

Challenge of the Soft-tissue to Device Interface:

A Rheology-based Approach to Biomaterial Development

By

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Philosophy in the Division of Engineering at Brown University

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Preface and Acknowledgments

The following dissertation represents the culmination of six years of research endeavor that were mostly instructive, at times frustrating, but ultimately fulfilling. As an undergraduate an astute professor once told me that success at the graduate level is 20% intellect and 80% determination. Well, I may not agree with exact numbers but the sentiment is true. Although, the work presented here is the product of my own determination and intellectual endeavor, the completion of the work would not have been possible without the support, guidance, insight, and motivation provided by my mentors and the love and devotion of my friends and family.

I would like to take this opportunity to thank Dr. Jeffrey R. Morgan for being an extraordinary mentor over the course of my graduate school experience, both personally and professionally. Jeff, throughout the trials and tribulations, successes and failures of my graduate school experience, whether the training moments were at the bench-top or in your office, via scientific jargon or the odd basketball related metaphor, the wisdom and truisms you imparted to me during our many discussions will last me a life-time. Thank you, Jeff for your support, and ultimately for your belief in my approach and methodology; even when a positive outcome did not always look likely. In a project as interdisciplinary as mine, you always made sure my course was true and my focus on acute. I will always be grateful to you for your guidance and mentorship.

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Finally, this dissertation is dedicated to James Holt Jr., my father. There are moments I ask for you, although I know you cannot hear me. There are moments I feel you, although I know you are not near me. There are moments I look to you, although I know you cannot see me. That said, there are never moments that I need your love and do not feel it surrounding me.

Table of Contents

Chapter 1

1.1	Introduction.....	1
1.2	Background	
1.2.1	Human Skin and Dermis.....	5
1.2.2	Wound Healing and Epidermal Regression	
1.2.2.1.	Cutaneous Wound Healing Process.....	6
1.2.2.2.	Cutaneous Wound Healing Process at the Soft-tissue to Device Interface.....	11
1.2.3	Percutaneous Medical Devices	
1.2.3.1.	Percutaneous Device Design Criterion.....	12
1.2.3.2.	Current Percutaneous Cuff Designs: Advantages and Disadvantages.....	19
1.2.3.3.	Percutaneous Devices to Benefit from Mechanics-based Approach to Device Design	
	Enteral Catheters.....	20
	Parental Catheters.....	21
	Peritoneal Dialysis Catheter.....	21
	Bone Lengthening External Fixators.....	22
	Osseointegration Dental Implants.....	23
1.2.3.4.	Percutaneous Device Failure Modalities.....	24
1.2.4	Role of Mechanics in Tissues.....	27
1.2.5	Soft-tissue Biomaterial Design.....	29
1.2.5.1.	Role of Mechanics in Biomaterial Design....	31
1.2.5.2.	Poly(2-hydroxyethyl methacrylate) [pHEMA] biomaterials and devices.....	32

1.2.6	Viscoelasticity and Rheology	
1.2.6.1.	Viscoelasticity in Shear.....	35
1.2.6.2.	Viscoelasticity of Human Skin and Dermis...	44
1.2.6.2.	Rheology.....	47
1.2.7	Dissertation Objectives.....	49

Chapter 2

2.1	Abstract.....	70
2.2	Introduction.....	72
2.3	Materials & Methods.....	74
2.4	Results and Discussion.....	77
2.5	Conclusions.....	85
2.6	Acknowledgements.....	89
2.7	References.....	90
2.8	Figures.....	94

Chapter 3

3.1	Abstract.....	102
3.2	Introduction.....	104
3.3	Materials and Methods.....	106
3.4	Results and Discussion.....	108
3.5	Conclusions.....	116
3.6	Acknowledgements.....	120
3.7	References.....	121
3.8	Figures.....	125

Chapter 4

4.1 Abstract.....	135
4.2 Introduction.....	136
4.3 Materials and Methods.....	137
4.4 Results and Discussion.....	143
4.5 Conclusions.....	149
4.6 Acknowledgements.....	151
4.7 References.....	152
4.8 Figures.....	156

Chapter 5

Conclusion and Future Directions.....	166
---------------------------------------	-----

Table Legends

Table 4.1: Quantitative comparison of untreated and gelatin modified, micro- and macro-porous substrates and human skin from isothermal (37°C), constant oscillatory strain (0.1%) experiments between 0.628 and 75.40 rad/s. Power law curves ($G' \sim G'' \sim \omega^x$) were fit to G' and G'' values within the low (≤ 10 rad/s) and high (> 10 rad/s) frequency regimes for comparison of the rate of increase of frequency dependent viscoelastic behavior. All pHEMA substrates are with normal human fibroblast cells..... 164

Table 4.2: Qualitative comparison of experimental data from isothermal (37°C), constant oscillatory strain (0.1%) experiments between 0.628 and 10 rad/s on untreated, micro- and macro-porous substrates without cells and protein modified, micro- and macro-porous substrates with normal human fibroblasts to an adapted soft-solid model. Experimental data and model predictions were compared using percent difference (accuracy) and correlation coefficient (trend-matching). All substrates are with NHF cells..... 165

Figure Legends

Figure 2.1: Preparation and rheological measurements of human skin and dermis. **(A)** Human skin specimens were sectioned using a 6mm dia. biopsy punch and then debrided for rheological characterization. Dermal specimens were obtained from whole skin samples using a six hour, dispase-incubation @ 37°C. **(B)** Circular skin samples were placed into the rheometer, epidermis side up, onto water proof sandpaper secured with double side tape. After loading with a small normal force compressive force (0.02 and 0.06N), each sample was subjected oscillatory shear forces..... 94

Figure 2.2: Oscillatory strain sweep. Whole skin and dermis-only biopsies were subjected to isothermal (37°C) percent strains ranging from 0.01 to 5% at 6.283rad/s in order to determine the optimal percent strain value for the oscillatory response experiments. The resultant storage modulus, G' was measured for both soft-tissues and changes in those values noted..... 95

Figure 2.3: Linear viscoelastic response. (A) Whole skin and (B) dermis-only biopsies were subjected to isothermal (37°C) oscillatory strains of 0.5% and 0.04%, respectively between frequencies of 0.628 and 75.398rad/s. The resulting G' and G'' and $\delta = \tan^{-1}(G''/G')$ values at various frequencies were measured..... 96

Figure 2.4: Creep compliance as a function of time. (A) Whole skin and (B) dermis-only biopsies were subjected to isothermal (37°C) shear creep loads of 5 and 10Pa and the resulting strain and compliance data was measured in order to quantify the low magnitude shear stress creep behavior for the two types of soft-tissue..... 97

Figure 2.5: Recoil after steady flow as a function of time. Whole skin (A) and dermis-only (B) biopsies were subjected to no shear-stress and the resulting strain and compliance data was measured in order to quantify the low magnitude shear stress recovery behavior for the two types of soft-tissue..... 98

Figure 2.6: (A) Schematic of Kelvin-Voigt viscoelastic solid model. (B) Whole Skin, 10Pa Kelvin-Voigt model simulation; (C) Whole Skin, 5Pa Kelvin-Voigt model simulation; (D) 10Pa Kelvin-Voigt model fit applied to oscillatory response data; (E) 5Pa Kelvin-Voigt model fit applied to oscillatory response data These models were intended to mimic the inertio-elastic oscillations observed during the creep experiments in order to determine the G' and G'' values associated with the two soft-tissue's shear creep response. Parameters were optimized using an iterative simulation process, allowing for the back-calculation of the respective G' and G'' values..... 99

Figure 3.1: Preparation and rheological measurements of porous pHEMA substrates. (A) Micro-porous pHEMA substrates were polymerized by UV-cross linking using NaCl as a diluent and EGDMA as a cross-linker. (B) Micro-porous substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% between 0.6283 and 75.398 rad/s, a physiologically relevant frequency range. The resulting G' and G'' values at various frequencies were measured..... 125

Figure 3.2: Determination of optimal NaCl concentration in micro-porous pHEMA substrates intended to mimic human skin. Rheological characterization was conducted on micro-porous pHEMA substrates with ethylene glycol dimethacrylate cross-linker were photo-polymerized with ultraviolet light containing 0.6, 0.7, 0.8, and 0.9M aqueous NaCl diluent. The equilibrium modulus, G_o , values were compared in order to determine a viscoelastic behavior related threshold for diluent molarity. Substrates with greater than 0.9M NaCl did not successfully polymerize under the given protocol..... 126

Figure 3.3: Strain sweep experiments of micro-porous substrates. pHEMA substrates were subjected to isothermal (37°C) oscillatory strain sweep experiments between 0.01 and 3 % strain at 1 Hz (6.283 rad/s). Micro-porous substrates had various monomer to diluent ratios: 1:4 (■); 1:8 (●); and 1:10 (▲); as well as various EGDMA cross-linker concentrations: 0.0001875 (◆) and 0.0075 (◆). Independent of monomer to diluent ratio and cross-linker concentration, pHEMA substrates show linear viscoelastic behavior across wide range of physiologically relevant strains. Whole thickness human skin is also shown for reference (◆)..... 127

Figure 3.4: Oscillatory shear stress response of micro-porous pHEMA substrates. Micro-porous pHEMA substrates were subjected to isothermal (37°C) oscillatory stress sweep experiments between 1 and 100 Pa at 1 Hz (6.283 rad/s) and the resulting G' and G'' values measured for comparison [*closed and open symbols, respectively*]. Despite differences in magnitude, substrates with 1:4 (■), 1:8 (●), and 1:10 (▲) monomer to diluent ratios showed similar degrees of linear viscoelastic behavior, becoming non-linear at loads ≤ 10 Pa. Substrates with 0.001875 (◆) and 0.0075 (●) EGDMA concentrations showed similar behavior..... 128

Figure 3.5: Physiologically relevant rheological characterization of micro-porous pHEMA substrates. Micro-porous substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% and the resulting G' (A) and G'' (B) values were measured [*closed and open symbols, respectively*]. pHEMA substrates with 1:4 (■), 1:8 (●), and 1:10 (▲) monomer to diluent ratios were examined between 0.1 and 12 Hz (0.6283 – 72.4 rad/s). Identical measurements were taken on pHEMA substrates with 0.001875 (◆) and 0.0075 (●) EGDMA concentrations. Whole thickness human skin is also shown for reference (◆)..... 129

Figure 3.6: Temperature dependent rheological characterization of micro-porous pHEMA substrates. pHEMA substrates were subjected to oscillatory strains of 0.1 % between 1 - 10 Hz (6.283 – 62.83 rad/s) from 20 - 50°C at 10°C intervals; the resulting G' (closed) and G'' (open) values were measured. The viscoelastic response of micro-porous pHEMA substrates with 1:4 (A), 1:8 (B), and 1:10 (C) monomer to diluent ratios were compared as were their corresponding $\tan \delta$ values (D, E, and F, respectively).....130

Figure 3.7: Iso-frequency comparison of temperature dependent rheological characterization of micro-porous pHEMA substrates. To further emphasize the effect of temperature on the viscoelastic response of micro-porous pHEMA substrates, G' (closed) and G'' (open) values from substrates with differing monomer to diluent (aqueous NaCl) ratios (A) and EGDMA concentrations (B) were compared at 1 Hz (6.283 rad/s). Monomer to diluent ratios of 1:4 (■), 1:8 (●), and 1:10 (▲) showed clear trends with increasing temperature. However, the effect of temperature on the viscoelastic response of substrates with 0.001875 (◆) and 0.0075 (●) EGDMA concentrations proved to be negligible..... 132

Figure 3.8: Micro-mechanical characterization of micro-porous pHEMA substrates with differing monomer to diluent ratios. Decreasing monomer to diluent ratio has a significant effect on mechanical behavior of micro-porous pHEMA substrates.133

Figure 4.1: Preparation and rheological measurements of porous pHEMA substrates. (A) Micro- and macro- porous pHEMA substrates were polymerized by UV-cross linking. Micro pores and macro pores were created using NaCl and NaCl plus alginate beads, respectively. For cell growth, substrates were modified with CDI enabling gelatin attachment. (B) Micro- and macro-porous substrates, on which were cultured normal human fibroblasts, were subjected to an isothermal (37°C) oscillatory strain of 0.1% between 0.6283 and 75.398 rad/s, a physiologically relevant frequency range. The resulting G' and G'' values at various frequencies were measured..... 156

Figure 4.2.: Alginate bead porogen preparation. (A) Cross-linked alginate was used to create macro-scale pores in pHEMA substrates. Alginate beads were cross-linked in a drop-wise fashion by exposure to 4% (w/v) BaCl₂ stirred at 300 rpm. (B) From scanning electron micrographs, alginate beads were estimated to between 200 – 400 µm in diameter.....157

Figure 4.3: Rheological characterization of substrates mimicking the mechanical behavior of human skin. pHEMA substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% and the resulting G' (A) and G'' (B) values were measured. Micro-porous substrates had various concentrations of NaCl: No NaCl (■, □); 0.6M (●, ○); 0.7M (▲, △); 0.8M (▼, ▽); and 0.9M (◀, ▶). Macro porous substrates had 0.8M NaCl plus 1.5% (w/v) dried alginate beads (►, ►). Human skin is also shown for reference (◆, ◇). 158

Figure 4.4: Kinetics of swelling and water uptake of pHEMA substrates. Micro-porous (■) and macro-porous (●) substrates were dried, weighed and then added to de-ionized water equilibrated at 37°C over 180mins. At specific time intervals, substrates were removed and weighed. (A) The resulting weights were used to calculate the swelling factor (closed symbols) and equilibrium water content (open symbols). (B) Linear curve fitting to a reduced $\ln\text{-}\ln$ swelling factor v. time plot revealed information about the swelling kinetics of the micro- and macro-porous substrates. 159

Figure 4.5: Functionalization and cell growth on pHEMA substrates. (A) pHEMA substrates were functionalized via 1,1' carbonyldiimidazole-mediated hydroxyl group activation and gelatin attached. (B) Human fibroblasts were seeded on various control and modified pHEMA substrates and cell proliferation was measured using the WST-1 assay 72 hours after seeding cells. Cell growth was significantly increased when pHEMA were treated with both CDI and gelatin..... 160

Figure 4.6: Viscoelastic response of micro- and macro- porous substrates with and without NHFs. Cells were grown on micro- (A, B) and macro-porous (C, D) substrates for 14days, after which substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% between 0.628 and 75.40 rad/s and G' (A, C) and G'' (B, D) were measured. Untreated substrates, with (●,○) and without (■,□) cells and substrates treated CDI and gelatin with cells (▲,△) were measured and compared to human skin (◆,◇). Note, cellular activity on modified substrates increased the magnitude of G' and G'' at low frequencies while also altering the degree of high frequency dependence, indicating that cellular interactions with the micro-pore infrastructure has a profound effect on the viscoelastic behavior of the substrate..... 161

Figure 4.7: Adapted soft-solid model – A soft-solid viscoelastic model was applied to the rheological data on control, micro-porous and macro-porous substrates without cells (■,□) and modified micro-porous and macro-porous substrates on which normal human fibroblasts were cultured (▼,▽ and ▲,△, respectively). [*open symbols denote G'' values*] (A, B)The soft-solid model simulates viscoelastic solids with an internal structure that liquefies at higher frequencies. The resulting G' and G'' values predicted by the model (dashed lines) were compared qualitatively to experimental data. A model sensitivity analysis showed G' (C) response of the Caswell model was essentially a one parameter fit in G_e while G'' (D) required a more complex three parameter fit..... 162

Figure 4.8: Scanning electron microscope [SEM] analysis of poly(2-hydroxyethyl methacrylate) [pHEMA] substrates – SEM analysis of freeze-dried micro-porous (A-C) and macro-porous (D-F) pHEMA substrates shows a dense, interconnected pore network, characteristic of hydrogels and ideal for biomaterial related, soft-tissue applications..... 163

Chapter 1:

Introduction and Background

1.1. Introduction

The formation and maintenance of a proper soft-tissue seal, or interface region, is essential to the millions of percutaneous medical devices implanted annually in the United States [Jacobsson et al., 1991; NNIS, 2004; Edwards et al., 2009]. The majority of indwelling percutaneous devices fail due to epidermal regression mediated infection, leading to the development of acute or chronic non-nosocomial or nosocomial/healthcare-related infections [HAIs]. Therefore, epidermal regression-mediated infections represent a significant economic burden to health care institutions and quality of life reduction for patients [NNIS, 2004; Edwards et al., 2009]. The family of percutaneous medical devices is quite expansive, however within the context of clinically implanted devices capable of eliciting a wound healing response that leads to epidermal regression-mediated failure there are five specific devices that require the most attention: enteral catheters; parenteral catheters; peritoneal dialysis catheters; bone-lengthening external fixators; and osseointegrated dental implants. Despite advances in fixation and antimicrobial surface treatments, within the family of percutaneous medical devices there still exist profound limitations that exacerbate the disintegration of the soft-tissue to device interface [Winter, 1974; von Recum et al., 1984; Palacci et al., 1995; Pendegrass et al., 2006; Norowski et al., 2009; Morais et al., 2010]. The associated infections are as varied as the family of percutaneous devices but the predominant pathogens are *Staphylococcus aureus* [*Staph a*] and *Staphylococcus epidermidis* [*Staph e.*] [von Recum et al., 1984; Gristina, 1987]. Due to the exposure of underlying tissues, any infection poses a significant risk to the well-being of the patient. Additionally, the growing reliance on advanced treatment/drug delivery systems has further increased the prevalence of percutaneous devices in

contemporary medicine, creating the potential for exponential growth in the number of patients who will undergo procedures involving percutaneous devices. Thusly, as the number of devices implanted grows so too will the number of failures, leading to further increases in medical cost and a negative impact on patient quality of life.

Although a profoundly biochemical process, the initiation and persistence of epidermal regression is the product of disruptions to the mechanical integrity of the whole tissue, mediated by individual cells responding to shear-based changes in the micro- and macro-scale mechanical environment. The mechanical discontinuities at the soft to device interface cause stress concentrations within the skin that lead to cellular hyperproliferation and hyperactivity within the dermis. Changes in cellular morphology and phenotype eventually disrupt the normal wound healing process and begin the self-perpetuating cytokine cascade that leads to exit-site epidermal regression. Consequently, to avoid epidermal regression, it is necessary to develop an accurate way to describe, and then minimize, the contribution of micro-strain, shear induced stress concentrations to changes in the physiologic/metabolic processes that lead to tissue degradation.

Previous biomaterial design efforts have focused primarily on the biocompatibility and the biomimetic capacity of a given substrate. That said, advances in polymer synthesis and an improved understanding of the biophysics-related mechanisms behind cell-substrate interactions has created areas for additional consideration within the biomaterial development field. Add to these advances an improved ability to describe how soft-

tissues dissipate quasi-static and dynamic loads, and researchers are now capable of establishing more definitive connections between in vitro biomechanical experimentation and physiologically relevant loading conditions [Gardel et al., 2008; Humphrey, 2010]. Together these advances allow researchers to examine the mechanical biomimetic capacity of a given substrate, providing an additional design criterion with the potential to improve the efficacy of biomaterials in situ. This dissertation focuses on the development of a mechanics-based approach for the design of a soft-tissue related biomaterial capable of addressing the unique mechanical considerations present at the soft-tissue to device interface of percutaneous medical devices.

1.2. Background

1.2.1 Human Skin and Dermis

Human skin is composed of two primary sub-layers: the epidermis and dermis, whose thicknesses are dependent on anatomical location and percent water content. The epidermis is avascular and approximately 100 microns thick with only the deepest three layers of the five possessing metabolically active cells. The five layers of epidermis, from outer-most to innermost, are as follows: the stratum corneum, the stratum lucidum, the stratum granulosum, the stratum spinosum, and the stratum germinativum. The two primary functions of the epidermis are: 1) to serve as a continuous barrier to pathogens, water loss, and ultraviolet rays, and 2) to mediate thermoregulation. The cells of the epidermis are bound to one another within the five distinct layers by desmosomes, occluding junctions, and gap junctions. The majority of the mechanical strength of the epidermis is derived from these relatively strong intercellular adhesion structures.

The dermis is composed of two sub-layers: the papillary and reticular layers, which contain a connective tissue matrix with a self-orienting/organizing, interlaced collagen fiber infrastructure. The collagen fiber infrastructure is an important part of the extracellular matrix (ECM). The primary type of collagen in the dermis is collagen Type I, although type III collagen is also prevalent [Silver et al., 1992]. Collagen Type I, the most common in the human body, has a right-handed twist triple helical structure [Lynch et al., 2004] that gives the protein an inherent mechanical strength. The papillary layer, the thinner and uppermost layer, contains collagen fibrils on the order of 20 to 40 nm in diameter [Lynch et al., 2004]. The reticular layer, the thicker and lower layer, contains

collagen fibrils that are 60 to 100 nm in diameter [Lynch et al., 2004] and accounts for much of the skin's mechanical strength. Within this connective tissue matrix there are various types of cells, of which fibroblasts are the dominant cellular phenotype. Fibroblasts are responsible for the protein production associated with the formation of the collagen fibrils and aid in the coordination of the larger collagen fiber network. Additional mechanical constructs of the dermis are the dermal papilla, best described as macro-scale inter-digitations which mechanically fix the two sub-layers in the horizontal plane to resist and dissipate shear loads.

1.2.2. Wound Healing and Epidermal Regression

1.2.2.1. Cutaneous Wound Healing Process

The cutaneous wound healing process involves four distinct stages: 1) inflammation; 2) granulated tissue deposition and neovascularization; 3) epithelialization; and 4) wound site contraction/extracellular matrix remodeling [Singer et al., 1999; Gillitzer et al., 2001]. Cutaneous wound healing represents one of the most well-orchestrated biological processes in the human body, requiring the cooperation of a myriad of distinct cells at the wound site as well as immune cells recruited from the near and distant vasculature. In addition to cellular recruitment, the process also requires changes in cellular morphology and phenotype, to produce the full spectrum of cytokines, growth factors, and proteins involved in the precisely timed stages of the wound healing process.

The inflammatory stage is intended to prevent blood and complement loss and return the injured tissue to as near a homeostatic state as is possible, both physiologically and

mechanically. The inflammation that follows wounding is critical for the recruitment of macrophages and fibroblasts. The infiltration of inflammatory leukocytes, neutrophils, and macrophages in response to chemoattractants elicited by fragments of extracellular matrix protein, precedes the synthesis of transforming growth factor β [TGF- β], tumor necrosis factor- α [TNF- α], and monocyte attractant protein 1 [MCP-1] which initiate the production and release of platelet-derived growth factor [PDGF] [Singer et al., 1999; Spiekstra et al., 2007]. As an aside, TNF has been shown to have an active, beneficial role in human wound healing, while in murine excision models, blocking TNF pathways has resulted in improved angiogenesis, collagen production, and wound closure [Mori et al., 2002]. In wounds with minimal to severe blood loss, neutrophil recruitment is increased by the presence of additional platelets that produce neutrophil-activating peptide-2 [NAP-2] via proteolytic conversion of chemokine-connective tissue activating peptide-III [CTAP-III] [Gillitzer et al., 2001]. NAP-2 promotes neutrophil migration and activation, accelerating the inflammatory response of the tissue at the wound site while furthering PDGF production. PDGF is essential for fibroblast activation and proliferation within the wound site, helping to bolster and sustain the soft-tissue repair and remodeling process. Additionally, TGF- β 1, β 2, and β 3 produced by transforming growth factor α [TGF- α] activated keratinocytes at the wound margins, also play a critical role in the cutaneous wound healing activation of dermal fibroblasts. During the inflammatory stage macrophages are the most active cell type at the wound site, phagocytosing cellular debris, foreign matter, and bacterium in the wound while also secreting the cytokines that control the transition of the gross cellular activity from inflammatory response to reparative functionality [Singer et al., 1999].

