification of the rate of differential degradation of the two sets of fibrils (loaded and unloaded controls). The overall increase in eccentricity exhibited by experimental group 1 is approximately linear ($R^2=0.951$), with a slope of $8 \times 10^{-4}$ min$^{-1}$, which is directly related to the relative rate of preferential degradation of fibrils perpendicular/unaligned with load. To characterize this, we assume that after 40 minutes of exposure to BC, the amount of fibrils perpendicular to load has significantly decreased. This is partially justified by Figure 68b, in which after ~40 minutes exposure to BC, the scattering angle of the unloaded fibrils decreases almost to that of the incident laser beam, indicating that a very low number of fibrils are capable of scattering the beam.

From SALS dynamic imaging and calculation of the slope of the rate of change in eccentricity, it is thus possible to conclude that the internal set of unloaded control fibrils are being disrupted at a faster rate than the loaded fibrils within the same sample. The preferential disruption supports the idea that strain can be used to sculpt tissue in the presence of the highly active enzyme: bacterial collagenase. The two other control groups (0% strain, enzyme and 6%
strain, no enzyme) do not produce a significant difference in the eccentricity, further supporting
the conclusion.

High magnification TEM micrographs generally corroborate the SALS data and indicate
that there is preferential retention of loaded fibrils. In Figure 70, it is clear that there are more
fibrils, which are less degraded, remaining in the loaded sample compared to the unloaded
sample. Furthermore, within the loaded sample, fibrils aligned with the load appear to be
retained preferentially over those which were transverse to the load.

As outlined in detail in the introduction, there have been numerous investigations which
demonstrate that small applied loads have a marked effect on the rate of enzymatic degradation
of collagen at the tissue, reconstituted network and single molecule scales. However, the precise
mechanism behind the strain-dependent activity of the enzyme on collagen has yet to be
elucidated. In whole tissues such as the cornea and tendon, it is possible that applied mechanical
tension could exclude the enzyme preferentially from loaded fibers or lamellae through a pseudo
Poisson-effect. This could lead to what appears to be preferred cleavage of unloaded tissue.
However, volume exclusion of enzyme as the mechanism of preferential degradation in loaded
cornea was discounted in Zareian et al [162] through arguments based on porosity changes.
Nabeshima et al [159], through a separate series of diffusion experiments on loaded tendon,
showed that enzyme retention in the tissue was actually enhanced (not reduced) by the
application of mechanical strain. At the fibril and molecular level, applied strain could 1) expose
or conceal enzyme binding sites; 2) shift the alpha-chains to prevent enzymatic cleavage or 3)
enhance the stability of the helix itself (possibly by bringing the alpha chains into closer
proximity and increasing the hydrogen bonding). Currently, the mechanism of strain-stabilization
remains an open question.
In spite of the positive and statistically significant results that were obtained, there are several limitations of the approach used in this investigation. First, the measurement method cannot actually detect collagen fibrils in the cornea (~35 nm in diameter), but rather it is most likely detecting lamellae packed with hundreds to thousands of fibrils in uniform alignment. Therefore the perceived loss of signal is possibly related to the disruption of the lamellar structure in a particular direction and may not be due to actual collagen degradation. Second, experiments in our lab on single native collagen fibrils indicate that collagenase may cleave the monomers in the fibril, but that the fibril optics may not change appreciably on the time scale of the experiment. This is possibly because the cut monomer fragments do not diffuse away from the fibril surface after enzymatic conversion. Finally, the use of bacterial collagenase in lieu of physiologically relevant enzymes such as MMP limits the applicability of the observation of strain-directed remodeling. In a recent single molecule investigation, it has been shown that MMP-1 cuts collagen fragments at a higher rate when loaded in axial tension [184]. Whether or not this observation holds true for full collagen molecules, intact native fibrils or for whole tissues remains to be determined.

A6. Conclusion

The results strongly support previous work which suggests that mechanical strain stabilizes collagen against enzymatic attack by bacterial collagenase [156, 157, 162, 163, 185]. The detection of preferential enzymatic disruption of unloaded fibrillar/lamellar structures demonstrates that SALS may be a simple low cost method capable of dynamically tracking and quantifying distinct enzyme-induced changes in tissue engineered constructs and in native tissue, which has previously eluded researchers [162]. To produce load-bearing, tissue replacements
which require arrays of collagen fibrils that are highly-organized at the nanoscale, tissue engineers could potentially modulate strain and collagenase concentration in combination with SALS feedback to controllably sculpt collagen architecture. Such an advance could enable large scale production of engineered tissue replacements with optimized collagen organization.

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APPENDIX

Scanning Electron Microscope fixation protocol
1) Primary fixation (Karnovsky’s fixative): 2.5% Glutaraldehyde, 2.5% Formaldehyde, 0.1M Cacodylate Buffer pH 7.2 for 2 hours at 4°C.
2) Wash: three 15 minute washes of 0.1M Na Cacodylate Buffer pH 7.2 for 2 hours at 4°C.
3) Post fixation: 1.0% Osmium Tetroxide, 0.1M Cacodylate Buffer pH 7.2 for 2 hours at room temperature.
4) Dehydration (each for 10min): 30% Ethanol, 50% Ethanol, 70% Ethanol, 85% Ethanol, 95% Ethanol, 100% Ethanol (1 hour, 2 changes)

Scanning Electron Microscope critical point drying protocol
Use plated well buckets to place the hydrogel CL inside the chamber, making sure to keep track of which side is up/was in contact with the cornea. Flood the chamber with liquid CO₂, wait 2 minutes, purge, and repeat 10 times. Afterwards, turn on the heat until the critical temperature is reached, and then slowly vent the chamber. Remove the CL samples when the pressure reduces to atmospheric levels.
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