The granulated tissue deposition and epithelialization stages occur nearly simultaneously within the wound bed over the course of several days, involving the coordinated efforts of the keratinocytes of epidermis and the fibroblasts of the underlying dermis. Hours after injury, macrophages tethered to the remnants of the post-injury extracellular matrix [ECM], continue to produce PDGF and TGF- β 1 and begin to elicit colony stimulating factor 1 to stimulate fibroblast proliferation and the production of the intermediate procollagen lattice that will form a provisional matrix in the wound bed [Singer et al., 1999]. Note, macrophage cytokine production also ensures fibroblasts express alternative integrin complex conformations that allow the cells to migrate more freely throughout the entire wound bed [Singer et al., 1999]. As well, initial keratinocyte activity is directed by macrophage and monocyte derived interleukin-8 [IL-8]. At later phases of this stage, the up-regulation and endogenous production of IL-8 by keratinocytes serves to sustain and direct the migration of the wound edge [Gillitzer et al., 2001]. The provisional matrix contains fibrin, fibronectin, and hyaluronic acid, all of which are essential for epidermal wound margin propagation and subsequent epithelialization [Singer et al., 1999]. Structural molecules of the provisional matrix provide the topographical and biochemical cues necessary for fibroblast-mediated ECM deposition and remodeling within the wound bed.

The remodeling of the provisional matrix into a mature, collagenous extracellular matrix and the removal of the fibrin plug (i.e. eschar/clot) is accomplished by a coordinated effort of the keratinocytes of the epidermis and the fibroblasts of the dermis. The release

of plasminogen activators, collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, also known as matrix metalloproteinases 1, 2, 3, and 13, respectively [MMP-1, 2, 3, 13], by fibroblasts and keratinocytes allows the former to freely remodel the matrix and the latter to move from the distal edge of the wound site to the wound site center [Singer et al., 1999].

During the simultaneous granulated tissue deposition and epithelialization stage, the fibroblasts and keratinocytes work in concert to reproduce an ordered basement membrane and rebuild the tight junctions and desmosomes that characterize a healthy epithelium. The formation of a new basement membrane is an essential part of securing a continuous epithelial seal and ensuring wound closure. Throughout the later phases of this stage, keratinocyte growth factor [KGF], fibroblast growth factor-7 [FGF-7] and interleukin-6 [IL-6] work synergistically to modulate growth of the epidermis over the underlying dermis [Werner et al., 2007]. In murine wound healing models, IL-6 deficient subjects actually showed a severe retardation of the wound healing process [Werner et al., 2007]. In abnormal wound healing conditions and fibroproliferative disorders, variations in concentrations of fibrogenic cytokines, namely TGF- β 1, TGF- β 2, TGF- β 3, insulin-like growth factor 1 [IGF-1], and interleukin-1 [IL-1], have led to amorphous pro-collagen and/or collagen deposition leading to the disruption of the cutaneous wound healing process.

The final phase of the cutaneous wound healing process involves wound contraction and extracellular matrix reorganization occurring 8-10 days post-injury, depending on the

severity of the wound. The wound contraction and ECM reorganization phases of the final cutaneous wound healing process are marked by a pronounced change in fibroblast phenotype and slower rate of collagen lattice degradation. Fibroblasts become myofibroblasts, increasing the number and density of acto-myosin microfilaments within their cytoplasm to reinforce the cell-cell and cell-matrix linkages established following the epithelialization/wound closure stage [Singer et al., 1999]. Similar to previous stages PDGF and TGF- β dominate cytokine expression. The remodeling and reorganization is mediated by a delicate balance of MMP expression and MMP inhibitor expression. Synthesis of dermal collagen continues but the majority of fibroblast cellular activity is focused on the remodeling, bundling, and cross-linking of collagen fibers into more complex hierarchal structures capable of maintaining and restoring the mechanical integrity of the skin to near pre-injury capacity. In the concluding phases of ECM remodeling, the fibroblast population begins to decrease through a series of tissue initiated apoptotic biochemical cues. Fibroblast apoptosis further slows the matrix remodeling process as a homeostatic mechanical state is reached. Similar to the previous stages of the cutaneous wound healing process, excess and/or deficiencies in TGF- β 1, TGF- β 2, TGF- β 3, IGF-1 and IL-1 can lead to pathologic scarring and abnormal healing.

Neovascularization is a key component of the cutaneous wound healing process, beginning after the cessation of the inflammatory stage, continuing well into the wound contraction and ECM remodeling phases of the wound healing process. The importance of neovascularization and the energy utilized to reconstitute the damaged or destroyed vasculature in the hypoxic, post-injury wound site should not be disregarded. However,

due to the micro-biological and biochemical complexity of angiogenesis, the required discussion of the topic would be tangential to scope of the work presented in this thesis. Consequently, the discussion is omitted but not to discount the importance of neovascularization in the wound healing process.

1.2.2.2. Cutaneous Wound Healing Process at the Soft-tissue to Device Interface

The cutaneous wound healing process at the soft-tissue to device interface is not dissimilar to the normal cutaneous wound healing, particularly in its initial stages. An acute inflammatory stage follows the implantation of the device, lasting from hours to days [Morais et al., 2010]. The acute inflammatory stage is characterized by complement and protein exudation [Morais et al., 2010]. Like the normal cutaneous wound healing process, the acute inflammation is intended to limit fluid loss and begin the process by which biological and mechanical homeostasis will be returned through the development of a provisional matrix. Similar to the normal wound healing process, the inflammatory stage is characterized by the infiltration of mast cells and monocytes, namely neutrophils and macrophages, and the migration of fibroblasts to the wound site. Unlike the normal cutaneous wound healing process, once the provisional matrix is established and micro-scale debris cleared, a chronic inflammatory stage begins due to the persistence of the device at the wound site [von Recum et al., 1984; Joseph et al., 2010; Morais et al., 2010]. Although not characteristic of this stage, in some soft-tissue and percutaneous devices fluid and protein exudation may continue. The chronic inflammatory stage is marked by an increase in angiogenesis, monocyte to macrophage and fibroblast to myofibroblast differentiation, and fibroblast hyperplasia [Morais et al., 2010]. During this

stage the provisional matrix is continually supplemented by non-regular, disordered collagen deposition. The predominant collagen types at the soft-tissue to device interface are type I and III [von Recum et al., 1993]. The resulting amorphous provisional matrix is remodeled into a dense, more mature ECM-like capsule intended to separate the device from the internal corporeal space. Once formed this capsule becomes the reference substrate for epidermal regression, resulting in the failure of the device. Note, the stages and phases of the cutaneous wound healing process at the soft-tissue to device interface are all dependent on the composition, contact duration, degradation rate (if applicable), surface morphology, porosity, roughness, shape, size, sterility, and surface chemistry [Morais et al, 2010].

1.2.3. Percutaneous Medical Devices

Percutaneous devices are those devices or implants that breach the dermal and epidermal layers of the skin. Percutaneous devices are designed and intended to remain in place for intermediate indwell periods (three months to several years) or for long term/"permanent" indwell periods (three years to lifetime of patient with option for surgical removal). The devices' intimate contact with the cardiovascular and lymphatic systems of the recipient patient requires that increased caution be taken in the design and implementation of percutaneous devices.

1.2.3.1. Percutaneous Device Design Criterion

Contemporary percutaneous device design was first postulated in the 1970-80s, motivated by naturally occurring biological structures (e.g. nails, feathers, hooves, horns,

etc...) thought to be percutaneous in nature [Winters et al., 1974; Grosse-Siestrup et al., 1984]. The idealized percutaneous device is composed of six main components: 1) skin penetrating component; 2) stress-reduction area; 3) 3-phase junction; 4) subcutaneous anchor; 5) micro-anchor; and 6) covering [Grosse-Siestrup et al., 1984].

The skin penetrating component would be the conduit/channel into the intra-corporeal space and the access point for therapeutic intervention. In non-catheter related percutaneous devices the skin penetrating component represents the attachment/connection site for prostheses or fixators. Regardless of the nature of the clinical application, stresses and relative motion are translated to the surrounding tissues via the skin penetrating component. Consequently any attempt to reduce or limit stress translation should be position proximal to this component of the percutaneous device.

The stress-reduction area is an interfacial region in which the device and soft-tissue make direct contact. Ideally the device would possess a modulus of elasticity close to or lower than that of the surrounding tissue [Grosse-Siestrup et al., 1984]. The design emphasis for this region would focus on the reduction of stress concentrations mediated by material property discontinuities between the skin and the device. Functionally then, the stress-reduction area would be able to respond and dissipate dynamic loads in order to promote soft-tissue to device integration by decreasing localized shear.

The 3-phase junction is the critical intersection of the external environment, the synthetic device, and the skin [Grosse-Siestrup et al., 1984]. In order to ensure soft-tissue

integration and long-term implantation of the percutaneous device, adhesion and some degree of integration must be achieved. The nature of the wound-site created at this interface has made the “3-phase junction” the most significant obstacle in the development of truly successful percutaneous device. The stress reduction area and 3-phase junction are areas in which surface area is often increased by the addition of polymer brushes and/or weaves, increasing contact surface area while decreasing load density.

The subcutaneous anchor is the primary means of deep tissue fixation for the percutaneous device. Ideally placed near muscle or bone, the subcutaneous anchor is intended to retain and then dissipate dynamic mechanical loads, bending moments, and torques to the surrounding deep tissue. The subcutaneous anchor is intended to circumvent the limited mechanical load bearing capacity of the skin [Gross-Siestrup et al., 1984]. The micro-anchor is similar to subcutaneous anchor in function but is intended to merely increase the contact surface area of the deep tissue portion of the device, decreasing the stress applied directly to the subcutaneous anchor. The cover is the final component and is intended to isolate the device by shielding the 3-phase junction from direct mechanical loads and pathogen colonization [Grosse-Siestrup et al., 1984]. In large part these approaches and their related design considerations do not address the unique mechanics at the soft-tissue to device interface and consequently are not discussed here. However the contribution of these approaches to the advancement of a successful outcome for the prevention of epidermal regression around percutaneous devices should not be disregarded

As the fields of polymer chemistry, metallurgy, rheology, and molecular biology have advanced, so too has the ideal percutaneous device design. The most recent percutaneous device designs have focused on in vitro and in vivo assays intended to interrogate topographical surface alterations capable of improving soft-tissue integration and/or reducing bio-film adhesion. Note, chemical and pharmacological agents have also been investigated for percutaneous devices but these efforts are focused on biomaterial coating development and/or infection prevention as opposed to structural device development [Rizzi et al., 2004; Von Eiff et al., 2005; Smith et al., 2006; Chou et al., 2008; Norowski et al., 2009; Perry et al., 2009]. That said, development of a percutaneous device and/or biomaterial capable of improving soft-tissue integration, while not inducing bacterial colonization is a non-trivial design consideration and is thought to involve competitive cell migration and adhesion [Gristina et al., 1987].

The design and development of the 3-phase junction and subcutaneous anchor have dominated in vitro and in vivo studies related to percutaneous devices. Fibroblasts in culture for 24 hrs to 6 days have been shown to preferentially align parallel to ridges of grooved, fibronectin coated silicone surfaces of 2, 5, and 10 μm in vitro when compared to smooth fibronectin coated silicone surfaces [van Kooten et al., 1999]. Additionally, aligned human fibroblasts formed more mature fibronectin fibrils and focal adhesions when compared to coated, smooth silicone surfaces [van Kooten et al., 1999]. Similar studies have also been conducted with epithelium on polystyrene square groove topographies of 1 to 10 μm in width and 1 to 5 μm in depth examining the effect of

micro-grooves on cellular alignment and migration and showed groove depth had a profound effect on cellular migration [Dalton et al., 2001].

In vivo studies addressing the design of the 3-phase junction and subcutaneous anchor have also shown trends in cellular alignment and growth in response to surface topographies with and without micro-patterning. Ultrahigh molecular weight polyethylene pegs 10 mm in height, 1 mm in diameter with 1 mm thickness, 10 mm diameter circular flanges at one end were implanted dorsally in 71 mice for 11 weeks to validate the utility of subcutaneous anchors in the inhibition of epidermal regression around percutaneous devices [Heaney et al., 1996]. The devices were machined smooth in order to promote epidermal regression down the length of the implant. The circular base was implanted below the *panniculus carnosus* [PC], a layer of striated muscle just below the dermis in mice. Although differing degrees of epidermal regression occurred around the pegs of the implants after 1 week, the majority of the implants showed very little regression after 11 weeks [Heaney et al., 1996]. The majority of the implants showed evidence of fibrous granulated tissue in the area immediately surrounding the implants, in many cases replacing dermis and even striated muscle. The investigation proved the efficacy of subcutaneous anchors and challenged the emphasis on substrate surface modification over the utilization of in situ connective tissue in the prevention of migration/locomotion of the epidermis in percutaneous device design.

Additional work focused on epidermal regression at the 3-phase junction involved rats implanted with micro-machined two-part, subcutaneous and percutaneous, system of

titanium-coated epoxy resin hexagonal implants showed distinct epidermal trends after 24 weeks [Chehroudi et al., 2002]. Implant surfaces geometries were either smooth, micro-machined 30 μm width and 35 μm depth V-shaped grooves or 270 μm wide and 120 μm deep pitted surfaces with 115 μm square bases [Chehroudi et al., 2002]. Subcutaneous component implantation site excisions were sealed and allowed to heal for 8 weeks before the introduction of the percutaneous component. Implants with smooth subcutaneous components began to fail 1-2 weeks post implantation, a trend that continued as the study progressed. Following eight weeks of subcutaneous implantation, connective tissue within the micro-machined surfaces began to show some evidence of ectopic mineralization, indicative of abnormal wound healing [Chehroudi et al., 2002]. Following the addition of the percutaneous component and throughout the remainder of the study, experimental groups with grooved, micro-machined subcutaneous components showed the least amount of epidermal recession when compared to smooth and pitted subcutaneous groups [Chehroudi et al., 2002]. Micro-machined, grooved components possessed the densest connective tissue indicating the increase in contact surface area and orientation of the grooves promoted connective tissue development essential to the prevention of epidermal recession.

Additional work in rat models has combined topographical patterns with an additional titanium subcutaneous anchoring cuff [Walboomers et al., 2005]. Smooth silicone tubes or silicone tubes with 10 μm wide and 1 μm deep square grooves or 40 μm wide V-shaped grooves were placed in parallel or perpendicular orientations to the skin. Following implantation, the wound sites were graded using a clinical wound assessment scale and

examined histologically after 9 weeks of implantation [Walboomers et al., 2005]. Groove orientation had a more significant effect than groove geometry on epidermal regression. Independent of groove geometry, there was no significant difference between smooth tubes and grooves parallel to the surface of the skin, while perpendicular grooves showed the most tissue down-growth [Walboomers et al., 2005]. The contribution and role of grooves to improved epidermal retention at the in vivo soft-tissue to device interface is yet to be determined; however the authors proposed the histology indicated grooves increased contact surface area and thusly promoted better adhesion. An additional advantage of parallel grooves may be the disruption of shear and shear wave propagation promoting uninterrupted adhesion and reducing mechanical disturbances at the soft-tissue to device interface, although not postulated or addressed in the literature.

Additional in vivo studies have focused on the development of the stress reduction component of the percutaneous device. Sheep implanted with two different stress reducing cuff designs, intended to allow relative soft-tissue motion and attachment/reattachment on percutaneous skeletal extension bars [SEBs], showed positive soft-tissue to device interactions and sustained skin to device adhesion with minimal down-growth [Yu et al., 2003]. SEBs were made of stainless steel, with one stress reducing design made of VELCRO© hooks [LPD-III-F] and the other a circular stainless steel spring woven onto a nylon sleeve [LPD-III-S] [Yu et al., 2003]. Despite experimental limitations related to the growth of the animals and differing amounts and magnitudes of physical activity, both LPD-III-F and LPD-III-S devices demonstrated the ability of stress reducing cuffs to improve the soft tissue to device interface. Although

both cuff designs allowed the skin adhesion zone to move up and down the length of the SEB, ultimately the study was hampered by the length scales used in the designs of the cuffs. The study did establish three important factors for design considerations for the stress reducing component of percutaneous devices: 1) the binding strength of the connector; 2) the amount of movement anticipated at skin level; and 3) the stretching capacity of the skin proximal to the implantation site [Yu et al., 2003]. These three factors are essential to achieve success in not only the design of the stress reducing component, but also with regards to the optimization of the 3-phase junction and the subcutaneous anchor.

1.2.3.2. Current Percutaneous Cuff Designs: Advantages and Disadvantages

Current percutaneous cuff systems deal primarily with fixation and anchorage, without addressing the critical issues of relative motion, localized tissue matrix deformation, and stress-concentrations. The most common cuff designs involve a combination of hard or soft interior/exterior polymer bumpers and Dacron®, an engineering synthetic, fibrous matrix construct developed by 3M and common in tissue engineering applications. The Dacron® mesh is intended to promote local extracellular matrix production and soft tissue ingrowth. The primary role of the bumpers is to fix/anchor the device in a specified location, thereby preventing large-scale device migration and/or accidental device removal. The two device adaptations individually accomplish their intended goals with some success but are unable to improve the life-span of the device or the quality of life of the patient. Neither alone nor in combination have these device additions/adaptations come close to resolving the problems of infection or disruptions of the mechanical

integrity of the affected tissue. Other polymer and titanium meshes have also been added to improve fixation and/or integration with soft-tissue but with similarly mixed results [Yu et al., 2003; Walboomer et al., 2005].

1.2.3.3. Percutaneous Devices to Benefit from a Mechanics-based Approach to Device Design

Enteral Catheters

Enteral feeding catheters are used in medical care under several circumstances including malnourished patients following gastrointestinal surgical procedures, comatose or unconscious patients who have lost the swallowing reflex, patients with a high nutritional demand (Cystic Fibrosis or renal disease), and in extreme cases of anorexia nervosa. The most common forms of these catheters are the gastrostomy and jejunostomy, which are surgically or endoscopically implanted and anchored in the abdomen using a variety of techniques. The two procedures are commonly referred to as percutaneous endoscopic gastrostomies (PEGs) or percutaneous endoscopic transgastric jejunostomies (PEGJs). Most enteral feeding catheters rely on either an external/internal bumper arrangement and cuff to fix the tube in position or are simply taped down to prevent device movement and exit-site irritation. The problem of catheter exit site infection occurs in approximately 1-2% of the patient population (approximately 216,000 patients annually) [24]. The risk of exit site infection can be compounded by excessive fibrous encapsulation of the catheter by granular tissue, which leads to an increased susceptibility to catheter anchor dislocation and expulsion. Anchor dislocation leads to the formation of pockets of infected fluid, providing the infectious agent with numerous nutrient-rich breeding sites.

Parenteral Catheters

Parenteral catheters, commonly called central venous catheters (CVCs), supply nutrients or drugs via access to the vena cava or central venous system of the groin and upper leg to nearly 500,000 patients annually. CVC placement is largely determined by the vascular health of the patient. These CVCs are divided into two distinct groups based on the length of their indwell periods. The tunneled class of CVCs, which has a much longer indwell period, is susceptible to epidermal regression and infection. CVCs are most commonly fed through the subclavian vein using a minimally invasive surgical procedure. Once the catheter is positioned it is anchored by a sutured or stapled Dacron® cuff. The Dacron® cuff is thought to promote cellular in-growth and to provide a seamless soft tissue-to-device interface. However, this cuff represents the primary site of infection and is often the source of failure. The most common cause of infections is *Staphylococcus epidermidis* [Adal et al., 1996], an infection that only presents on the skin but often leads to cuff dislocation and catheter migration. Surface-treatments with antibiotics and anti-mitotic agents to prevent bacterial growth and epithelial hyperplasia are currently under investigation. However, the long-term success of such treatments is still unknown. Catheter migration caused by epidermal/dermal regression and infection is currently the dominant failure modality of CVCs.

Peritoneal Dialysis Catheter

Catheters utilized in peritoneal dialysis are similar in design to those previously discussed, however they are subjected to greatly increased fluid dynamic loading

conditions and possess markedly different anchor and skin/device interface configurations. These catheters are used to treat patients suffering from End Stage Renal Disease (ESRD). Peritoneal dialysis (PD) was developed as an alternative therapy in an attempt to improve the patients' perceived quality of life and free them from the constraints of the traditional treatments. The dialysate fluid is added through a "permanently" implanted percutaneous catheter. PD catheters are implanted endoscopically in the abdomen in a curved, coiled, or straight tube configuration. These catheters are made of silicone rubber or polyurethane, have outer diameters that range from 2.6mm to 5mm, and include Dacron® cuffs at the soft tissue-to-device interface. These catheters suffer from the same failure modalities seen in the enteral and parenteral catheter families.

Bone Lengthening External Fixators (Ilizarov Method)

Distraction osteogenesis uses circular external fixators and tension-wires in the treatment of traumatic non-union fractures and inoperable congenital bone deformities. The bone lengthening procedure involves the gradual dislocation and mending of bone, using an adjustable external fixator to increase the overall length of the bone. The external fixator consists of multiple circular rings, adjustable and interchangeable axial support bars, and tension wires. The circular rings circumscribe the limb and are fixed axially by metal bars of differing and interchangeable lengths. The percutaneous portions of the device are the tension wires that are affixed to the rings. The tension wires hold the manipulated pieces of bone in position until the gaps can be filled with new bone. Depending on the severity of the bone trauma or deformity, numerous tension wires may be inserted into one limb,

each one having its own exit site and potential for infection. Exit site infection and epidermal/dermal regression, are the primary modes of failure for the bone lengthening external fixator. This procedure involves a rigorous exit-site antiseptic cleaning regimen in order to ensure the relative sterility of the device. Epidermal/dermal regression in the case of bone lengthening external fixators can lead to tension wire migration, exit site “boring” or channel widening, and deep tissue infection

Osseointegration Dental Implants

Osseointegrated devices are those with at least 90% of the device’s surface being covered with bone and the remaining 10% protruding through the skin [Jacobsson et al., 1991]. Osseointegrated devices fail most often in the “interface zone [Palacci et al., 1995]” where the implanted device makes direct contact with soft-tissue. The earliest and most prevalent application of osseointegrated devices are dental implants and prostheses. The osseointegrated portion serves as a permanent anchor in hard tissue. The maintenance of force translation rests on the device-to-bone interface’s ability to transfer force. The osseointegrated portion is made of surgical grade titanium. The implantation procedure involves the incision and removal of overlying epithelial tissue and boring of a seating cavity into the bone. Once the cavity has been drilled, a titanium fitting is inserted and the entire assembly is covered by the removed flap. It is recommended that following the insertion of the titanium fitting, bone in-growth should be allowed to continue without mechanical interruption for three to five months [Palacci et al., 1995]. Following the bone tissue in-growth the underlying connective tissue and hair follicles of the skin are removed [Palacci et al., 1995] to ensure no relative motion between the skin and the

device. When relative motion occurs, the resulting mechanical disruption of the skin causes epidermal/dermal regression, promoting infection.

1.2.3.4. Percutaneous Device Failure Modalities

Five major failure modalities have been identified for percutaneous medical devices: 1) extrusion due to marsupialization; 2) extrusion due to permigration; 3) extrusion due to infection and abscess formation; 4) extrusion due to avulsion; and 5) extrusion due to any combination of the aforementioned [von Recum et al., 1984].

Extrusion by marsupialization is often cited as the chief failure modality of percutaneous medical devices, although this may be a misnomer. Extrusion by marsupialization involves the encapsulation of the percutaneous device by a dense fibrous tissue. The dense fibrous capsule facilitates the development of a sealed epidermis at the intra-corporeal surface of the device, rendering the once percutaneous/transcutaneous device completely extracutaneous [von Recum et al., 1984]. Marsupialization commonly occurs in percutaneous device whose external surface would be classified as non-porous.

Extrusion by permigration is a multi-phase extrusion process, primarily related to porous devices with a central, non-porous core. In the first phase following implantation, an eschar forms a loose fibrin plug around the device, tethering the soft-tissue to the device while the epidermal edge of the wound margin grows toward the device center. As the wound margin migrates, fibroblasts and dermal tissue begin to interact with the porous percutaneous surface of the device [von Recum et al., 1984]. In the second phase,

epidermal migration continues from the wound margin, encountering the inner-most, non-porous core of the device. Concurrently, dermal fibroblasts infiltrate the region proximal to the porous portion of the device surface. Fibroblasts begin to establish a provisional matrix around the soft-tissue to device interface region [von Recum et al., 1984]. In the third phase, the epidermal edge, having reached the inner-most, non-porous core of the device begins to grow down the length of the device toward the intracorporeal space, through the provisional matrix deposited by the dermal fibroblasts [von Recum et al., 1984]. The epidermal regression is similar to that seen in extrusion by marsupialization and is typical of all percutaneous device extrusions. The final phase of the permigration extrusion process occurs when the migrating epidermal edges on either side of the device meet below the device, rendering the device extracutaneous [von Recum et al., 1984]. Permigration refers to the migration of the epidermis through the provisional matrix. As the epidermal edge migrates through the provisional matrix and pores in the surface in the device, the edge encounters epidermal defects that prevent the tissue from attaining its hierarchical superstructure that would normally arrest epithelial cell proliferation and migration typical in normal cutaneous wound healing [von Recum et al., 1984]. The inability of the epidermis to attain its physiologic superstructure and its proximity to a highly active connective tissue allows cell migration-mediated or permigration marsupialization to occur.

Extrusion due to infection arises from the colonization of localized or systemic infections at the soft-tissue to device interface that prevent or hinder the normal cutaneous wound healing process [von Recum et al., 1984]. Extrusion due to infection

occurs similarly in porous and non-porous percutaneous devices. Whether acute or chronic in nature, the infection exacerbates the initial inflammatory stage of the wound healing process by up-regulating the recruitment of macrophages and neutrophils. The prolonged inflammatory stage prevents the development of the provisional matrix and vasculature within the soft tissue to device interface region, greatly slowing epidermal and dermal cell proliferation. The wound healing process around percutaneous devices is a competitive surface adherence interaction; thusly the decrease in epidermal and dermal cell proliferation allows pathogens to create a biofilm that further isolates the device and impairs integration irreversibly. Without the fibroblast and myofibroblast contraction associated with the cutaneous wound healing process the device begins to loosen, eventually requiring the removal of the device.

Extrusion due to avulsion is the product of mechanical stimuli that causes either the sudden removal of the device caused by a high intensity, instantaneous load application or the transmission of a high energy load through the device to the surrounding tissue resulting in severe soft-tissue damage and necrosis.

The final failure modality is the most common as it represents the combination of any of the aforementioned [von Recum et al., 1984]. Rarely does one failure modality alone account for the removal of the device. The combination of failure modalities exacerbates the inability of weak soft-tissue adhesions to the device to transfer the complex loading conditions present at the soft-tissue to device interface, preventing the soft-tissue to adherence and/or subsequent integration to the device surface [von Recum et al., 1984].

The inability of the soft-tissue linkages alone to transmit and/or resolve these loads indicates that balancing the stress discontinuities at the soft-tissue to device interface may provide a means of resolving the obstacles presented by the soft-tissue to device interface of percutaneous devices.

1.2.4. Role of Mechanics in Tissues

The ability of cells to identify, interpret, and alter their micro-mechanical environment is a firmly established paradigm within molecular biology, formally known as mechanotransduction. Integrin and cadherin-mediated cellular adhesion to a given substrate and/or neighboring cells is critical for proliferation and maintenance of phenotype in culture, particularly under mechanical load [Mackley et al., 2006]. Within more complex, hierarchical tissue structures, cells utilize cluster combinations of more than 20 trans-membrane integrin types to adhere to and work with extracellular matrix [ECM] proteins. Additionally, cells use cadherins and selectins to establish and maintain mechanical linkages between neighboring cells, to construct the cell-cell interactions that are necessary structurally for tissue formation. Thusly, integrins, cadherins, and selectins are commonly regarded as the mechano-receptors of cells [Ingber et al., 2003]. In human skin specifically, cells of the epithelium attach to neighboring cells and the extracellular matrix via tight junctions (zonulae occludens), adhesive zones, desmosomes, and hemidesmosomes [Jacobosson et al., 1991]. These transmembrane proteins help the cells to determine and synthesize the intra- and inter-cellular components necessary to create the primarily elastic/viscoelastic matrix that characterize their capacity to resist and

respond to a myriad of complex, short- and long-term, loading conditions [Ingber et al., 2003; Discher et al., 2005].

The mechanical stability and rigidity of the cell is controlled through remodeling of the cytoskeletal lattice, requiring the reorganization and coordination of microfilaments, microtubules, and intermediate filaments [Ingber et al., 2003]. Molecular reorganization of these elements allows the intra-cellular, actin-myosin filament complex to create or dissipate tension through activation or deactivation of myosin motors [Gardel et al., 2008]. The actin-myosin complex typically allows tissues to exhibit linear elastic or viscoelastic behavior up to 20% strain, further indicating a significant degree of inherent mechanical load adaptability [Discher et al., 2005]. Cell-matrix interactions of this nature are important for development and many homeostatic physiological processes [Mackley et al., 2006]. To further interrogate the role of cell-cell, cell-tissue, and cell-substrate interactions researchers have begun probing the mechanical responses of various cells types on a micro-mechanical scale utilizing atomic force microscopy [AFM] or through displacement field analysis on deformable or semi-deformable synthetic substrates with covalent bound proteins for improved cellular adhesion and traction force transmission [Van Citters et al., 2006; Ghosh et al., 2007]. Studies of this nature are crucial for understanding the initiators and mechanisms behind cell phenotype determination, differentiation, cellular gene and protein expression, the biophysics of cytoskeletal reorganization, organogenesis, and development of various disease states. With regards to disease specifically, angina, valve disease, scleroderma, irritable bowel syndrome, some forms of cancer, metastasis, glaucoma, osteoporosis, osteoarthritis, musculo-dystrophies,

asthma, pulmonary fibrosis, and pre-eclampsia may possess disease causality related to disruptions or dysfunction of mechanotransduction [Ingber et al., 2003]. Due to the linkages between biochemistry and mechanics, researchers within regenerative and restorative medicine are beginning to take note of the role of mechanics and mechanical behavior in cellular and tissue behavior [Ingber et al., 2003]. The widening of perspective within regenerative and restorative medicine has motivated further investigations into how mechanics might be applied to biomaterial design.

1.2.5. Soft-tissue Biomaterial Design

Biomaterials can be defined as polymer, metal, biologically-derived, or composite materials that evaluate, support, supplement, or replace in part or in whole the functionality of a diseased or impaired tissue through direct contact at biologic-synthetic interface [Roeder, 2010]. More than 2500 biomaterial-related research publications per year are motivated by more than \$240 million of annual sales in regenerative and restorative medicine [Lysaght et al., 2008; Hollister, 2009]. Within biomaterial research a specific class of materials known as hydrogels are commonly utilized to address soft-tissue applications. Hydrogels are biologically derived cellular or acellular matrices, or wholly synthetic substrates with mechanical and mass transport capacities that mimic the biological function of in situ soft-tissue [Mithieux et al., 2004; Place et al., 2009; Tibbit et al., 2009; Lai et al., 2010]. Synthetic, polymer hydrogels represent a spectrum of anionic, cationic, or neutrally charged blends, co-polymer systems, or interpenetrating networks [IPNs] [Slaughter et al., 2009]. Hydrogels have been used as tissue scaffolds,

wound healing barriers, drug delivery systems, and cell encapsulation matrices [Slaughter et al., 2009].

With regards to tissue regeneration, three design parameters have been identified as critical to the success of the biomaterial: 1) substrate surface modification for improve cell-substrate interaction; 2) controlled, bioactive protein and/or cytokine release; and 3) physiologically relevant mass transport [Hollister et al., 2009]. The aforementioned design parameters represent only three biomimetic properties that must be addressed in the design of biomaterials for soft-tissue applications. A comprehensive understanding of the mechanical behavior of the targeted soft-tissue is equally important to the design of hydrogels for soft-tissue applications [Hutmacher, 2001]. Mechanics has been shown to be critical in the development of avascular, semi-vascular, and highly vascular soft-tissue-related biomaterials [Freed et al., 2009]. The mechanical landscape of soft-tissues are quite complex and require that several unique mechanical conditions and states must be addressed. Those include: strain dependent linearity and non-linearity; elastic, inelastic, viscoelastic, and porelastic behavior; heterogeneity; anisotropy; finite strain regimes; major conformational and geometric changes between unloaded and loaded states; and time-dependent mechanical and material property changes [Stella et al., 2010]. In order to address these conditions and behaviors researchers can adapt hydrogel formulations by selecting different cross-linking densities, co-monomer/polymer candidates, and porogens [Anseth et al., 1996]. Due to the intrinsic adaptability and variability of constituent components, soft-tissue related biomaterials pose a significant

challenge to researchers focused on characterizing and then mimicking the in situ mechanical behavior of the affected soft-tissue.

1.2.5.1. The Role of Mechanics in Biomaterial Design

In biomaterial design mechanics and matching the mechanical behavior of hard- and soft-tissue has always been a component of the design process. However, not until researchers were able to more accurately characterize and describe the complex behaviors of biological tissues did mechanics become a focal point of the design process. It should be noted that there is a large group of biomaterial and tissue engineering applications that utilize mechanics predominantly as stimuli to guide cells or cell derived products into structurally advantageous conformations [Buschmann et al., 1995; Lee et al., 1997; Kim et al., 2000; Shelton et al., 2003].

Mechanically matching the soft-tissue the device is intended to replace is most common in cartilage, muscle, and bone tissue applications [Kim et al., 2000; van der Meulen et al., 2002; Levental et al., 2007; Oliveira et al., 2009]. Gellan gum, a polysaccharide produced by the fermentation of *Sphingomonas paucimobilis*, has shown great promise for a wide variety of soft-tissue applications, most notably cartilage [Oliveira et al., 2009]. Mechanical characterization has shown gellan gum possessed similar load bearing capacity to other biomaterials intended to support and supplement encapsulated chondrocytes for cartilage repair [Oliviera et al., 2009]. Additionally, differing concentrations of polymer within porous, biodegradable polyurethane scaffolds, without cells were shown to have failure strains comparable to or greater than human femoral and

popliteal arteries, indicating efficacy in vascular replacement applications [Guan et al., 2004]. In a combination of tissue engineering and a mechanics-based biomaterial design approach, rat aortic smooth muscle cells seeded on mechanically characterized polyglycolic acid [PGA], PGA with bound poly(L-lactic acid) [PLLA], and type 1 collagen sponges were loaded under cyclic mechanical strain to determine which cell-substrate combinations would yield the most physiologically relevant cellular phenotype [Kim et al., 2000]. Importantly, these substrates were characterized prior to cell seeding to ensure they possessed mechanical behavior similar to that of the host tissue they were ultimately intended to replace. In a similar combined tissue engineering/mechanics-based biomaterial design process, dextrin-HEMA based biomaterials optimized for mechanical biocompatibility have shown positive cell-substrate biological responses when exposed to murine fibroblast cells in culture [Carvalho et al., 2009]. These studies represent a small sample of the research effort dedicated by the biomaterial design field to the inclusion of mechanical characterization in the development process and serves as a further indicator of the ability of mechanics to have an impact on wide range of soft-tissue related biomaterial applications.

1.2.5.2. Poly(2-hydroxyethyl methacrylate) [pHEMA] biomaterials and devices

Cross-linked poly(2-hydroxyethyl methacrylate) [pHEMA] is a degradable or non-degradable, highly adaptable hydrophilic polymer, commonly used in ophthalmic applications (e.g. contact lenses), but recently has been used in many more biomedical applications [Slaughter et al., 2009]. Biomedical applications include central nervous system nerve regeneration, bone tissue, wound healing, and endothelial tissue scaffolds,

as well as keratoprotheses, bio-mimetic coatings, and porous drug delivery systems [Peluso et al., 1997; Dziubla et al., 2001; Sathian et al., 2003; Giavaresi et al., 2004; Carone et al., 2006; Hicks et al., 2006; Cabodi et al., 2007; Campillo-Fernandez et al., 2007; Brynda et al., 2009; Li et al., 2009]. The inherent mechanical and mass-transport adaptability of soft-tissue related pHEMA substrates has been connected to cross-linker type and cross-linker concentration [Mabilleau et al. 2006]. Alteration of pore size, density, and interconnectivity has also been found to significantly effect the mechanical and mass-transport/drug delivery capacities of pHEMA substrates [Liu et al., 2000; Dziubla et al., 2001; Monleón Pradas et al., 2001; Karabanova et al., 2006; Cabodi et al., 2007; Kulygin et al., 2007; Bajpai et al., 2008]. Note, past studies have also indicated a minimum pore-size for soft-tissue integration in percutaneous devices, approximately 40 μm [Winter et al., 1974]. Also important for soft-tissue applications, pHEMA is considered minimally protein adherent, inducing a reduced inflammatory response [Mabilleau et al., 2004; Slaughter et al., 2009]. PHEMA substrates have also been shown to support cellular activity through chemical surface modification that promote protein adherence [Merrett et al., 2001; Fukano et al., 2006; Brynda et al., 2009]. The aforementioned attributes make pHEMA-based biomaterials an ideal choice for cutaneous wound healing applications and percutaneous medical device design.

The soft tissue to device interface is critical for not only percutaneous devices but also for wound healing applications. In vivo, fibrin with 2-hydroxyethyl methacrylate, in a biologic + polymer composite spray, has been shown to decrease scar area and increase wound site collagen content in rat models over 16 days when compared to controls and

fibrin sponges alone [Sathian et al., 2003]. In vitro, PHEMA has demonstrated a positive impact on wound beds as the mass transport interface in active wound dressings [AWDs] [Cabodi et al., 2007]. As the basal layer of a poly(dimethyl siloxane) [PDMS], pHEMA bi-layer active wound dressing construct, the diffusive and convective capacity of pHEMA efficiently removed model wound bed solutes, benzoic acid, FITC-BSA, and fluorescein, from solid and soft substrates without the introduction of adverse flow related stresses [Cabodi et al., 2007]. Wound healing applications tend to focus on the mass transport capability of pHEMA substrates, deprioritizing the importance of biocompatibility of the substrate, a design consideration essential to percutaneous device design.

Within the field of percutaneous device development, many groups have utilized pHEMA-based biomaterials to investigate and interrogate the soft-tissue to device interface. A novel in vitro percutaneous device model comparing pHEMA to polytetrafluoroethylene [PTFE] placed substrates trans-cutaneously through excised human skin samples and co-cultured up to 14 days showed very little difference in epidermal behavior between experimental groups [Knowles et al., 2005]. The absence of a discernable difference between epidermal and dermal tissue behavior on pHEMA and PTFE substrates was attributed to the intrinsic minimization of protein adherence on pHEMA and not to an inherent inability of the substrate to serve as an appropriate biomaterial for the soft tissue to device interface. The same group repeated the study with pHEMA substrates modified with 1,1' carbonyldiimidazole to promote protein attachment and observed improved epidermal attachment and soft-tissue integration after

7 days in culture [Fukano et al., 2006]. A further in vivo study placed pHEMA unmodified, modified, and modified substrates with bound laminin 5 dorsally in mice for 7 days to investigate the skin response at the soft tissue to device interface [Isenhath et al., 2007]. The pHEMA implants showed no evidence of marsupialization, permigration, or avulsion across all experimental groups. Additionally, no appreciable difference in epidermal integration was seen between modified substrates with and without laminin 5. The lack of integration was attributed to the use of human- rather than mouse-derived laminin 5 and the in situ production of laminin 5 by the host keratinocytes [Isenhath et al., 2007]. That said, the in vivo model demonstrated the efficacy of pHEMA in percutaneous device related applications. Note, none of the aforementioned groups addressed mechanics or mechanically matching the load response of skin in the design process.

1.2.6. Viscoelasticity and Rheology

1.2.6.1. Viscoelasticity in shear

Many materials within biomedical research are classified as linear or nonlinear viscoelastic materials [Ferry, 1980; Ehrenstein, 2001]. Viscoelasticity is often presented in response to shear loads but can also be referenced in response to uniaxial or biaxial tension or compression. Shear is the application of a force tangent to the surface of interest. Viscoelasticity is a composite material response that combines elements of a classical Hookean solid:

$$\varepsilon = \frac{\sigma_E}{E} \tag{1.1}$$

and a Newtonian fluid:

$$\dot{\gamma} = \frac{\sigma}{\eta} \quad (1.2)$$

where ε is the deformation, $\dot{\gamma}$ is the rate of shear deformation, σ is the shear stress, σ_E is the tensile stress, E is the elastic modulus, and η is the viscosity. The elastic modulus, E , is defined as the ratio of the stress to the strain within the linear material behavior regime when a material is under a given force. The viscosity, η is a measure of a fluid's resistance to shear or flow. The elastic and viscous components of a material work in concert to store and dissipate energy in a load and time dependent fashion.

Viscoelastic materials are characterized by a condition known as stress relaxation. In perfectly elastic materials, when shear history, $k(t)$, is applied to a uniform deformable solid using a unit step function, $H(t)$, at time, t equal to zero, the corresponding stress history is of the form,

$$\sigma(t) = \sigma_o H(t) , \quad (1.3)$$

constant for all time, t greater than zero (positive) [Pipkin, 1972]. In an ideal viscous fluid exposed to the same instantaneous shear load, the stress history would be infinite at time, $t = 0$ and then zero for all times greater than $t = 0$ [Pipkin, 1972]. In reality, the response of solids and fluids is a mix of the two conditions. When the load is removed the stress decreases quickly at first but then decelerates, reaching a limiting value, $\sigma(\infty)$. When the limiting value of a material is not zero the material is usually regarded as more “solid-like”, but if the limiting value is zero or approaches zero quickly the material is described as more “fluid-like” [Pipkin, 1972]. Theoretically, the limiting value is often regarded as experiment or material-type dependent because as $t \rightarrow \infty$, limiting values of all

materials would equal zero. Critical to the understanding of stress relaxation and the comparison of materials is the concept of relaxation time. Although this quantity will have more relevance following the presentation of viscoelastic models below.

Viscoelastic materials undergo an additional unique mechanical response known as creep. In a perfectly elastic material subjected to stress history, $\sigma(t) = \sigma_o H(t)$ the resultant shear is constant, $k(t) = k_o H(t)$, for all times, $t \geq 0$ [Pipkin, 1972]. In a viscous fluid the shear would increase linearly, proportional to the applied stress, $k(t) = \sigma_o(t/\eta)$ where η is the viscosity [Pipkin, 1972]. In viscoelastic solids the shear increases to a plateau or limiting value, $k(\infty)$, while in viscoelastic fluids after an initial rapid increase the shear begins to increase linearly.

Establishing the connection between stress relaxation and creep is essential for understanding viscoelastic behavior and provides insights into the connections between solid and viscous material behaviors. Assume $R(k, t)$ is the stress relaxation function and $C(\sigma, t)$ is the creep function, where k, σ , and t are the shear, stress, and time, respectively. Assume R and C are zero for all $t < 0$ [Pipkin, 1972]. In an isotropic material, making R and C odd in k and σ , respectively, and assuming small k and σ ,

$$R(k, t) = G(t)k + O(k^3) \quad (1.4)$$

$$C(\sigma, t) = J(t)\sigma + O(\sigma^3) \quad (1.5)$$

where, $G(t)$ is the linear stress relaxation modulus and $J(t)$ is the linear creep compliance [Pipkin, 1972]. At times $t = 0^+$ and $t = \infty$ the stress relaxation modulus and creep compliance are denoted as G_g , G_e , J_g , and J_e , respectively [Pipkin, 1972]. The g and e

stand for *glass* and *equilibrium*, respectively. Understanding that the instantaneous response and the equilibrium response of viscoelastic materials is independent of whether or not the stress or strain in the system is held constant establishes $G(t)$ and $J(t)$ are reciprocal at those two instances and in most others. Therefore, the following relationship can be written

$$J_g G_g = 1 \quad (1.6)$$

$$J_e G_e = 1 \quad (1.7)$$

To further the understanding of viscoelastic materials it is necessary to introduce three basic linear viscoelastic models: the Maxwell body, the Voigt model, and the Kelvin model. The models contain differing arrangements of linear springs with spring constants μ and dashpots with viscosity η [Fung, 1993]. When a force, F is applied to the elements the resulting responses are seen in the spring and dashpot, respectively,

$$F = \mu u \quad (\text{Spring}) \quad (1.8)$$

$$F = \eta \dot{u} \quad (\text{Dashpot}) \quad (1.9)$$

where, u is the displacement/extension of the spring and $\dot{u}$ is the velocity of the deflection of the dashpot [Fung, 1993].

The Maxwell model is a spring and dashpot in series, the Voigt model is a spring and dashpot in parallel, and the Kelvin model or standard linear solid is a dashpot and spring in series with an additional spring in parallel [Fung, 1993].

In the Maxwell model the same force is experienced by both the spring and the dashpot, creating displacements of F/μ and F/η for the spring and dashpot, respectively, and a velocity of $\dot{F}/\mu$ in the spring. With those relationships in mind, the Maxwell model is often represented as a summation of the velocities of the individual components,

$$\dot{u} = \frac{\dot{F}}{\mu} + \frac{F}{\eta} \quad (1.10)$$

where at time, $t = 0$ the displacement in the spring is zero, as is the deflection of the dashpot [Fung, 1993].

In the Voigt model, the applied force, F is seen equally by both elements at the same instant. Drawing from the description of displacements in the Maxwell model, the forces within the spring and dashpot are μu and $\eta \dot{u}$, respectively. Consequently, summing the forces of the system yields,

$$F = \mu u + \eta \dot{u} \quad (1.11)$$

where the initial deflection in the system is zero because the dashpot resists the extension of the spring [Fung, 1993].

In the Kelvin model the response to an applied force is a bit more complex. First, recognize that the Kelvin model contains a Maxwell body component in parallel to another spring. Deconstructing the displacements and forces of the various elements will improve the understanding of the behavior of the whole system and simplify the derivation of the total force for the system. The displacement of the dashpot of the Maxwell body will be labeled u_1 and the displacement of the spring of the Maxwell body will be labeled u_2 . Note, the displacement of the spring in parallel, u_3 represents the

displacement of the whole system and is simply, u . Additionally, the total force of the system, F is the sum of the force of spring, F_{spring} and the force of Maxwell body, $F_{Maxwell}$. It is also important to note that transmission of force seen in the Maxwell body alone applies within the Kelvin model. With those descriptions in mind the following relationships can be established [Fung, 1993],

$$u = u_1 + u_2 = u_3 \quad (1.12)$$

Total displacement is equal to the displacement of the Maxwell body

$$F = F_{spring} + F_{Maxwell} \quad (1.13)$$

Total force of the system is the sum of the spring and Maxwell body

$$F_{spring} = \mu_3 u \quad (1.14)$$

Force in the spring is the product of its spring constant the total displacement

$$F_{Maxwell} = \eta_1 \dot{u}_1 = \mu_2 u_2 \quad (1.15)$$

Total force seen by the Maxwell body is also seen in its component elements

By substitution then [Fung, 1993],

$$F = \mu_3 u + \mu_2 u_2 = (\mu_3 + \mu_2) u - \mu_2 u_1 \quad (1.16)$$

which becomes,

$$F + \frac{\eta_1}{\mu_2} \dot{F} = (\mu_3 + \mu_2)u - \mu_2 u_1 + \frac{\eta_1}{\mu_2} (\mu_3 + \mu_2)\dot{u} - \eta_1 \dot{u}_1 \quad (1.17)$$

replacing the final term with $\mu_2 u_2$ and simplifying using the relationship $u = u_2 + u_1$, yields [Fung, 1993]

$$F + \frac{\eta_1}{\mu_2} \dot{F} = \mu_3 u + \eta_1 \left(1 + \frac{\mu_3}{\mu_2}\right)\dot{u} \quad (1.18)$$

and,

$$\tau_\varepsilon = \frac{\eta_1}{\mu_2} \quad \text{Relaxation time for constant strain} \quad (1.19)$$

$$\tau_\sigma = \frac{\eta_1}{\mu_3} \left(1 + \frac{\mu_3}{\mu_2} \right) \quad \text{Relaxation time for constant stress} \quad (1.20)$$

$$E_R = \mu_3 \quad \text{Relaxed elastic modulus} \quad (1.21)$$

further reducing Equation (1.18) to [Fung, 1993],

$$F + \tau_\varepsilon \dot{F} = E_R u + (u + \tau_\sigma \dot{u}) \quad (1.22)$$

Which in response to applied load and displacement at time, $t = 0$ becomes,

$$\tau_\varepsilon F(0) = E_R \tau_\sigma u(0) \quad (1.23)$$

With the development of the force relationships for each of the models the creep functions and relaxation functions may be derived. Creep is defined as the gradual deformation of a material under an applied load or its own weight [Daniels, 1989]. Relaxation is the response of the same material following the removal of load. By solving the force relationship equations for $u(t)$ when the force $F(t)$ is a unit step function, $\mathbf{1}(t)$ defined as 1 when $t > 0$, $\frac{1}{2}$ when $t = 0$, and 0 when $t < 0$, the following three creep functions result [Fung, 1993],

$$c(t) = \left(\frac{1}{\mu} + \frac{1}{\eta} t \right) \mathbf{1}(t) \quad \text{Maxwell Model} \quad (1.24)$$

$$c(t) = \frac{1}{\mu} \left(1 - e^{-(\mu/\eta)t} \right) \mathbf{1}(t) \quad \text{Voigt Model} \quad (1.25)$$

$$c(t) = \frac{1}{E_R} \left[1 - \left(1 - \frac{\tau_\varepsilon}{\tau_\sigma} \right) e^{-t/\tau_\sigma} \right] \mathbf{1}(t) \quad \text{Kelvin Model} \quad (1.26)$$

Conversely, by solving the force relationship equations for $F(t)$ when $u(t)$ is a unit step function, $\mathbf{1}(t)$ the relaxation functions for the three models can be derived [Fung, 1993].

The relaxation functions are,

$$k(t) = \mu e^{-(\mu/\eta)t} \mathbf{1}(t) \quad \text{Maxwell Model} \quad (1.27)$$

$$k(t) = \eta \delta(t) + \mu \mathbf{1}(t) \quad \text{Voigt Model} \quad (1.28)$$

$$k(t) = E_R \left[1 - \left(1 - \frac{\tau_\sigma}{\tau_\varepsilon} \right) e^{-1/\tau_\varepsilon} \right] \mathbf{1}(t) \quad \text{Kelvin Model} \quad (1.29)$$

Note the inclusion of the Dirac-delta or unit-impulse function $\delta(t)$ which is 0 at times greater than or less than 0 and $\int_{-\varepsilon}^{\varepsilon} f(t)\delta(t)dt = f(0)$ for all strains greater than 0, where $f(t)$ is an arbitrary function [Fung, 1993]. The relaxation times for the Maxwell and Voigt models are η/μ while the relaxation time for the Kelvin model is dependent on whether a constant deflection or load, strain or stress is applied [Fung, 1993]. The creep and relaxation functions can be applied to experimental data in order to determine viscoelastic material parameters for the empirical comparison of materials.

Many biomedical applications occur in highly dynamic setting or involve ambulation, consequently the materials developed to create biomedical devices need to be characterized in a similarly dynamic fashion. Thusly, the interrogation of a material's mechanical response to harmonic/sinusoidal/oscillatory loading conditions is essential to the development of viscoelastic materials for biomedical applications. An application specific presentation of relevant equations for the description of the viscoelastic behavior of materials is included below but the theoretical basis for those relationships is presented here.

Within the linear viscoelastic regime, when exposed to oscillatory loads, the strain within a material will vary sinusoidally, although out of phase, with the applied stress [Ferry, 1980]. The oscillatory response can be described as

$$\gamma = \gamma^o \sin \omega t \quad (1.30)$$

where γ^o represents the maximum strain amplitude. Differentiating Equation 1.30 with respect to time yields,

$$\dot{\gamma} = \omega \gamma^o \cos \omega t$$

At point it is necessary to introduce the linear constitutive equation for simple shear [Ferry, 1980],

$$\sigma_{21}(t) = \int_{-\infty}^t G(t-t') \dot{\gamma}_{21}(t') dt' \quad (1.31)$$

Substituting Equation 1.30 into Equation 1.31 and s for $t - t'$ produces [Ferry, 1980],

$$\sigma(t) = \int_0^{\infty} G(s) \omega \gamma^o \cos[\omega(t-s)] ds \quad (1.32)$$

=

$$\gamma^o [\omega \int_0^{\infty} G(s) \sin \omega s ds] \sin \omega t + \gamma^o [\omega \int_0^{\infty} G(s) \cos \omega s ds] \cos \omega t$$

The above equation indicates that γ is in phase with $\sin \omega t$ and 90° out of phase with $\cos \omega t$ and from above σ is periodic with respect to ω and out of phase to γ [Ferry, 1980]. For ease of presentation, Equation 1.32 becomes,

$$\sigma = \gamma^o (G' \sin \omega t + G'' \cos \omega t) \quad (1.33)$$

where, G' and G'' are defined as the shear storage and loss moduli or elastic and viscous moduli, respectively [Ferry, 1980]. To further the description of viscoelastic materials the shear storage and loss moduli can be written in terms of the amplitude of the applied stress, $\sigma^o(\omega)$ and phase angle, $\delta(\omega)$ between the stress and strain [Ferry, 1980].

$$G' = \left(\frac{\sigma^o}{\gamma^o} \right) \cos \delta \quad (1.34)$$

$$G'' = \left(\frac{\sigma^o}{\gamma^o} \right) \sin \delta \quad (1.35)$$

$$G''/G' = \tan \delta \quad (1.36)$$

Equations 1.33 and 1.34 – 1.36 are related by the following trigonometric function,

$$\sigma = \sigma^o \sin(\omega t + \delta) = \sigma^o \cos \delta \sin \omega t + \sigma^o \sin \delta \cos \omega t \quad (1.37)$$

Further, the complex shear modulus can be related to the shear storage and loss moduli through,

$$G^* = \sigma^* / \gamma^* = G' + iG'' \quad (1.38)$$

Equation 1.38 represents the critical connection between the elastic and viscous components of the viscoelastic behavior of biomaterials materials.

1.2.6.2. Viscoelasticity of Human Skin and Dermis

Human skin is a complex system of interdependent mechanical load-carrying elements. Whole human skin's pre-loaded state is described as a tension plane [Diridollou et al., 2000]. From a material science point-of-view, human skin is an anisotropic, multilayered viscoelastic solid. Anisotropic materials are those with direction dependent material properties. Viscoelastic materials, by definition, possess the ability to store and dissipate energy depending on the dynamic or quasi-static loading conditions to which they are subjected. Viscoelastic solids are modeled mathematically using a system of springs and dampers, in either series or parallel circuit orientations, to describe the material's ability to store (the “spring” element) or dissipate (the “damper” element) energy under different load phases. Viscoelastic materials are described by a complex modulus that accounts for the material's elastic (storage) behavior and viscous (loss) behavior under various load regimes. The nature of viscoelastic solids allows for the material to exhibit creep deformation and a material response known as hysteresis.

Creep is a type of deformation commonly occurring in conditions of high stress and temperature variation. More specifically, creep in viscoelastic solids is described as a “time-dependent” deformation in which there is a cumulative strain condition over time. Prolonged strain conditions can occur at room temperature, which is why the deformation type is important in examining the mechanical behavior of soft tissues. The hysteresis curve is a representation of how a viscoelastic solid can store and dissipate energy under a given load history.

Additionally, living human skin exhibits a type of mechanical behavior commonly referred to as stress relaxation. Stress relaxation is an important component of the mechanical behavior of viscoelastic solids because it describes the material’s ability to dissipate prescribed loads, simple or complex, over time. The stress relaxation function, a product of stress relaxation experiments, is the chief means of classification and comparison in the study of viscoelastic solids. In addition, the stress relaxation function is an essential piece of the puzzle used in the development of a mathematical viscoelastic model.

Many groups have conducted viscoelastic characterization of human and animal skin [Edwards et al., 1995; Corr et al., 2009]. However, due to the variance in mechanical load application, mechanical behavior, and specimen anatomic sourcing, no directly applicable data is available in current literature. Skin requires various assumptions to constrain the system to less complex solid mechanics and material science principles. One such assumption is transverse isotropy. This reduces the anisotropy of skin to a set of depth

dependent layers. In other words, no two layers are alike vertically and within individual layers the material properties are independent of the transverse directions. Another important assumption is related to the skin's capacity to translate load through the collagen fiber network of the reticular dermis.

Collagen fibers of the dermis are able to self-organize along the lines of action of the applied load in order to dissipate the load before a failure stress is developed. There is a constant remodeling of the fiber network during loading. This quality allows skin to gain mechanical strength over time by “remembering” previous load situations in order to dissipate similar loads more efficiently in the future. More specifically, it is believed that skin has a three-phase, time-dependent response to load: an “immediate” response, a “short-term memory”, and a “long-memory” [Pioletti et al. 2000]. These terms account for the elastic, viscous, and coupled mechanical behavior, respectively, of living human skin under a number of complex loading conditions. Properly relating these three terms, their invariants, and related assumptions (incompressibility, thermodynamic constraints, symmetry, etc...) will produce a viscoelastic model that avoids discontinuities at shifts or boundaries in the load translation.

To address viscoelasticity and deformation within skin there are more than thirty-six guidelines [Medalie et al., 1997], some more relevant than others, that are used to describe what are commonly referred to as relaxed lines of tension. Early researchers noticed the elliptical deformation of incisions and holes in the skin when they were made in distinct planes and orientations. With these observations, early investigators

hypothesized there was direction dependence to the natural tension in human skin. These early experiments lead to the creation of a map of the “cleavage lines” or “Langer lines” [Medalie et al., 1997] for the human body. These cleavage lines supported the creation of guidelines for surgical incision based on the concept of relaxed tension lines in skin. These guidelines lead to reduced scarring from medical procedures by preventing discontinuities to skin’s inherent pre-tensed condition. Cleavage line guidelines have been essential to the development of surgical techniques such as z-plasty and skin flaps.

1.2.6.3. Rheology

Rheometers, also called viscometers, are used to describe the flow characteristics and fluid properties of various viscous and semi-viscous liquids. However, with different load-cell geometries this instrument is capable of measuring a viscoelastic solid’s material behavior under a given shear loading condition. Either a cone and plate geometry or parallel plate geometry can be used to create a loading condition that mimics the driven horizontal displacement of one parallel-plate relative to another, with the material of interest resting between the two plates. Although the cone and plate geometry is ideal from a loading condition perspective, based on the dimensions of the tissue and the need to maintain a constant shear rate throughout the specimen, the parallel plate geometry is more than capable of providing the level of detail required to fully capture the material’s response to a shear load. The material’s resistance to shearing is then used to determine various material parameters such as the relaxation time of the material, the storage (elastic) modulus, and the loss (viscous) modulus. The quantities are then used to formulate the complex modulus of the material. The complex modulus in conjunction

with the stress relaxation function can be used to develop a unique viscoelastic model capable of describing the non-linear, time-dependent mechanical behavior of a viscoelastic solid under load. Rheometers are ideal for the mechanical characterization of soft-tissues and soft-tissue related biomaterials.

Dissertation Objectives

Dissertation Objective 1:

To characterize the viscoelastic properties of whole skin and dermis under physiologically relevant loading conditions.

Quantifying the viscoelastic properties of human skin and dermis is critical to understanding the in situ mechanical landscape and capacity of human skin, the body's largest organ. The mechanical characterization of soft-tissue, and human skin specifically, is centuries old [Medalie et al., 1997]. The vast majority of mechanical characterization has focused primarily on the age-related mechanical response to uniaxial or biaxial tension, indentation, suction, and torsional loading modalities [Edwards et al., 1995]. Although the native mechanical state of human skin is tension-related, shear is a particularly destructive loading modality in many biological and medical applications. Despite the breadth of mechanical properties available in the literature for human skin, few studies have attempted to quantify the viscoelastic behavior of human skin and dermis-only under physiologically relevant dynamic shear loading conditions. The physiologically relevant frequency range has been defined between 0.1 and 20Hz [Aamer et al., 2004]. However, appropriate stresses and percent strains for loading are less defined, requiring researchers to develop percent strain regimes specific to the anatomic location and function of the soft-tissue under study.

In Chapter 2, an in-depth physiologically relevant, viscoelastic characterization of whole human skin and dermis-only, was conducted with a stress controlled rheometer. The

examination of the skin and dermis-only response provided a “snap-shot” of the mechanical behavior of the collagen lattice within the respective tissues. Skin and the dermis are highly dynamic and adaptable tissues, capable of reorganizing and remodeling their collagen lattice in response to changes in loading conditions. To account for this inherent variability, the viscoelastic characterization data of numerous skin samples were compiled to formulate an approximation of the bulk mechanical behavior of human skin and dermis only samples. The viscoelastic properties provided by the characterization are limited by anatomical location and age of the donor subjects, however the representative behavior of the tissue studied here may be extended to other anatomical locations and age groups through scaling; although not discussed in this study. This approach is supported by discussions in the literature on the effects of age and anatomical location on changes in the elasticity and mechanical behavior of human skin. The material properties gathered during the viscoelastic characterization of skin and dermis-only formed the foundation for a mechanics-based biomaterial design template.

Dissertation Objective 2:

To develop and characterize a novel biomaterial that is mechanically matched to whole, human skin.

The success of biomaterials is linked to three primary design criteria: physical/mechanical properties; mass transport properties; and biocompatibility/biomimetic capacity [Gheduzzi et al., 2006; Kohane et al., 2008; Tibbit et al., 2009; William et al., 2009]. A substrate's ability to mimic the mechanical behavior of the in situ tissue it is intended to replace or supplement is critical for long-term device implantation [von Recum, et al., 1984]. The ability of cells and tissues to adapt to changes in loading conditions and mechanical stimuli is well established [Gardel et al., 2008]. Cutaneous wound healing relies heavily on the ability of cells to detect changes in the mechanics of their environment [Singer et al., Wang et al., 2003; Hoffman et al., 2009]. Although cutaneous wound healing is a profoundly bio-chemical process, discounting the mechanical component disregards the cue that initiates and dictates the wound healing process. Unfortunately, little effort has been made to synthesize biomaterials with mechanical properties that match the soft-tissue, with even fewer attempting to mimick the viscoelastic behavior of human skin.

In Chapter 3, the data from the viscoelastic characterization of human skin (*Specific Aim 1*) was used to optimize the formulation of a novel, poly(2-hydroxyethyl methacrylate) [pHEMA]-based biomaterial. The viscoelastic response of dermis-only was not included in the optimization process because the ultimate target applications were related to whole

human skin implantation. A comparative rheology-based optimization process was developed to better understand the critical formulation components (e.g. micro-porosity, cross-linker density, monomer to diluent ratio, etc...) essential for matching the viscoelastic response of the substrate to human skin. The product of the optimization process would be utilized in further studies reinforcing the importance of a biomaterial's mechanical biomimetic capacity.

Dissertation Objective 3:

To interrogate the effect of in vitro cellular activity on novel biomaterial for insights into the development of a polymer-based solution to epidermal regression around transcutaneous/percutaneous medical devices.

Biomaterials are often remodeled or altered following implantation in the human body. The post-implantation alterations are chemically and mechanically related, and can occur on a micro and macro-scale level [Slaughter et al., 2009]. The biophysics and molecular biology fields have identified the importance of cell-substrate interactions and how those interactions alter cellular morphology and phenotype [Singer et al., 1999; Gardel et al., 2008]. However, few groups have extended this approach to whole tissues and biomaterial design. Developing a biomaterial intended to reproduce the viscoelastic behavior of an in situ soft-tissue is only a portion of the design process. To ensure a biomaterial continues to reflect the tissue it is intended to replicate and ultimately replace or supplement, the effect of cellular activity on the biomaterial must be quantified.

In Chapter 4, novel, micro- and macro-porous pHEMA substrates developed to match the viscoelastic behavior of human skin were co-cultured with human fibroblasts to quantify the effect of cellular activity on the rheological behavior of the substrates. Following 14days of co-culture the rheological behaviors of pHEMA substrates with various polymerization and surface treatments were compared and the effect of cellular activity qualitatively and quantitatively described. The resulting data was used to further the

development of a biomaterial intended to minimize stress-discontinuity related epidermal regression at the soft-tissue to device interface of percutaneous medical devices.

Manuscript

The following thesis focuses on the development of a synthetic substrate that mechanically matches the viscoelastic behavior of human skin. The following three (3) chapters represent three individual manuscripts published in, submitted to, or prepared for submission to peer-reviewed academic journals.

The thesis contains the following manuscripts:

Chapter 2: Holt, B., Tripathi, A., Morgan, J.R. Viscoelastic response of human skin to low magnitude physiologically relevant shear. Journal of Biomechanics 2008; 41: 2689 – 2695.

*Chapter 3: Holt, B., Tripathi, A., Morgan, J.R., A Physiologically Relevant Rheological Characterization-based Approach for the Development of Biomaterials that Mimic the Viscoelastic Response of Soft Tissue
(Prepared for Submission)*

*Chapter 4: Holt, B., Tripathi, A., Morgan, J.R., Designing PolyHEMA Substrates that Mimic the Viscoelastic Response of Soft Tissue.
(Submitted to Journal of Biomechanics, August 2010)*

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Chapter 2:

Viscoelastic Response of Human Skin to Low Magnitude Physiologically Relevant Shear

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Abstract

Percutaneous implants are a family of devices that penetrate the skin and all suffer from the same problems of infection because the skin seal around the device is not optimal. Contributing to this problem is the mechanical discontinuity of the skin/device interface leading to stress concentrations and micro-trauma that chronically breaks any seal that forms. *In situ* human skin is under biaxial tension but relative motion at the skin/device interface creates shear. In this paper, we have quantified the mechanical behavior of human skin under low-magnitude shear loads over physiological relevant frequencies. Using a stress-controlled rheometer, we have performed isothermal (37°C) frequency response experiments between 0.628 to 75.39rad/s at 0.5% and 0.04% strain on whole skin and dermis-only, respectively. Step-stress experiments of 5 and 10Pa shear loads were also conducted as were strain sweep tests (6.28rad/s). Measurements were made of whole human skin and skin from which the epidermis was removed (dermis-only). At low frequencies (0.628 to 10rad/s), the moduli are only slightly frequency dependent, with approximate power-law scaling of the moduli, $G' \sim G'' \sim \omega^\beta$, yielding $\beta = 0.05$ for whole skin and $\beta = 0.16$ for dermis-only samples. Step-stress experiments revealed three distinct phases. The intermediate phase included elastic “ringing” (damped oscillation) which provided new insights and could be fit to a mathematical model. Both the frequency and step stress response data suggests that the epidermis provides elastic rigidity and dermis provides viscoelasticity to the whole skin samples. Hence, whole skin exhibited strain hardening while the dermis-only demonstrated stress softening under step-stress conditions. The data obtained from the low magnitude shear loads and

frequencies that approximate the chronic mechanical environment of a percutaneous implant should aid in the design of a device with an improved skin seal.

1. Introduction

Percutaneous medical devices that penetrate the skin include indwelling catheters, dialysis ports, feeding tubes, and external bone fixation devices [Jacobsson 1991, Paquay, et al. 1996]. There are over a million percutaneous devices implanted annually in the United States and all are designed to function for relatively short periods of time (weeks to months) because of the well known risk of infection of this entire family of devices [Jacobsson 1991]. The skin seal around these devices is not stable, nor secure. There are several instances where medical devices needed for long term implantation have undergone re-engineering to circumvent this problem of the skin interface such as cochlear implants which are now wireless [Goto, et al. 2001]. Another percutaneous device under consideration for long term implantation is a transcutaneous osseointegrated device for limb restoration [McClarence 1993]. Integration of this device with bone is acceptable and osseointegration is a well established phenomenon in orthopedic implants [Palacci 1995]. However, integration of the device with soft tissue (skin) suffers from the same problems as those limiting all percutaneous devices. Thus, a solution to the problem of skin integration could improve the safety/performance of current devices and enable the design of new devices for long term implantation.

The problem of the skin seal is multi-faceted. Within weeks after implantation, when the wound healing response has subsided, the area around a percutaneous device is still inflamed and the epidermis has not formed a tight seal around the device. In most cases, the epidermis has begun to grow downward around the device forming a sinus tract that helps to sustain inflammation and provides a pathway for future infection [Knabe, et al.

1999]. Likewise, the adjacent dermal tissue is not well attached and contains abundant inflammatory cells [Hadgraft 2004, Jacobsson 1991, Knabe, et al. 1999, Presland, et al. 2002, Tregear 1966, Wang, et al. 2003]. This unstable state persists for the lifetime of the device and has similarities with chronic wounds of the skin [Jacobsson 1991, Presland, et al. 2002, Wang, et al. 2003]. Several factors contribute to this instability including motion of the device and inadequate adhesion of skin cells. Progress has been made to stabilize the gross motion including fixation and anchorage designs [Chehroudi, et al. 2002, Jacobsson 1991, Knabe, et al. 1999, Paquay, et al. 1996, Shalabi, et al. 2004] and new materials that promote cell adhesion is an active area of investigation [Chehroudi, et al. 2002, Paquay, et al. 1996].

One area not usually considered is the mismatch in the mechanical properties of the device and the skin as well as the stress concentrations that occur as a result of this mismatch. The compliance mismatch of a synthetic graft and a native artery is thought to be a factor that leads to failure of vascular grafts [Conklin, et al. 2002, Kim, et al. 1993]. In skin, the stress concentrations at the skin/device interface due to this mismatch facilitate repeated micro-trauma breaking any seal and leading to chronic wounding [Cacou, et al. 1995, David, et al. 2004, Jacobsson 1991, von Recum 1984, von Recum, et al. 1981, Wang, et al. 2003]. Biaxial tension is the dominant load in human skin and has applications with regards to various biomedical devices. However in percutaneous systems the relative motion at the skin/device interface caused by device movement and soft-tissue inertia during physical activity fosters shearing that prevents the wound healing process, cellular adhesion, and tissue integration. Shear loading in skin related

biomedical applications is another area not often investigated. Shear, even at low-magnitudes, is a highly destructive loading modality, especially when applied over the long time periods of an implant. Although the viscoelastic properties of skin are well established from torsional, uni- or bi-axial measurements, there is a wide range of elastic moduli reported and the tests do not approximate the chronic physiologically relevant shear conditions that exist at an implant [Diridollou, et al. 1998, Grahame, et al. 1969, Khatyr, et al. 2006, Panisset, et al. 1992, Silver, et al. 2001].

In this study, we measured the mechanical behavior of human skin subjected to low-magnitude shear loads over a range of physiologically relevant frequencies. Strain sweep, frequency response, as well as creep and recovery measurements were made on whole human skin and human skin from which the epidermis was removed (dermis-only). In addition to providing a range of highly accurate values of elastic (G') and viscous (G'') moduli in response to physiologically relevant chronic shear stresses, the step stress measurements revealed an interesting ringing or damped oscillation. This ringing is commonly seen with polymer-based fluids and provides new insight. The step-stress data was fit to a mathematical model which should aid the design of percutaneous devices that might minimize these shear conditions at the skin/device interface.

2. Materials and Methods

2.1. Skin Preparation

Human skin (foreskin) obtained at the Women & Infants Hospital of Rhode Island, Providence, RI, USA (approved by the Institutional Review Board) were collected in

50mL centrifuge tubes with approximately 25mL of keratinocyte medium (KCM) and stored on ice ($\sim 4^{\circ}\text{C}$). Specimens were emptied into 20mm Petri dishes and rinsed several times with Dulbecco's phosphate buffered saline (PBS) (Cellgro, Mediatech Inc., Herndon, VA)(**Figure 2.1**). Specimens were laid flat, dermal-side up and an incision was made at the thinnest part of the specimen using straight, 10.5 cm fine iris scissors. This allowed the sample to lay flat exposing the full dermal layer and clear longitudinal and transverse directions. A 6mm biopsy punch (Miltex, York, PA) was used to obtain uniform samples for measurement. Time between collection and experimentation was approximately 4 hours. A total of 20 skin samples (whole skin and dermis-only) were obtained for rheological testing.

To separate the epidermis from the dermis, 6mm biopsies were incubated at 37°C for six hours in 25mL grade II Dispase 2.4U/mL (Roche Applied Science, Indianapolis, IN). The specimens were rinsed with versene, PBS with 0.5 mM ethylene diamine tetra acetic acid (EDTA), to neutralize the enzyme, at which point the epidermis could be carefully separated from the dermis. Upon separation, the dermal specimens were placed in 20mL PBS (room temperature) in preparation for testing.

2.2. Frequency response measurements

Small amplitude oscillatory measurements were performed on uniform samples (6 mm punch biopsies) of whole human skin or skin from which the epidermis was removed (dermis only) using a controlled-stress rheometer (AR-2000N, TA Instruments, Newcastle, DE) with parallel plate geometry (8 mm diameter) and specimen-dependent

gap lengths. Samples were placed, dermal side down, on waterproof sandpaper (~20 by 10 mm) (Norton 320 Grit, Saint-Gobain Inc, Worcester, MA) held to the bottom plate by double sided adhesive tape (Scotch 666, 3M, St. Paul, MN) for no-slip shear [Tsubouchi, et al. 2006].

The 8mm driven plate of the rheometer was lowered to a gap of 400 to 780 μm , depending on the thickness of the biopsy. Critical to this loading protocol was the maintenance of a compressive normal force between 0.02 and 0.06N. Within this range sample spreading, uninhibited by boundary constraints (i.e. ring-guards), ensured the complete straining of the material and a pre-loading condition that mimicked skin's in situ tensile state. Additionally, it was determined an oscillatory torque greater than 10 $\mu\text{N} \cdot \text{m}$ was required for accurate characterization. Within this range, sufficient contact was maintained between the upper, driven plate and the epidermis to ensure constant shearing without slippage. The no slip boundary condition between sample and rheometer geometry was confirmed by monitoring the magnitude of shear stress and its repeatability over different samples. The biopsies were wet with 1mL of PBS and all measurements performed at 37°C. For oscillatory testing, strains of 0.5% were applied over a frequency range of 0.628 to 75.398rad/s that were divided into two frequency sweeps to ease data collection; a higher range (6.283 to 75.398rad/s) and a lower range (0.628 to 6.283rad/s). Specimens were loaded from high to low frequencies to maintain the optimal oscillatory torque and ten data points were sampled per decade.

2.3. Creep and recovery measurements

For creep and recovery experiments, biopsies were loaded in two phases: first a creep phase and then a recovery phase with no equilibration step between phases, ensuring an accurate viscoelastic step-stress measurement. Each phase was approximately 300s long, ensuring complete creep and recoil. The creep phase was conducted with two applied shear stresses, 5 and 10 Pa (~ 0.0007 and 0.0014 psi). The oscillatory torque range for the creep phase was determined from the oscillatory experiments and torque values were 0.0503 and 1.0053 $\mu N \cdot m$ for the 5 and 10 Pa creep experiments, respectively. The recovery experiments were conducted with no applied shear stress. Upon completion of the two phases, the specimens were visually inspected to ensure that there had not been macro-scale destruction of the specimens that would affect the reliability of the creep and recovery data.

3. Results and Discussion

3.1. Strain sweep measurements

The biopsies were placed on the rheometer and loaded with a small normal compressive force (0.02 to 0.06N). In compressing the biopsies from the punch size of 6mm to the aperture diameter of 8 mm, the soft-tissues endured an expansion of approximately thirty-three percent but without any normal forces. This expansion eliminated residual shrinkage resulting from preparation steps. In addition to ensuring shearing without slippage, this loading also mimics the expanded state of human skin *in vivo*.

To determine the optimal percent strain (% angular displacement) for loading during frequency response experiments, biopsies were subjected to strain sweep measurements from 0.1 to 3% strain at 6.28rad/s (**Figure 2.2**). The strain sweep experiments ensured that the regime of small amplitude straining was valid and that equation was applicable (1) (i.e. linear viscoelastic regime). The dependence of the loss and the elastic moduli for whole skin and dermis-only on γ_0 was measured for $\omega = 6.28\text{rad/s}$. The loss and elastic moduli of whole skin displayed almost no strain dependence up to approximately 3% shear strain. For dermis-only samples, the moduli showed very little strain dependence up to 1% strain, after which some strain-induced reduction was observed. The strain sweep measurements suggest that the elastic modulus of whole skin is about three times that for the dermis only. Hence, it appears that the epidermis provides the stiff elastic properties to the whole skin. For oscillatory testing, the applied strain was limited to amplitudes $\gamma^0 = 0.05$ in order to ensure that the tests were within the small amplitude straining regime. No slippage was noticed for these strains.

3.2. Frequency response measurements

Constant strain was applied to the whole skin or dermis only samples over frequencies ranging from 0.628 to 75.40rad/s (**Figure 2.3**). The whole skin samples were investigated at 0.5% strains. However, strains of 0.05% could only be achieved in the dermis only samples due to slip between the top plate and samples. The data from ten whole skin biopsies and ten dermis-only biopsies shows clearly that the elastic modulus (G') and viscous modulus (G'') increases with frequency of oscillation. The viscoelasticity has two distinct contributions. At low frequencies (0.628 to 10rad/s), the rate of increase for G'

and G'' is gradual ($G' \sim G'' \sim \omega^{0.05}$) for both whole skin and dermis-only. This behavior is nearly frequency independent, consistent with observations in F-actin gels and keratin/intermediate filament systems [Gardel, et al. 2004, Ma, et al. 1999, Ma, et al. 2001, Shin, et al. 2004]. The rate of increase of G' and G'' ($G' \sim G'' \sim \omega^{0.16}$) is more pronounced for the dermis-only suggesting that the epidermis provides elastic rigidity. At higher frequencies ($>10\text{rad/s}$), the rate of increase in G' and G'' for whole skin and dermis-only both accelerated ($G' \sim G'' \sim \omega^{0.5-1.0}$) with the effect more pronounced for the dermis-only, especially G' . This suggests that the viscoelastic components of the dermis dominate at higher frequencies due to the inability of the viscous components to adapt to the rate of load application.

Across the entire frequency range tested, the G'' and G' curves did not intersect showing that under low magnitude oscillatory shear the viscoelastic behavior of the human skin is primarily elastic in nature. This is further supported by the calculated phase angle for whole skin and the dermis, with mean values of approximately 12.39° and 13.12° , respectively. Both of these values are within the elastic range for phase angles. Also consistent with observations in F-actin gels and keratin/intermediate filament systems [Gardel, et al. 2004, Ma, et al. 1999, Ma, et al. 2001, Shin, et al. 2004]. This finding supports the description of the skin as a viscoelastic solid and suggests that the thermal fluctuations of the skin network dominate the frequency-dependent mechanical response of the network.

Lastly, the dynamic viscosities $\eta'(\omega) = G''/\omega$ of skin samples can be evaluated using equation (1). Both whole skin and dermis-only showed shear thinning behavior, approximating power law scaling for dynamic viscosity, $\eta' = \nu\omega^{n-1}$, yielded $n = 0.125$ and $\nu = 85.37 \text{ Pa} \cdot \text{s}^n$ for whole skin samples and $n = 0.219$ and $\nu = 130.61 \text{ Pa} \cdot \text{s}^n$ for dermis only samples.

3.3. Creep measurements

To measure creep, we applied a time dependent shear stress and measured the resulting shear strain. A constant stress σ_0 was applied at $t = 0$ to the biopsy and time related compliance, as given by:

$$J(t) = \frac{\gamma(t)}{\sigma_0}, \quad (3)$$

was measured. For very small values of σ_0 , the compliance contains the same information as G' and G'' .

Shear loads of 5Pa and 10Pa were applied to whole skin and dermis-only for 300s at 37°C and creep compliances were measured (**Figure 2.4**). Interestingly, we found that the creep response followed three distinct phases; an initial elastic creep, an intermediate viscoelastic ringing and a long-term viscous creep. The initial elastic creep phase represents the elastic capacity of whole skin in response to the application of a step-stress. The whole skin biopsies showed an initial elastic response of $\sim 1.74\text{s}$ for both applied stresses of 5Pa and 10Pa. In contrast, the dermis-only showed $\sim 2.84\text{s}$ of the

initial elastic response for both applied stresses. Noticeably, the dermis-only showed higher compliance for larger stresses suggesting that the dermis suffered the permanent strains in the non-linear regime. The intermediate viscoelastic creep ringing phase with its characteristic oscillating behavior in the compliance $J(t)$ data is the product of both a specific instrument's inertia [Ewoldt, et al. 2007] and a material's specific inertio-elastic oscillation. In the creep experiments, this value is time-dependent. From approximately 1.7s to 7s for both stresses (5Pa and 10Pa), this inertio-elastic oscillation dominated the mechanical behavior of whole-skin and dermis-only. The amplitude of the oscillations and the number of oscillations was greater for whole skin versus dermis-only, but the time to fully dampen the oscillations was approximately the same (~8s). The third phase (>8s) of prolonged viscous creep was present in both whole skin and dermis-only and showed a ramped increase in compliance under load until the end of the experiment.

Based on the creep data, the dermis is significantly more compliant than whole skin, again implying that much of the elastic rigidity is provided by the epidermis. The whole skin shows ~20% of the compliance demonstrated by the dermis. Thus, whole skin can be treated as a two part keratin/polymer composite, consisting of an elastic solid (epidermis) and a viscous elastic fluid-like component (dermis). This depiction is further supported by the whole skin and dermis-only recovery data (See Supplemental Section).

Also of note is the observation that the creep compliance of whole skin was greater in response to the lower (5Pa) shear stress, whereas the opposite was true for the dermis-only, in which the creep compliance was greater in response to the application of the

higher shear stress (10Pa). This again suggests that for whole skin samples, which have a stiff elastic epidermal layer, the modulus increases with applied stress. This type of strain hardening behavior is also observed in vimentin biopolymer networks[Janmey, et al. 1991]. On the contrary, the dermis-only showed an increase in modulus with applied stress suggesting that the dermis layer shows a strain softening effect which is common in cross-linked actin networks[Tharmann, et al. 2007].

3.4. Recovery measurements

An abrupt removal of shear stress from samples that are undergoing steady shear flow will cause them to recoil to some previously occupied position. This recovery measurement is the shear strain during recoil which is measured relative to the time when the shear stress was removed. Whole skin and dermis-only were subjected to shear stresses of 5Pa or 10Pa to establish steady shear flow. Shear was terminated and the decrease in recovery strain $\gamma_r(t)$ measured over time (**Figure 2.5**). Both whole skin and dermis showed two regimes in the recovery process: a rapid viscoelastic recoil ($3 \leq t \leq 9$ s) and a long ultimate recoil (>9 s). The rapid viscoelastic recoil is a measure of the recovery compliance $J_r(t) = \gamma_r(t)/\sigma_0$ of a sample. The recovery response depends on the inertia of the instrument, a sample's materials resistance and response to shear loading. Both whole skin and dermis-only relaxed about 50% of the imposed strain in 5 seconds. Overall, the rigid whole skin samples relaxed about 72% and 84% (%ultimate recoil) of the imposed strain for 10Pa and 5 Pa of imposed stresses, respectively. The dermis-only relax about 64% and 90% (ultimate recoil) of the imposed strain for 10Pa and 5 Pa of imposed stresses, respectively. This clearly, shows that the strain softening of the dermis

is more prominent than the strain hardening of whole skin. Note that the plastic deformation (100 - %ultimate recoil) can be evaluated using this data. Lastly, the time required to attain ultimate recoil was approximately the same as the time to reach steady state in the creep experiments, validating the identification of single time scale of viscoelastic response.

3.5. Mathematical models

To apply these findings to more complex loading conditions, a mathematical model used to describe viscoelastic materials was fit to the strain data of the shear-based creep experiments. A modified Kelvin-Voight model was chosen based on previous studies that used this modified model to describe the damped inertio-elastic oscillations (ringing) observed in other viscoelastic solid materials [Ewoldt, et al. 2007]. The Kelvin-Voight model is a two parameter system consisting of a Newtonian dashpot (viscosity η_k) and Hookean spring (modulus G_k) in parallel (**Figure 2.6A**). Even though the storage modulus G' does not capture the frequency response observed in this study, the model can still be used to understand elastic modulus of skin samples. A back calculation method was applied to the damped inertio-elastic oscillation (due to instrument inertia I) regions of the creep experiments to determine the relevant values of springs and dashpots. The creep strain behavior, $\gamma(t)$ of whole skin and dermis-only biopsies was fitted using the following expression for Kelvin-Voight strain:

$$\gamma(t) = \gamma_K \{1 - e^{-A_K t} [\cos(\omega_K t) + \frac{A_K}{\omega_K} \sin(\omega_K t)]\}, \quad \text{where} \quad \gamma_K = \frac{\tau_o}{G_K}, \quad A_K = \frac{\eta_K^2 b}{2I},$$

$$\omega_K = \sqrt{\left(\frac{G_K b}{I} - A_K^2\right)} \quad \text{and} \quad b = \frac{\pi R^4}{2h}. \quad \text{Here, } R \text{ and } h \text{ are the radius and thickness of}$$

samples, respectively. For whole skin, this model reasonably captures both elastic and ultimate creep compliance for the 10 and 5 *Pa* simulations (**Figure 2.6B and C**). By optimizing the equations for the best-fit, values for η_K (coefficient of viscosity) and G_K (spring constant) were determined. The mean η_K and G_K ($\pm$ SD) values calculated from the 10 *Pa* shear creep experiments were $26.62 \pm 7.26 \text{ Pa} \cdot \text{s}$ and $271.1 \pm 111.1 \text{ Pa}$, respectively. The mean η_K and G_K ($\pm$ SD) values obtained from the 5 *Pa* shear creep experiments were $22.86 \pm 4.71 \text{ Pa} \cdot \text{s}$ and $276.7 \pm 135.3 \text{ Pa}$, respectively. These values were applied in the following expressions: $G' = G_K$ and $G'' = \eta_K \omega$ to solve for the elastic (G') and viscous (G'') moduli of whole skin. After obtaining η_K and G_K , the model utilized a frequency-based simulation to calculate a value for G' and a range of G'' values. To verify the accuracy of the model, we applied a frequency range similar to the earlier oscillatory experiments (0.628 to 11.95 rad/s). For simulations based on the 10 *Pa* shear creep experiments on whole skin, the calculated mean G' ($\pm$ SD) was $257.5 \pm 111.6 \text{ Pa}$ and the mean range for G'' ($\pm$ SD) over the frequency range of 0.628 to 11.95 rad/s was 16.72 ± 4.56 to $318.1 \pm 86.81 \text{ Pa}$ (**Figure 2.6D**). For simulations based on the 5 *Pa* shear creep experiments of whole skin, the calculated mean G' ($\pm$ SD) was $255.4 \pm 150.5 \text{ Pa}$ and the mean range for G'' ($\pm$ SD) over the same frequency range was 14.08 ± 2.92 to $252.3 \pm 93.02 \text{ Pa}$ (**Figure 2.6E**). While the Kelvin-Voigt model accurately predicts the elastic and ultimate compliance phase of the step-stress experiments, it does not completely capture the damped interio-elastic oscillatory phase (viscoelastic creep ringing) and, consequently, the viscous creep phase. When the model was used to

determine the storage and loss moduli of human skin during oscillatory loading, the percent error between the G' data and the model fit is approximately 35%. But with a model of this simplicity applied to a material of this complexity this fit is encouraging. The linear relationship between G'' and the applied frequency diverge quickly from the mechanical behavior measured during experimentation. In order to fully capture the viscoelastic behavior of human skin a more complex viscoelastic model will need to be developed.

4. Conclusions

Biaxial tension is the dominant load in human skin, however at the interface of skin and a percutaneous device, the relative motion of the device and the inertia of skin during routine physical activity fosters shearing that prevents the formation of an adequate skin seal. We measured the viscoelastic response of human skin to low magnitude shear loads over a range of physiologically relevant frequencies (0.628 to 75.39rad/s at 37°C). The mean elastic (G') and viscous (G'') moduli in whole skin increased over the frequency range from $325.0 \pm 93.7 Pa$ to $1227.9 \pm 498.8 Pa$ and $68.5 \pm 21.2 Pa$ to $189.9 \pm 56.0 Pa$, respectively. Dermis-only showed a similar trend with mean G' and G'' values increasing from $434.9 \pm 122.1 Pa$ to $6620.0 \pm 849.5 Pa$ and $126.3 \pm 34.5 Pa$ to $458.6 \pm 134.9 Pa$, respectively. Hence, in oscillatory response experiments, G' and G'' increased gradually over the entire frequency range with values of G' consistently higher than G'' for both whole skin and dermis only samples. Values of G' and G'' were slightly higher for dermis only samples compared to whole skin. Across this range of frequencies and under these low magnitude shear loads, skin is a complex viscoelastic composite with a

rigidly elastic upper epidermal layer and an underlying viscoelastic dermal layer. In shear step-stress experiments, whole skin showed strain hardening, while the dermis-only showed stress softening. The data could be fit to a Kelvin-Voigt model with parameters, η_K and G_K , that could approximate G' and G'' over the same oscillatory response frequency range.

Several approaches have been used to measure the biomechanics of human skin including tensile (uni- or bi-axial), torsion and suction tests. Tensile studies performed in vitro on excised human skin used uni-axial and biaxial load regimes and vary significantly in applied loads and strains (Marks 1991; Silver, Freeman et al. 2001). The load regimes in these studies do not approximate the chronic shear environment of the skin around percutaneous devices. With those qualifiers in mind, the studies available for a comparative discussion were primarily reduced to in vivo studies. Torsion tests have also been applied to skin *in vivo*. Small angular displacements have been used to measure the resultant strain allowing for the quantification of skin's elasticity and extensibility. Sanders et. al., applied an oscillatory torque of $0.83 \mu N \cdot m$ to investigate the step-stress behavior of human forearms (ages 6 to 61 years) and reported modulus of elasticity values ranging from 2.3×10^{-2} to $6.2 \times 10^{-2} MPa$ [Sanders 1973]. Agache et. al., applied an oscillatory torque of $28.6 \mu N \cdot m$ (ages 3 to 89 years), measured immediate distension, final distension of loading, delayed distension, and immediate retraction [Agache, et al. 1980]. Moduli of elasticity for young and old individuals were 0.42 and 0.85 MPa, respectively [Agache, et al. 1980]. Escoffier et. al. applied oscillatory torques of 2.3 and $10.4 \mu N \cdot m$ (ages 8 to 98 years) measured immediate deformation, viscoelastic

deformation, immediate recovery, and creep relaxation time and reported a modulus of elasticity of 1.12 MPa [Escoffier, et al. 1989]. Each of these studies applied only one or two oscillatory torque values and did not test the skin's response to the full range of values that simulates loads associated ambulation. The values obtained in these studies were based on a mathematical approximation that treats the skin as an elastic isotropic plate, this technique differs from our direct measure of G' and G'' . Additionally, Agache et al. and Escoffier et al. included a guard ring that restricts sample spreading, a boundary condition restriction not present in this study.

Suction based tests have also been used. Grahame et. al. applied negative pressures ranging from ~ 7 to $\sim 40 \text{ kPa}$ through an aperture (100 mm) (ages 19 to 83 years) and calculated a comparative modulus of elasticity ranging from $20\text{-}100 \text{ MPa}$ for females and $9\text{-}52 \text{ MPa}$ for males [Grahame, et al. 1969]. Diridollou et. al., applied a negative pressure of 10 kPa through an aperture (6 mm) and reported a modulus of elasticity of $0.129 \pm 0.088 \text{ MPa}$ [Diridollou, et al. 2000]. Suction-based experiments isolate the epidermal and dermal layers, making them ideal for *in vivo* experimentation. However, like tensile and purely torsional experiments, these studies did not focus on the cyclic load regimes relevant to medical devices, nor did they examine repetitive shear loads.

The frequency range tested in our oscillatory shear experiments (0.628 to 75.39 rad/s) corresponds to a range of every day activities (0.1 to 12 Hz) that covers the principle levels of ambulatory activity of walking, jogging, and running [Danion, et al. 2003,

Farley, et al. 1996, Gutmann, et al. 2006, Kokshenev 2004]. Over this range, we observed a reproducible and concise range of values for G' and G'' . Similarly, the low magnitude step-stress experiments yielded important insight into the viscoelastic behavior of skin. We observed strain hardening in the whole skin and stress softening in the dermis-only, suggesting that the epidermis provides the more rigidly elastic behavior of human skin, while the dermis provides a viscous, fluid-like foundation. This description of human skin as a two part solid-like fluid composite is also supported by the inertio-elastic ringing seen in our measurements. It should be noted that inertio-elastic ringing relies heavily on the instrument used for mechanical characterization and may vary from one instrument to another. That being said, inertio-elastic ringing has been observed in step-stress rheological studies of biopolymer gels, but has not been reported for human skin [Ewoldt, et al. 2007]. A viscoelastic model, modified for this ringing behavior, provided a very accurate fit to the inertio-elastic ringing observed when measuring a mucous biopolymer [Ewoldt, et al. 2007] and may have applications for human skin.

The aim of this study was to investigate the low magnitude shear loading behavior of human skin as a means to understand the mechanics of skin around percutaneous devices. Although skin can attach to these devices, an adequate and long lasting seal is never formed due in part to the stress concentrations at the skin/device interface and the chronic low level shear forces that break this seal. While other studies have measured the mechanical behavior of skin, none have examined the response to low magnitude shear forces over a range of frequencies that cover the normal range of physical activity. An important finding of this study is the relatively narrow range of G' and G'' values in

response to this range of frequencies. This narrow range of gradually increasing moduli will be helpful for the development of a finite element based simulation of the soft-tissue-to-device interface. And, this data indicates that it may be possible to design an interface that can dissipate stress at this interface. A second finding is the inertio-elastic ringing behavior which has never been reported for skin. In rheometry, creep ringing is often ignored in data analysis. But, if properly interpreted, ringing has been shown to provide a valuable extended data set in that critical transition phase between elastic creep and viscous creep [Ewoldt, et al. 2007]. Moreover, we've used the ringing data as a means to verify our viscoelastic measurements in the forced oscillations experiments and a modified Kelvin Voight model that accounts for ringing was used in our simulations. Thus, the ringing data will help improve our understanding and modeling of the complex viscoelastic behavior of skin in the magnitude loads and frequencies critical to improving the skin seal around percutaneous devices.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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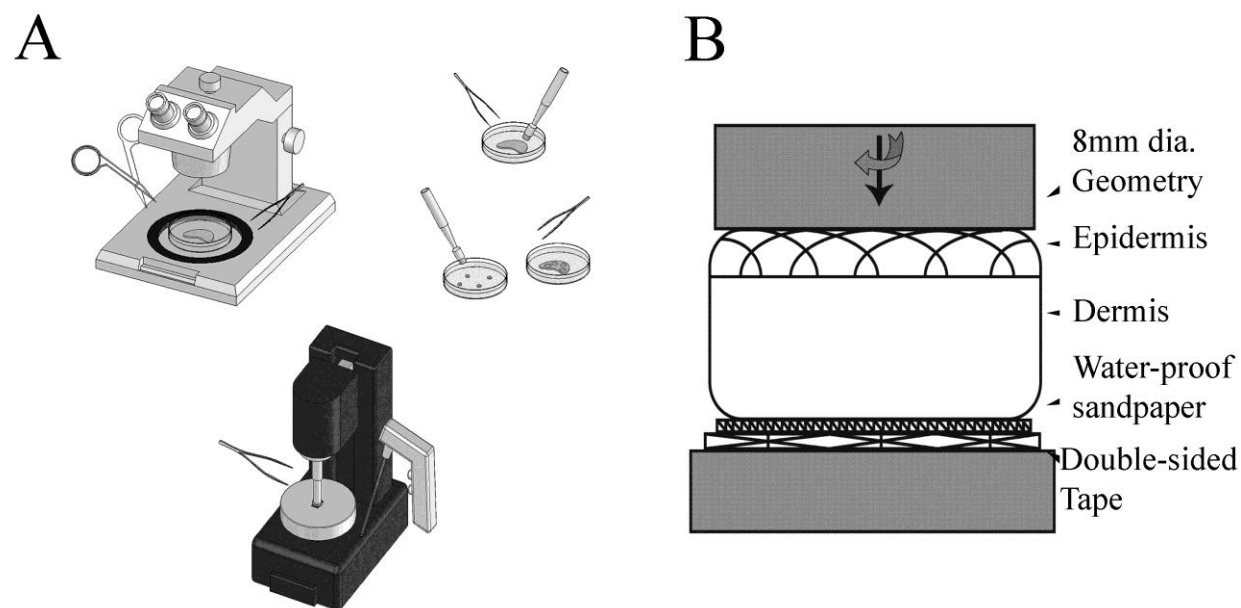


Figure 2.1: Preparation and rheological measurements of human skin and dermis. (A) Human skin specimens were sectioned using a 6mm dia. biopsy punch and then debrided for rheological characterization. Dermal specimens were obtained from whole skin samples using a six hour, dispase-incubation @ 37°C. (B) Circular skin samples were placed into the rheometer, epidermis side up, onto water proof sandpaper secured with double side tape. After loading with a small normal force compressive force (0.02 and 0.06N), each sample was subjected oscillatory shear forces

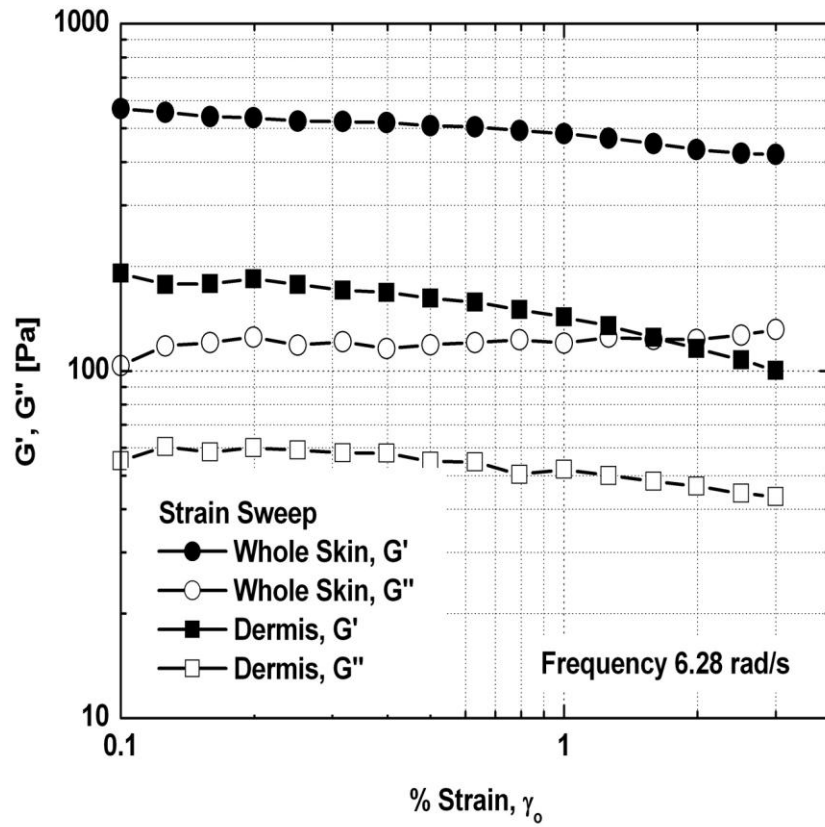


Figure 2.2: Oscillatory strain sweep. Whole skin and dermis-only biopsies were subjected to isothermal (37°C) percent strains ranging from 0.01 to 5% at 6.283rad/s in order to determine the optimal percent strain value for the oscillatory response experiments. The resultant storage modulus, G' was measured for both soft-tissues and changes in those values noted

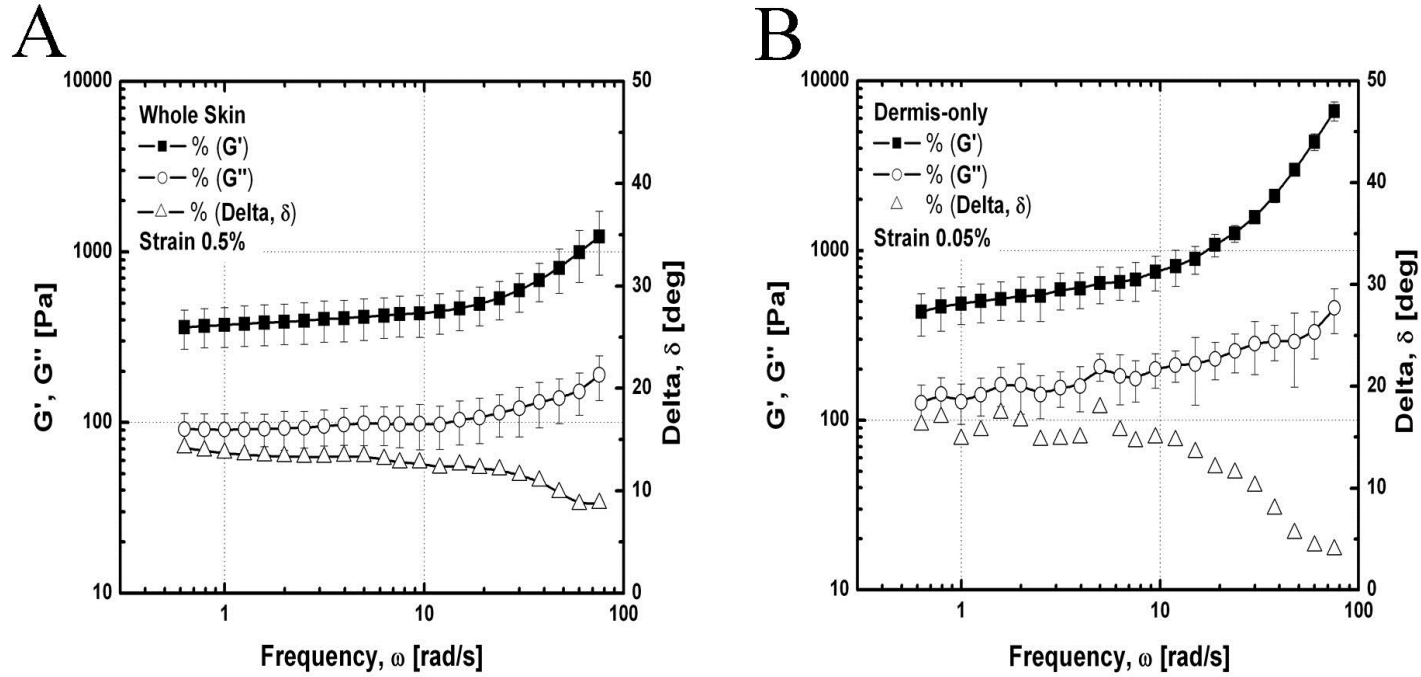


Figure 2.3: Linear viscoelastic response. (A) Whole skin and (B) dermis-only biopsies were subjected to isothermal (37°C) oscillatory strains of 0.5% and 0.04%, respectively between frequencies of 0.628 and 75.398 rad/s. The resulting G' and G'' and $\delta = \tan^{-1} \left(G'' / G' \right)$ values at various frequencies were measured.

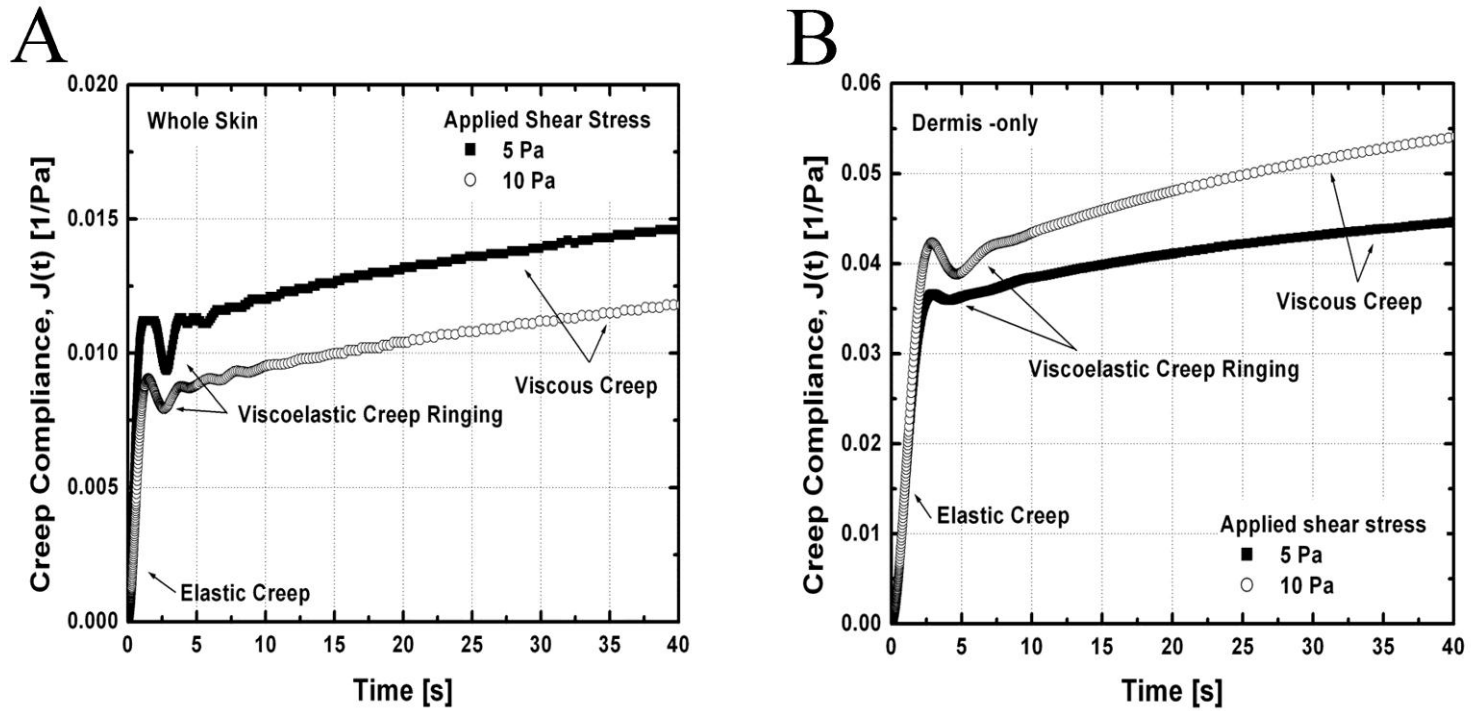


Figure 2.4: Creep compliance as a function of time. (A) Whole skin and (B) dermis-only biopsies were subjected to isothermal (37°C) shear creep loads of 5 and 10Pa and the resulting strain and compliance data was measured in order to quantify the low magnitude shear stress creep behavior for the two types of soft-tissue

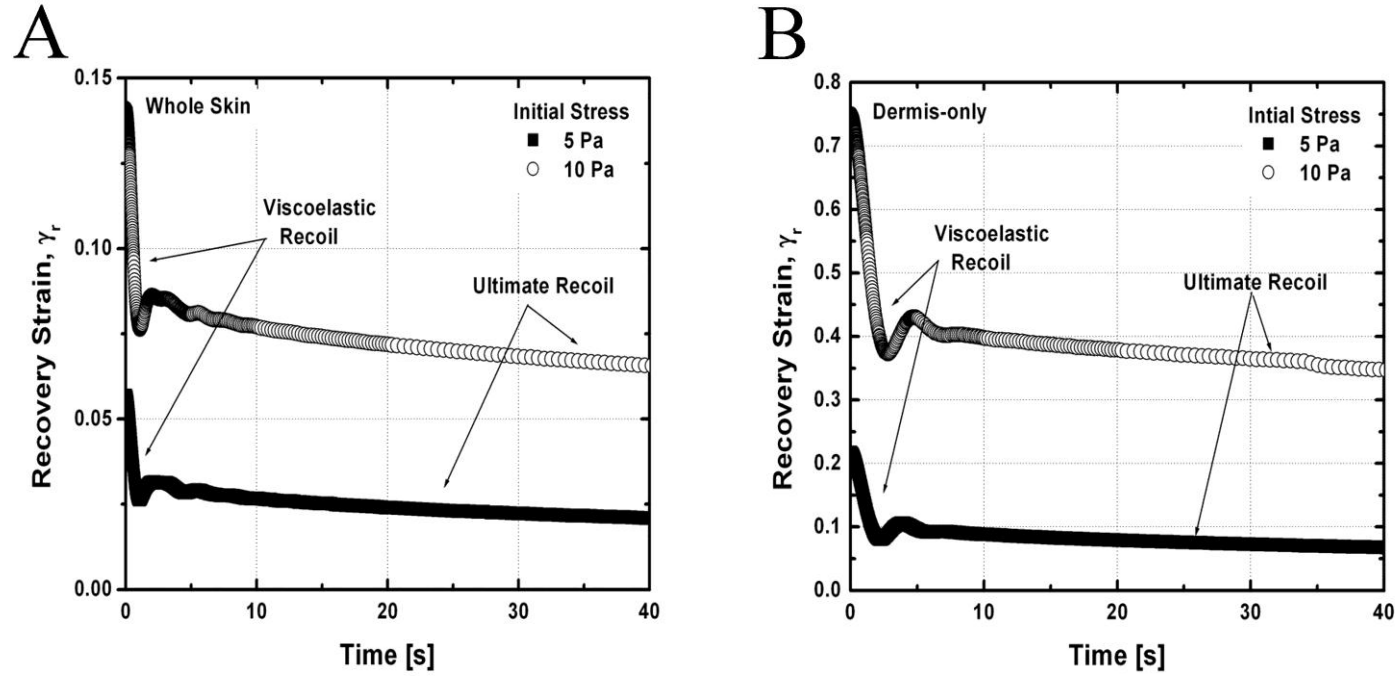


Figure 2.5: Recoil after steady flow as a function of time. Whole skin (A) and dermis-only (B) biopsies were subjected to no shear-stress and the resulting strain and compliance data was measured in order to quantify the low magnitude shear stress recovery behavior for the two types of soft-tissue

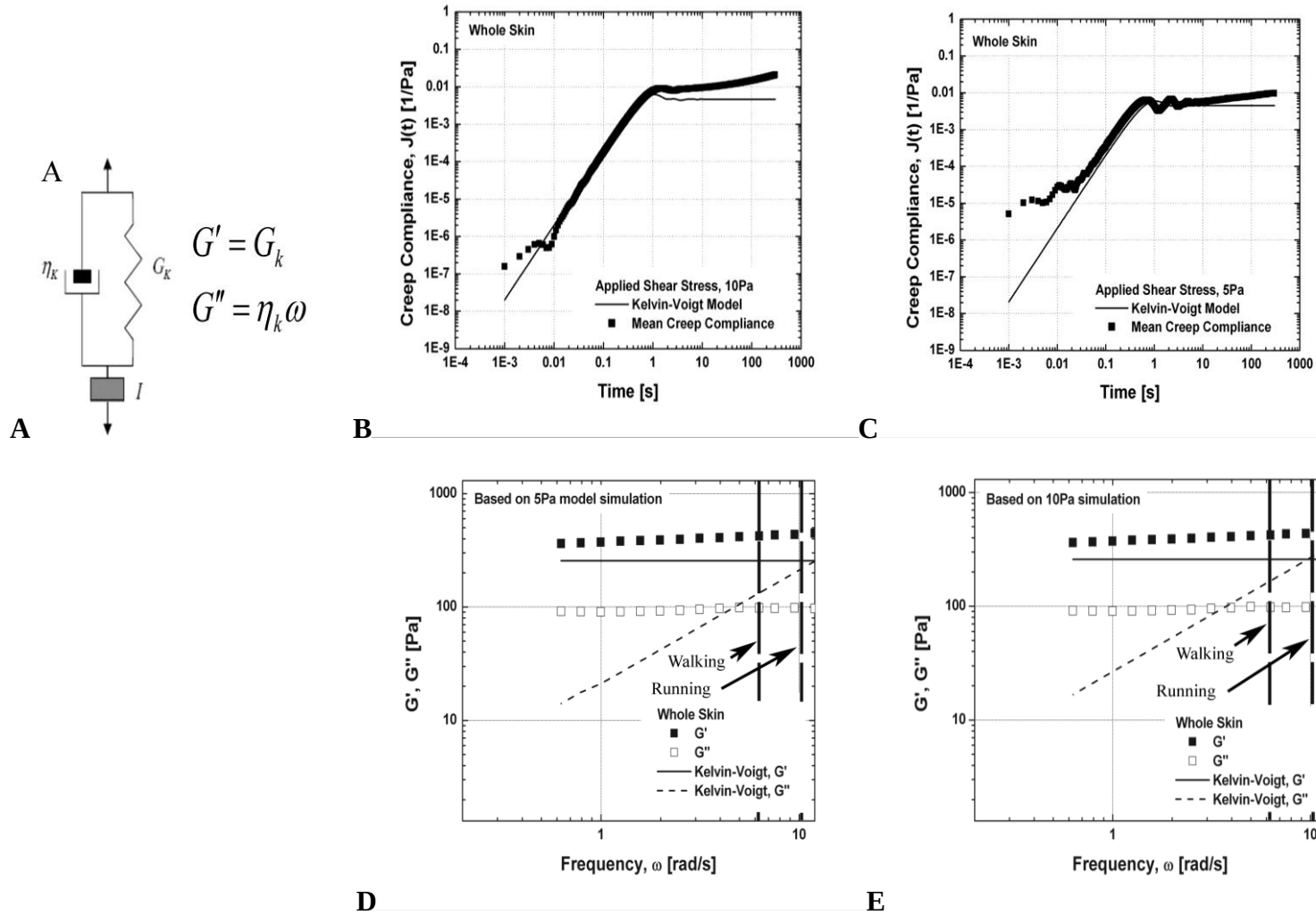


Figure 2.6: (A) Schematic of Kelvin-Voigt viscoelastic solid model. (B) Whole Skin, 10Pa Kelvin-Voigt model simulation; (C) Whole Skin, 5Pa Kelvin-Voigt model simulation; (D) 10Pa Kelvin-Voigt model fit applied to oscillatory response data; (E) 5Pa Kelvin-Voigt model fit applied to

oscillatory response data These models were intended to mimic the inertio-elastic oscillations observed during the creep experiments in order to determine the G' and G'' values associated with the two soft-tissue's shear creep response. Parameters were optimized using an iterative simulation process, allowing for the back-calculation of the respective G' and G'' values

Chapter 3:

A Physiologically Relevant Rheological Characterization-based Approach for the Development of Biomaterials that Mimic the Viscoelastic Response of Soft Tissue

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Abstract

The adaptability and inherent hydrophilicity of poly(2-hydroxyethyl methacrylate) [pHEMA] make this polymer ideal for many soft-tissue related applications. In this paper, rheological characterization was utilized to determine the optimal monomer to diluent ratio [HEMA : NaCl] and ethylene glycol dimethacrylate [EGDMA] cross-linker concentration necessary to develop micro-porous pHEMA substrates capable of reproducing the viscoelastic behavior of human skin. Using a stress-controlled rheometer, physiologically relevant, isothermal (37°C) oscillatory strain experiments between 0.01 and 3 % strain, oscillatory stress experiments between 1 – 100 Pa, frequency response experiments between 0.628 and 75.4 rad/s [0.01-12Hz] at 0.1% strain, and temperature-dependent frequency response experiments between 20 and 50°C were conducted. Independent of substrate composition, micro-porous pHEMA substrates were predominately elastic, possessed a narrow range of G' and G'' values, and exhibited stable linear viscoelastic behavior across a wide range of shear strains and stress; mimicking the response of human skin. Physiologically relevant frequency sweep experiments showed decreasing the [HEMA : NaCl] concentration effected rheological behavior. Comparative power law scaling ($G' \sim G'' \sim \omega^x$) showed 1:4 [HEMA : NaCl] ratio best replicated the viscoelastic trends of human skin. Increasing temperature also had an effect on the rheological behavior of micro-porous substrates. The effect of EGDMA concentration on rheological behavior was minimal. An improved understanding of how changes to polymer formulation affect the mechanical behavior of biomaterials within physiologically relevant load regimes will provide invaluable insights

for the design of new materials intended to address the biological and mechanical needs of host soft-tissues.

1. Introduction

Poly(2-hydroxyethyl methacrylate) [pHEMA]-based biomaterials have shown efficacy in many soft-tissue and hard tissue applications [Slaughter et al., 2009]. More specifically, porous pHEMA substrates have been utilized in the interrogation of the soft-tissue to device interface, a challenge that plagues the majority of cutaneous wound healing and percutaneous devices [Sathian et al., 2003; Knowles et al., 2005; Fukano et al., 2006; Cabodi et al., 2007; Isenhath et al., 2007]. The use of phase separation and phase emulsions has been shown to be an effective means of controlling pore size and interconnectivity [Liu et al., 2000; Kulygin et al., 2007]. Porosity is one of the structural parameters that has been found to significantly affect the mechanical behavior of pHEMA substrates in soft-tissue related biomaterial applications [Dziubla et al., 2001; Monleón Pradas et al., 2001; Karabanova et al., 2006; Cabodi et al., 2007; Bajpai et al., 2008]. PHEMA substrates are also known to have swelling/mass transport profiles and minimal inflammatory responses that are advantageous to soft-tissue biomaterial design [Dziubla et al., 2001; Mabileau et al., 2004; Bajpai et al., 2008]. The literature has addressed the biocompatibility and biomimetic capacity of pHEMA-based substrates however, few groups have chosen to emphasize the importance of mechanically matching host tissue beyond a cursory characterization; a characterization that rarely includes loading conditions with any physiological relevance. Consequently, the development of pHEMA-based biomaterials, motivated exclusively by mechanical characterization, represents a unique approach to the design and optimization of soft-tissue related biomaterial substrate.

In this study, we made a series of micro-porous pHEMA substrates for a rheology-based biomaterial optimization approach aimed at the development of synthetic substrates intended to mimic the dynamic viscoelastic behavior of human skin. The concentration of polymerization diluent, monomer to diluent ratio, and crosslinker concentration were varied and the elastic (G') and viscous (G'') moduli of the pHEMA substrates measured under low-magnitude shear stress and strain loads over a range of physiologically relevant frequencies and temperatures. These studies helped determine the diluent concentration threshold at which the viscoelastic response of the substrates tended toward the rheological behavior of human skin. A diluent molarity above this threshold was selected for the interrogation of the proper monomer to diluent ratio and cross-linker concentration. Across a wide range of physiologically relevant strain and stress values the substrates possessed consistent linear viscoelastic behavior. Physiologically relevant frequency and temperature sweep studies yielded the most significant insights for substrate composition and indicated the parameters most critical to a mechanically matched, soft-tissue related biomaterial. Lastly, we conducted a preliminary study that examined the micro-mechanical behavior of the substrates using atomic force microscopy to interrogate the localized mechanical landscape presented by the substrates. Again, changes in monomer to diluent ratios showed significant decreases in the recorded moduli. The macro-scale and micro-scale rheological characterization provided an improved understanding of which components of the substrate formulation were critical for controlling the mechanical properties of a biomaterial intended to mimic the viscoelastic response of soft-tissues.

2. Materials and methods

2.1. Preparation of micro-porous pHEMA substrates

To make micro-porous substrates, 1.0mL of stock solution containing 40mL of ophthalmic grade 2-hydroxyethyl methacrylate [HEMA] (Polysciences, Warrington, PA), 160mL of aqueous NaCl, ethyleneglycol dimethacrylate [EGDMA] (Polysciences, Warrington, PA), azobisisobutyronitrile [AIBN] (Aldrich Chemical, Allentown, PA) were added to a 12 well polystyrene cell culture plate (**Figure 3.1**). Once mixed, the solution was cross-linked with ultraviolet light for 60 minutes (South New England Ultra Violet Company, Branford, CT). The experimental groups were created by varying the molar concentration of aqueous NaCl (0.0 to 0.9M NaCl), the monomer to diluent ratio (1:4, 1:8, and 1:10), and the concentration of EGDMA cross-linker (18.75 and 75 mL). This technique is similar to a protocol presented in work related to soft-tissue biomaterial development and active wound dressings [Liu et al., 2000; Cabodi et al., 2007].

2.2. Rheological characterization

Small amplitude oscillatory measurements were performed using a controlled-stress rheometer (AR-2000N, TA Instruments, Newcastle, DE) with parallel plate geometry (8mm diameter) and specimen-dependent gaps [Holt et al. 2008] (**Figure 3.1**). Circular punch sections (8mm) of the substrates were placed, top side up, on waterproof sandpaper (~20 by 10mm) (Norton 320 Grit, Saint-Gobain Inc, Worcester, MA) held to the bottom plate by double sided adhesive tape (Scotch 666, 3M, St. Paul, MN) for no-slip shear [Tsubouchi, et al. 2006]. The 8mm parallel plate geometry was lowered to a gap of 1600 to 2200 μm , depending on the thickness of the sample and the maximum

value of the normal force [Holt et al. 2008]. Critical to this loading protocol was the maintenance of a consistent compressive normal force. Samples were uninhibited by boundary constraints ensuring strain transmission throughout the material. The maintenance of the no slip boundary condition was determined by monitoring the magnitude of shear stress and its repeatability [Holt, et al. 2008]. Samples were hydrated during testing with 1mL of PBS and all measurements were performed at 37°C. All data shown represents the mean of four samples taken at random, from 8 substrates with the same amount of time in storage.

2.2.1. Oscillatory stress and strain sweep experiments

Isothermal (37°) stress and strain sweeps were conducted at 1Hz (6.283 rad/s). Shear stress values ranged from 1 – 100Pa and percent (%) strain values ranged from 0.01 – 3 %. Samples were loaded from the low to high magnitude stresses and strains, collecting ten data points per decade. Data collected from the strain sweep experiments also helped determine the boundaries of the linear viscoelastic response regime for the different substrate formulations.

2.2.4. Physiologically relevant dynamic shear experiments

Oscillatory tests were conducted at strains of 0.1% over a frequency range of 0.628 to 75.398rad/s, divided into two isothermal sweeps to ease data collection; a higher range (6.283 to 75.398rad/s) and a lower range (0.628 to 6.283rad/s). Samples were loaded from high to low frequencies to maintain the optimal oscillatory torque, collecting ten data points per decade.

2.2.3 Temperature sweep experiments

Temperature sweep measurements were conducted at 0.1% strain from 1 to 10 Hz (6.283 – 62.8 rad/s) between 20 and 50°C, increasing in 10°C intervals. Test samples were hydrated at the beginning of each temperature sweep with 1mL PBS and allowed to equilibrate within each temperature interval for 2 minutes before load application.

2.2.5. Micro-mechanical characterization of porous pHEMA substrates

Force-indentation curves were collected using an atomic force microscope (MFP-3D, Asylum Research, Santa Barbara, CA). Borosilicate glass spheres (5 μm diameter) were attached to the tip of AFM cantilevers ($k \sim 0.3 \text{ N/m}$, Novascan Technologies, Inc., Ames, IA) to facilitate microscale testing. Elastic indentation curves were sampled at 5 kHz, with a force trigger of $\sim 5 \text{ nN}$ prescribing the point at which the cantilever approach was stopped and then retracted. Indentation tests (100 curves/region, 15 $\mu\text{m/s}$ indentation velocity) were applied over three, 50x50 μm regions for each sample. Elastic moduli, E , were determined by fitting a modified Hertz model to force-indentation curves as described previously [Darling et al, 2008]. For data analysis, the Poisson's ratio of pHEMA samples was assumed to be 0.5.

3. Results and discussion

3.1. Increasing aqueous NaCl diluent concentration decreases viscoelasticity of pHEMA substrates

To determine the effect of aqueous NaCl concentration on the viscoelastic response of the pHEMA substrates, constant strain (0.1%) was applied to micro-porous pHEMA substrates containing 0.6, 0.7, 0.8, and 0.9M aqueous NaCl over frequencies from 0.628 to 75.40 rad/s, a physiologically relevant range (Data not shown here). From this data G_o , the equilibrium shear modulus, values for each condition was calculated and then compared (**Figure 3.2**). Increasing the molarity of aqueous NaCl, in a constant monomer to diluent ratio formulation showed a pronounced decrease in G_o values. The decrease in G_o with an increase in the molarity of aqueous NaCl indicated the existence of a threshold for the effect of NaCl concentration on the viscoelastic capacity of the substrates. Above 0.9M NaCl, photopolymerization was impeded by a yet to be determined mechanism. Between 0.6 and 0.9M aqueous NaCl, micro-porous pHEMA substrates with constant monomer to diluent ratios possessed G_o values similar to other material properties reported for whole human skin [Edwards et al., 1995].

3.2 PHEMA substrates possess stable viscoelastic response at low magnitude oscillatory stresses and strains

To determine the effect of monomer to diluent ratios and cross-linker concentrations on the viscoelastic response of micro-porous pHEMA substrates across a range of physiologically relevant dynamic shear stresses and strains, micro-porous substrates were loaded with shear strains ranging from 0.01 – 3% and shear stresses ranging from 1 – 100 Pa at 1Hz (6.283 rad/s) at 37°C. Micro-porous pHEMA substrate experimental groups included 1:4, 1:8, and 1:10 monomer to diluent [HEMA : NaCl] ratios with constant EGDMA cross-linker concentrations, and substrates with 0.001875 and 0.0075 mL of

EGDMA/mL of HEMA with the same monomer to diluent ratio [1:4]. Other cross-linker concentrations above and below the two discussed here were attempted but failed to yield complete substrates under the current photo-polymerization protocol. Micro-porous pHEMA substrates across all three monomer to diluent ratios showed a significant degree of linear viscoelastic response across the entirety of the strain regime (**Figure 3.3**). Additionally, all three monomer to diluent ratios showed similar magnitudes of elastic response under the given strain regime. Similar responses have also been seen in other soft-tissue related biomaterials [Aamer et al, 2004; Geerligs et al., 2008]. Although not shown here, similar trends are seen in the viscous component of the substrates' viscoelastic response. Previous characterization of human skin showed nonlinearity at % strains greater than 0.1 [Holt et al., 2008]. On a comparative basis then micro-porous pHEMA substrates are capable of sustaining predictable viscoelastic behavior across physiologically relevant strains well beyond that of the soft-tissue they are intended to mimic.

The linear viscoelastic behaviors exhibited by the substrates in response to increasing % strain are not seen with increasing physiologically relevant shear stresses (**Figure 3.3**). The viscoelastic behavior of 1:4, 1:8, and 1:10 [HEMA : NaCl] ratio micro-porous pHEMA substrates became non-linear at oscillatory shear stresses great than 10Pa. These data indicated a shared mechanism of viscoelastic behavior that may be related to polymer chain density and/or stretching within the substrates, however the mechanism was not interrogated further in this study. Similarities in elastic moduli magnitudes of the 1:8 and 1:10 HEMA : NaCl ratio substrates across the applied stress regime indicated a

threshold for monomer to diluent ratio as it relates to the viscoelastic response of the substrates. Similar trends were seen in physiologically relevant shear strain and stress behavior of micro-porous substrates with differing concentrations of EGDMA (**Figure 3.3**). Note, substrates with increasing cross-linker concentration show a lesser degree of non-linearity at shear loads greater than 10Pa. The viscous components of the experimental groups showed increases in magnitude with increasing shear stress.

3.2. Increasing monomer to diluent ratios produces pHEMA substrates that mimic human skin.

To determine the effect of HEMA : NaCl ratio and cross-linker concentration on the development of a biomaterial mechanically matched to human skin, rheological measurements of micro-porous pHEMA substrates under physiologically relevant dynamic shear loads were conducted (**Figure 3.5**). A decrease in monomer to diluent ratio had a profound effect on the viscoelastic response of the micro-porous pHEMA substrates, and resulted in nearly a 1^o magnitude decrease in G' and G'' across the entire frequency range. The reduction was most evident when between 1:4 and 1:8 [HEMA : NaCl] ratio substrates. When 1:8 and 1:10 [HEMA : NaCl] substrates were compared there was no significant difference between their rheological behavior. In the high frequency regime ($10 < \omega \leq 72.4$ rad/s), all the micro-porous substrates showed some degree of frequency dependence, due in part to fluid flow within the intra-pore network of the substrates. The physiologically relevant rheological response of the substrates indicated the concentration of cross-linker had a negligible effect on the viscoelastic behavior of micro-porous pHEMA substrates (**Figure 3.5**). These observations indicate

that under physiologically relevant dynamic shear loads the variance of monomer to diluent ratio had the most pronounced effect on the rheological response of micro-porous substrates.

Micro-porous substrates with 1:4, 1:8, and 1:10 HEMA : NaCl ratios showed similar trends in G' and G'' . Despite differences in magnitude of the elastic and viscous moduli, at low frequencies, $\omega \leq 10 \text{ rad/s}$, micro-porous substrates with 1:4 [HEMA : NaCl] ratios showed the slowest rate of increase in elastic and viscous response ($G' \sim \omega^{0.04}$ and $G'' \sim \omega^{0.03}$) when compared to other monomer to diluent ratios ($G' \sim \omega^{0.07}$, $\omega^{0.09}$ and $G'' \sim \omega^{0.26}$, $\omega^{0.19}$, respectively). In human skin at low frequencies ($\leq 10 \text{ rad/s}$), the rate of increase for G' and G'' was gradual ($G' \sim G'' \sim \omega^{0.05}$) [Holt et al., 2008]. Comparing human skin to the micro-porous substrates indicated the 1:4 [HEMA : NaCl] monomer to diluent ratio created substrates that best replicated the viscoelastic trends seen in human skin. At higher frequencies ($\omega > 10 \text{ rad/s}$), the rate of increase in G' and G'' of all micro-porous substrates increased ($G' \sim G'' \sim \omega^{0.40}$), but the elastic response of substrates with 1:4 HEMA : NaCl ratio maintained a more gradual increase ($G' \sim \omega^{0.1}$) when compared to the other monomer to diluent ratio substrates. These observations indicated that decreasing the monomer to diluent ratio improved the ability of the substrates to mimic the magnitude of the viscoelastic response of human skin, but substrates with higher monomer to diluent ratios are more capable of reproducing the frequency independence observed in the rheological behavior of human skin. The development and inclusion of an alternative means for matching the magnitudes of the G' and G'' in higher monomer to diluent ratio pHEMA substrates would produce the optimal formulation.

3.2. PHEMA substrates with differing monomer to diluent ratios show temperature-dependent viscoelastic behavior.

To determine the effect of temperature on the rheological behavior of micro-porous pHEMA substrates intended to mechanically match human skin with differing [HEMA : NaCl] ratios and EGDMA concentrations, physiologically relevant rheological measurements were conducted at temperatures between 20 and 50°C (**Figure 3.6**). Within the experimental temperature range the magnitudes of G' within experimental groups were very similar, however the magnitudes of G'' varied more significantly. To further interrogate the intra- and inter-group temperature dependent differences in G' and G'' , the data was examined at 1Hz (6.283 rad/s), roughly the median of the physiologically relevant frequency range. Intergroup comparison better illustrated the differences in magnitudes and rate of change of rheological response (**Figure 3.7**). The influence of temperature on the rheological behavior of micro-porous pHEMA substrates increases with decreasing monomer to diluent ratio. Micro-porous pHEMA substrates with 1:4 HEMA : NaCl ratios showed the least temperature dependent reduction in mean viscoelastic response ($G' \sim t^{0.12}$ and $G'' \sim t^{0.40}$) when compared to the 1:8 and 1:10 HEMA : NaCl ratio substrates ($G' \sim t^{0.04}, t^{0.12}$ and $G'' \sim t^{0.24}, t^{0.25}$, respectively). The effect of temperature on the viscoelastic response of micro-porous pHEMA substrates with differing cross-linker concentrations was negligible when compared to the aforementioned observations (**Figure 3.6**). Note, the temperature range examined here is below the known glass transition temperature, T_g for homopolymer, pHEMA-based substrates [Çaykara et al., 2003].

The effect of temperature on micro-porous substrates with differing monomer to diluent ratios is illustrated further by the examination of the $\tan \delta$, the ratio of G''/G' (**Figure 3.6**). $\tan \delta$ is a common means of comparison between viscoelastic materials and an indicator of the viscoelastic capacity of a given material. All the micro-porous pHEMA substrates show a temperature dependent decrease in $\tan \delta$ values across the entire frequency range. Additionally, $\tan \delta$ frequency dependence was shown to increase with decreasing monomer to diluent ratios (**Figure 3.6**). Within each temperature interval, decreases in monomer to diluent ratio and increases in frequency lead to more fluid-like behavior. When compared over the entire temperature range, increases in temperature lead to more solid-like behavior. These data indicated that higher monomer to diluent ratio micro-porous substrates become more rheologically stable (i.e. show less frequency dependence) between 20 and 50°C (**Figure 3.6**). However, when comparing the responses within the narrower physiologically and clinically relevant temperature ranges between 30 and 40°C, the difference in mean rheological behavior between the substrate types is reduced.

3.3 Micro-mechanical characterization provides insights for cell-substrate interactions

To better understand the micro-mechanical environment presented by the micro-porous pHEMA substrates with differing monomer to diluent ratios, a preliminary atomic force microscope based force-indentation mechanical characterization was conducted (**Figure 3.8**). Decreasing monomer to diluent concentration had a significant effect on the micro-scale mechanical properties of micro-porous pHEMA substrates. A greater than 7-fold

decrease in elastic modulus, E was detected between 1:4 and 1:8 [HEMA : NaCl] ratio substrates. The micro-mechanical characterization further supported the importance of monomer to diluent ratios in the mechanics-based development of biomaterials intended for skin-related applications.

There is an inherent AFM cantilever probe size to substrate grain/pore size limitation for characterizations on this size scale. Further micro-mechanical characterization should utilize a dynamic texture analyzer to avoid any cantilever probe size related complications. Although, the load sensitivity of texture analyzers lack the acuity of AFM-based studies, the probe size may be more appropriate for a substrate with the grain-size and pore interconnectivity implicit to micro-porous pHEMA substrates. The probe-size to pore-size limitation prevented the examination of 1:10 [HEMA : NaCl] substrates. Additionally, an improved technique for the polymerization of thin membranes would ensure smoother surfaces for testing and increase the clarity of the results. Describing the micro-mechanical behavior of pHEMA substrates on the cellular level is non-trivial [Kaufman et al., 2008]. Further studies examining the micro-mechanical behavior of human skin must be completed before more analytical, comparative conclusions can be made about the role of micro-mechanical characterization in a rheology-based biomaterial design process. Micro-mechanical characterizations were not conducted on micro-porous pHEMA substrates with differing EGDMA concentrations.

4. Conclusions

Understanding the rheological implications of changes to biomaterial formulation is critical to achieving success in the development of biomaterials for soft-tissue applications. The viscoelasticity of micro-porous pHEMA substrates were predominately elastic in nature and showed trends in rheological behavior similar to skin. At low magnitude shear strains, micro-porous substrates showed distinctly linear viscoelastic behavior, independent of monomer to diluent ratio and/or EGDMA concentration. Under physiologically relevant dynamic shear loads (1 – 100 Pa), all substrates showed non-linear viscoelastic behavior at loads ≤ 10 Pa. At low frequencies ($\omega \leq 10$ rad/s), micro-porous substrates with 1:4, 1:8, and 1:10 HEMA : NaCl ratios showed very similar trends in viscoelastic behavior differing only in magnitude ($G' \sim \omega^{0.06}$ and $G'' \sim \omega^{0.25}$). Neglecting differences in magnitude, the trends were similar to those seen in skin ($G' \sim G'' \sim \omega^{0.5-1.0}$). At high frequencies ($10 < \omega \leq 72.4$ rad/s), despite differences in monomer to diluent ratio and EGDMA concentration, the viscoelastic behavior of micro-porous substrates showed similar degrees of frequency dependence ($G' \sim \omega^{0.31}$ and $G'' \sim \omega^{0.42}$). Only substrates with 1:4 [HEMA : NaCl] ratios maintained the trends seen at low frequencies, ($G' \sim \omega^{0.10}$). Examining the viscoelastic response of micro-porous substrates under physiologically relevant dynamic shear loads at different temperatures showed that a reduction in monomer to diluent ratio made substrates behavior more solid-like with increasing temperature. Substrates with 1:4 [HEMA : NaCl] ratios showed more stable rheological behavior when compared to trends seen in other monomer to diluent ratio substrates. This was supported by an examination of the viscoelastic frequency sweep behavior at 1 Hz (6.283 rad/s) and an analysis of $\tan \delta$ values of the individual substrates.

Temperature, it was determined had very little effect of substrates with differing EGDMA concentrations under these loading conditions. Lastly, we examined the effect of varying monomer to diluent ratio on the micro-mechanical behavior of the substrates and found a nearly 7-fold decrease in the mechanical response of the substrates with decreases in monomer to diluent ratio.

Soft-tissues are complex, highly dynamic biological and mechanical systems. The use of mechanical characterization data to develop synthetic substrates capable of capturing that dynamism is non-trivial. Although the micro-porous pHEMA substrates developed here replicate the trends seen in human skin, to improve the accuracy of the biomimetic mechanical capacity of the substrates an additional means of effecting mechanical response must be developed and investigated. Previous work with pHEMA varied the cross-linking agent, namely divinyl benzene [DVB], ethylene glycol dimethacrylate [EGDMA], tetraethylene glycol diacrylate [TEGDA], and polyethylene glycol diacrylate [PEGDA], and showed the intrinsic length of the cross-linking agent has a significant effect on the mechanical response of the resultant substrates [Mabilleau et al., 2006]. With regards to micro-porous substrates then, the type of cross-linker may have a more profound effect on rheological behavior than simply changing cross-linker concentration. Dynamic shear experiments conducted at 0.05 shear strain from 0.01 – 10 Hz at 16°C and oscillatory shear temperature sweep experiments between 15 and 50°C at 1Hz performed on methacrylamide modified gelatin showed pronounced temperature-, matrix substitution- and cross-linking initiator dependent rheological behavior based on changes to the physical structure of the material [Van Den Bulcke et al., 2000]. The study by Van

Den Bulcke et al. highlights the importance of cross-linking initiator, a parameter which may prove crucial to any subsequent rheological characterization of micro-porous pHEMA substrates. Note, the rheology-based optimization approach utilized here relied on porosity adaptation, in order to advance this approach an additional means of pore initiation, beyond phase separation, must be developed and utilized.

Although the ultimate objective of this study, the development of a biomaterial that mechanically matched human skin under small amplitude, physiologically relevant loads, was not achieved the reader should note the investigation of other soft-tissue related biomaterials has yielded similar rheological results. When exposed to small magnitude strain, oscillatory shear experiments between 0.01 - 10 Hz at 25, 37, and 50°C and strain and shear sweep experiments triblock copolymers of poly (L-lactic acid) [PLLA] and poly (ethylene oxide) [PEO] intended for soft-tissue applications have shown similar trends and magnitudes in G' and G'' ; as well as similar degrees of linear viscoelastic behavior across a wide range of shear strains and stresses [Aamer et al., 2004]. Additionally, the use of small strain oscillatory rheological experiments between 0.01 and 10 Hz at 34°C on porous gelatin hydrogel scaffolds intended for corneal cell transplantation has shown gelatin concentration dependent viscoelastic responses that are similar to the rheological behavior presented here [Lai et al., 2010].

The aim of this study was to use rheology as the quantitative basis for the optimization of a novel, porous pHEMA-based substrate intended to approximate the mechanical behavior of human skin. Our rheological measurements show that pHEMA substrates are

predominately elastic in nature with a narrow range of G' and G'' values that mimic the response of human skin. Furthermore, our measurements demonstrate that within physiologically relevant loading and temperature conditions, micro-porous pHEMA substrate formulations possess relatively stable rheological behavior. Additionally, the rheological optimization of micro-porous pHEMA substrates highlighted monomer to diluent ratio as a key design parameter in matching the mechanical response of synthetics to biological tissue. More specifically, that changing the monomer to diluent ratios has a profound effect on the bulk and micro-scale viscoelastic response of the substrates. At a micro-mechanical level this was further reinforced, revealing an additional obstacle commonly encountered during any mechanics related biomaterial design process: design for the cell-substrate interaction; or design for the tissue-substrate interaction. The ideal biomaterial would account for both interactions. An improved quantitative understanding of the mechanics of synthetic materials and how the viscoelastic behavior of those substrates may be tailored to match a specific soft-tissue provides a very powerful tool for the development of more advanced biomaterials that address the cell and tissue response. The design insights provided by this study will aid in the development of new materials that will utilize mechanics as the foundation for therapeutic improvement or enhancement of damage or diseased soft-tissues.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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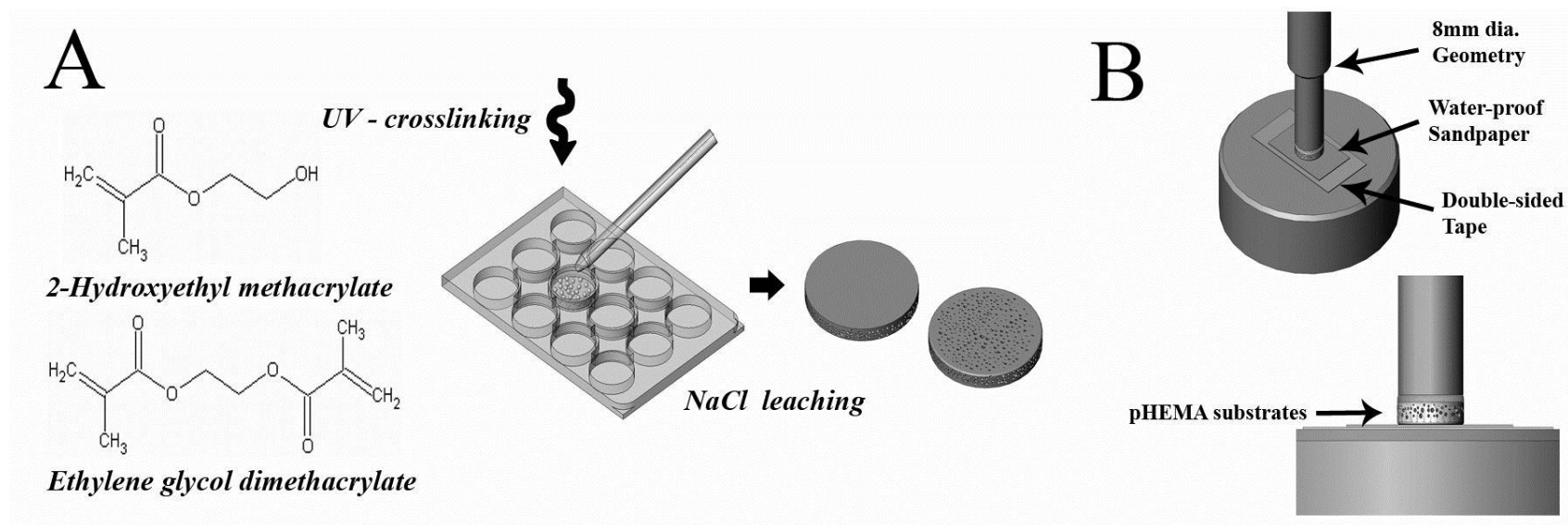


Figure 3.1: Preparation and rheological measurements of porous pHEMA substrates. **(A)** Micro-porous pHEMA substrates were polymerized by UV-cross linking using NaCl as a diluent and EGDMA as a cross-linker. **(B)** Micro-porous substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% between 0.6283 and 75.398 rad/s, a physiologically relevant frequency range. The resulting G' and G'' values at various frequencies were measured

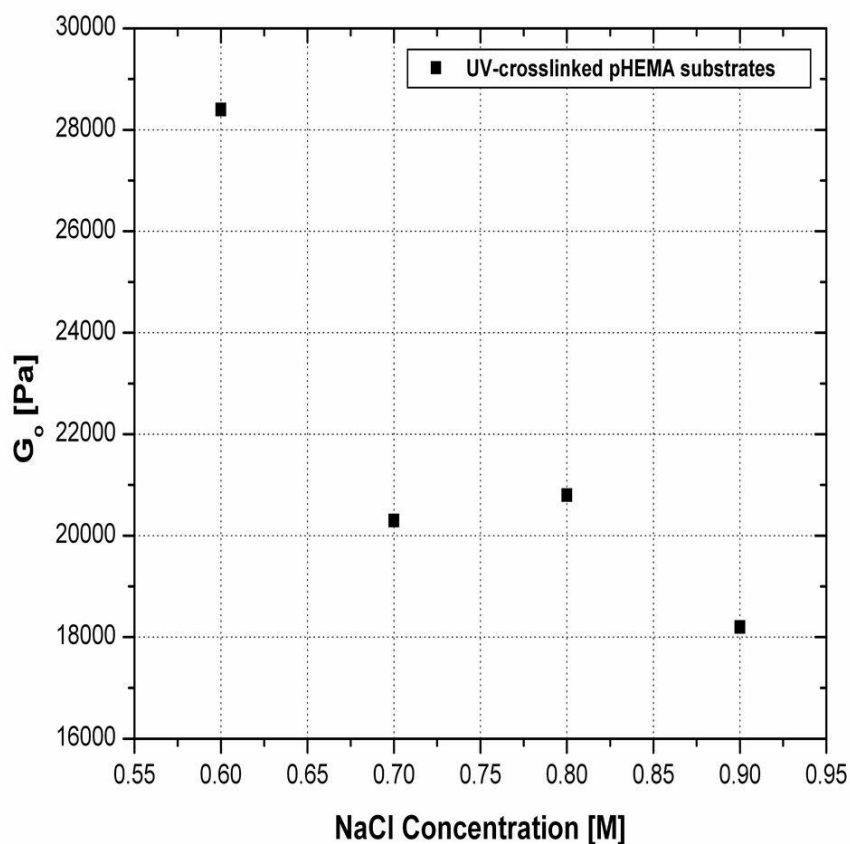


Figure 3.2: Determination of optimal NaCl concentration in micro-porous pHEMA substrates intended to mimic human skin. Rheological characterization was conducted on micro-porous pHEMA substrates with ethylene glycol dimethacrylate cross-linker were photo-polymerized with ultraviolet light containing 0.6, 0.7, 0.8, and 0.9M aqueous NaCl diluent. The equilibrium modulus, G° , values were compared in order to determine a viscoelastic behavior related threshold for diluent molarity. Substrates with greater than 0.9M NaCl did not successfully polymerize under the given protocol

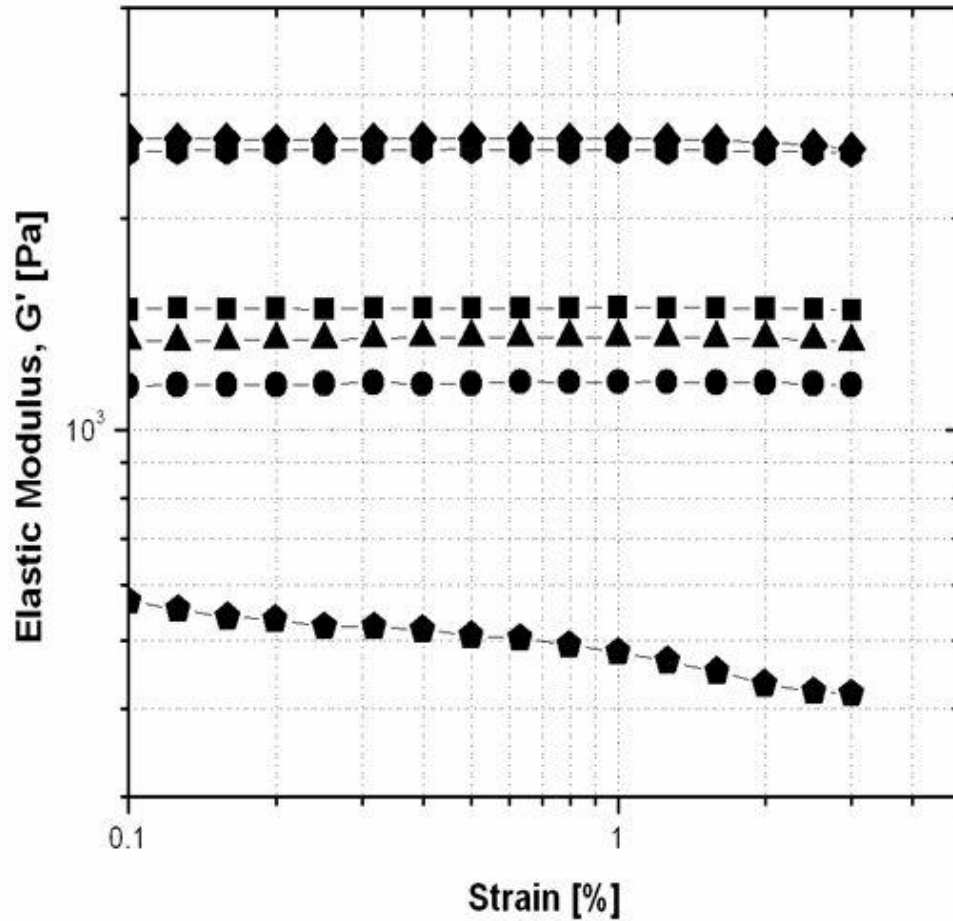


Figure 3.3: Mean strain sweep experiments of micro-porous substrates. pHEMA substrates ($n=6$) were subjected to isothermal (37°C) oscillatory strain sweep experiments between 0.01 and 3 % strain at 1 Hz (6.283 rad/s). Micro-porous substrates had various monomer to diluent ratios: 1:4 (■); 1:8 (●); and 1:10 (▲); as well as various EGDMA cross-linker concentrations: 0.0001875 (◆) and 0.0075 (●). Independent of monomer to diluent ratio and cross-linker concentration, pHEMA substrates show linear viscoelastic behavior across wide range of physiologically relevant strains. Whole thickness human skin is also shown for reference (◆).

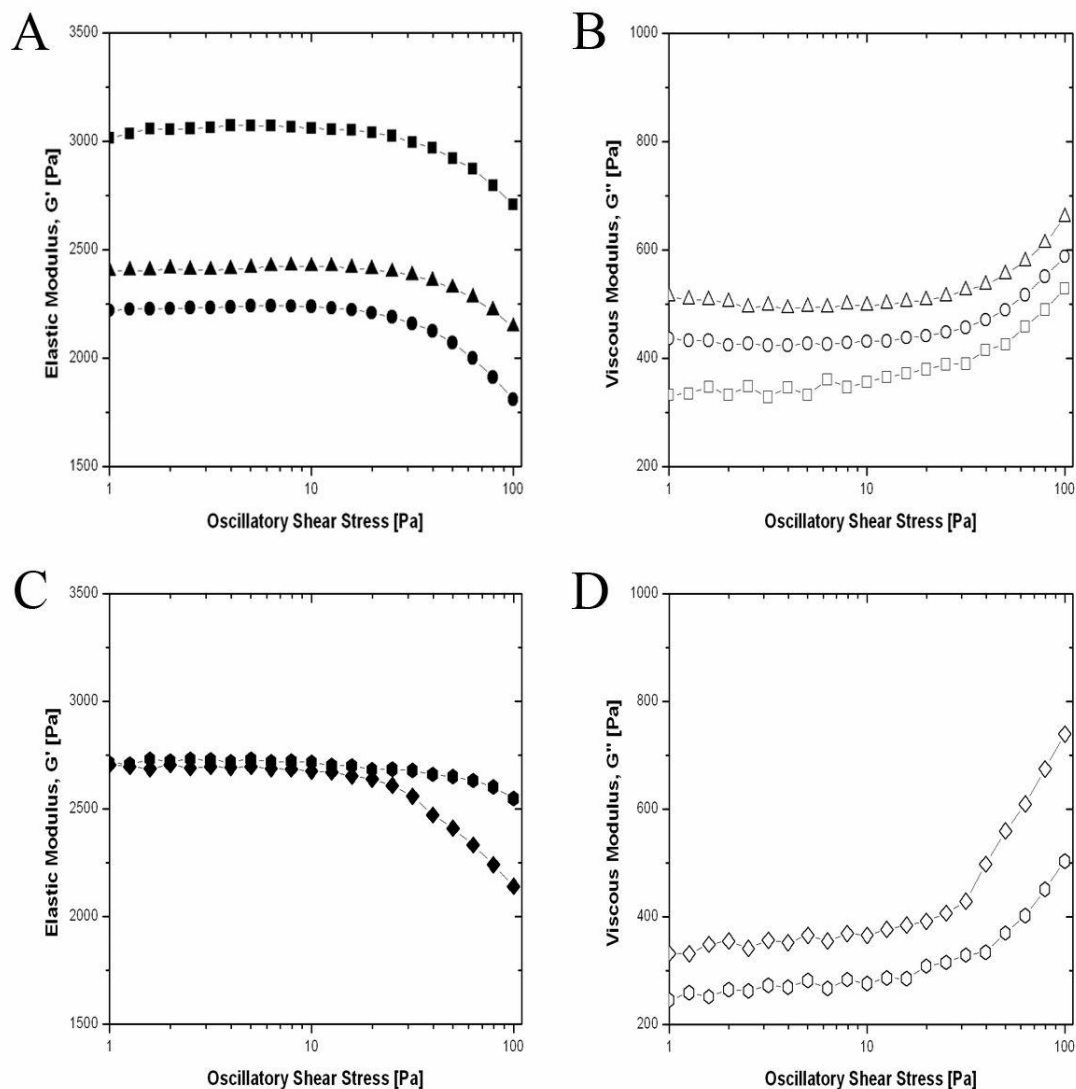


Figure 3.4: Mean oscillatory shear stress response of micro-porous pHEMA substrates. Micro-porous pHEMA substrates ($n=6$) were subjected to isothermal (37°C) oscillatory stress sweep experiments between 1 and 100 Pa at 1 Hz (6.283 rad/s) and the resulting G' and G'' values measured for comparison [*closed and open symbols, respectively*]. Despite differences in magnitude, substrates with 1:4 (■), 1:8 (●), and 1:10 (▲) monomer to diluent ratios showed similar degrees of linear viscoelastic behavior, becoming non-linear at loads ≤ 10 Pa. Substrates with 0.001875 (◆) and 0.0075 (●) EGDMA concentrations showed similar behavior

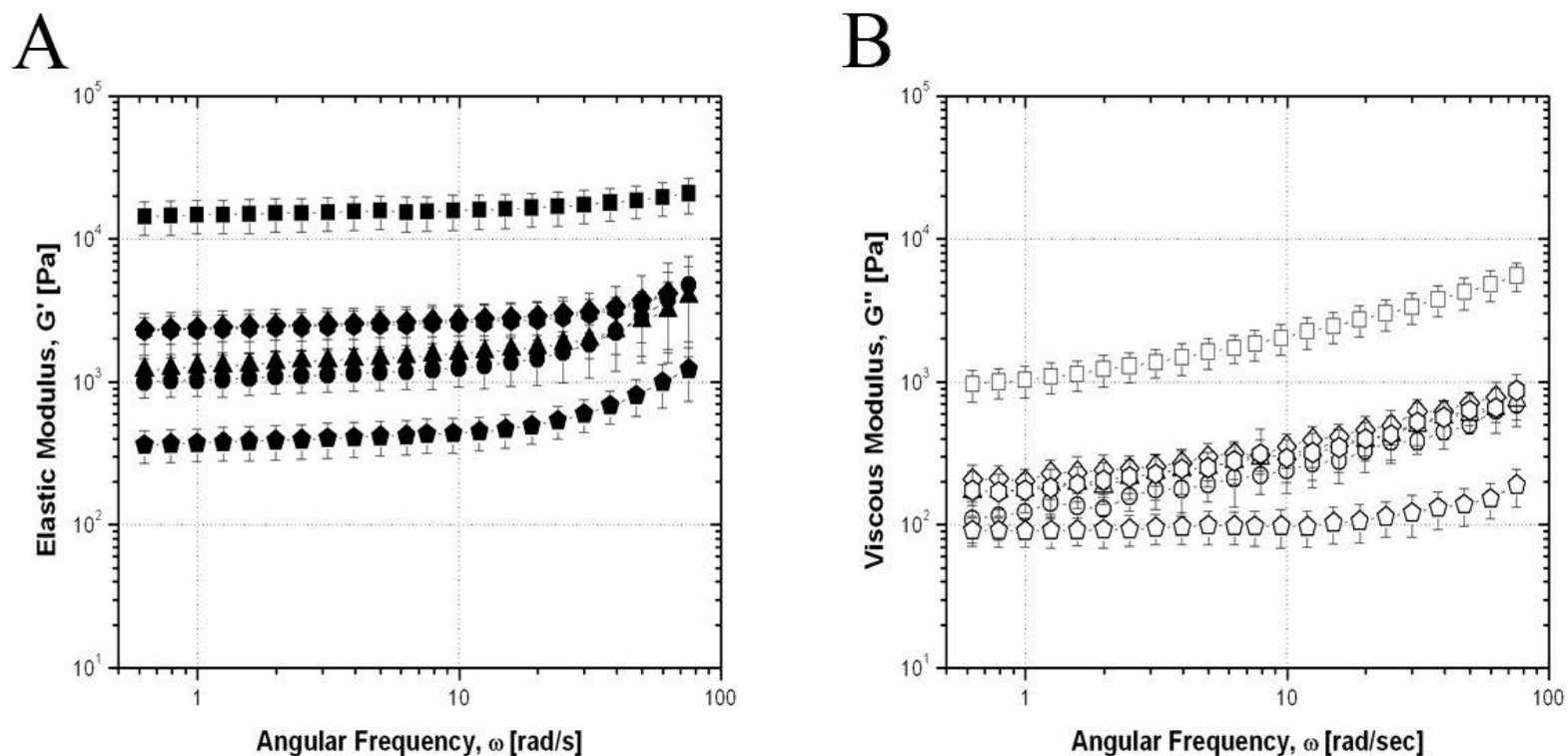


Figure 3.5: Physiologically relevant rheological characterization of micro-porous pHEMA substrates. Micro-porous substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% and the resulting G' (A) and G'' (B) values were measured [*closed and open symbols, respectively*]. pHEMA substrates with 1:4 (■), 1:8 (●), and 1:10 (▲) monomer to diluent ratios were examined between 0.1 and 12 Hz (0.6283 – 72.4 rad/s). Identical measurements were taken on pHEMA substrates with 0.001875 (◆) and 0.0075 (●) EGDMA concentrations. Whole thickness human skin is also shown for reference (◆)

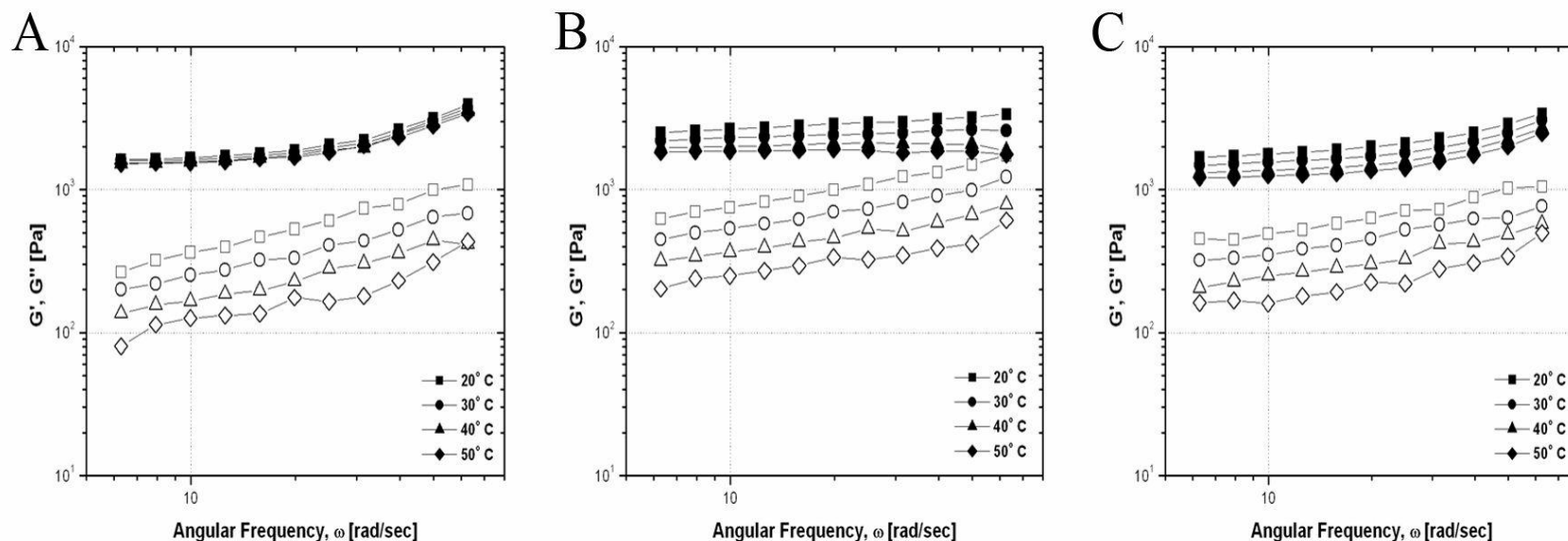


Figure 3.6: Mean temperature dependent rheological characterization of micro-porous pHEMA substrates. pHEMA substrates ($n=6$) were subjected to oscillatory strains of 0.1 % between 1 - 10 Hz (6.283 – 62.83 rad/s) from 20 - 50°C at 10°C intervals; the resulting G' (closed) and G'' (open) values were measured. The viscoelastic response of micro-porous pHEMA substrates with 1:4 (A), 1:8 (B), and 1:10 (C) monomer to diluent ratios were compared as were their corresponding $\tan \delta$ values (D, E, and F, respectively) [NEXT PAGE]

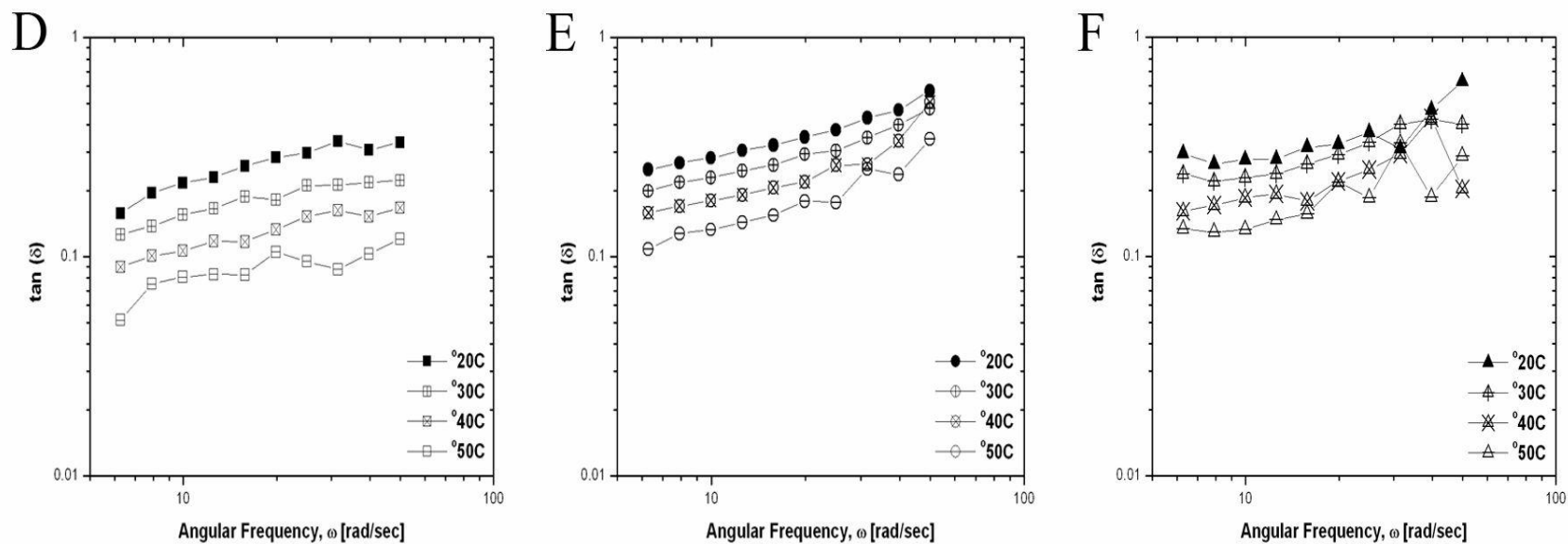


Figure 3.6: Mean temperature dependent rheological characterization of micro-porous pHEMA substrates. pHEMA substrates ($n=6$) were subjected to oscillatory strains of 0.1 % between 1 - 10 Hz (6.283 – 62.83 rad/s) from 20 - 50°C at 10°C intervals; the resulting G' (closed) and G'' (open) values were measured. The viscoelastic response of micro-porous pHEMA substrates with 1:4 (**A**), 1:8 (**B**), and 1:10 (**C**) monomer to diluent ratios were compared as were their corresponding $\tan \delta$ values (**D**, **E**, and **F**, respectively)

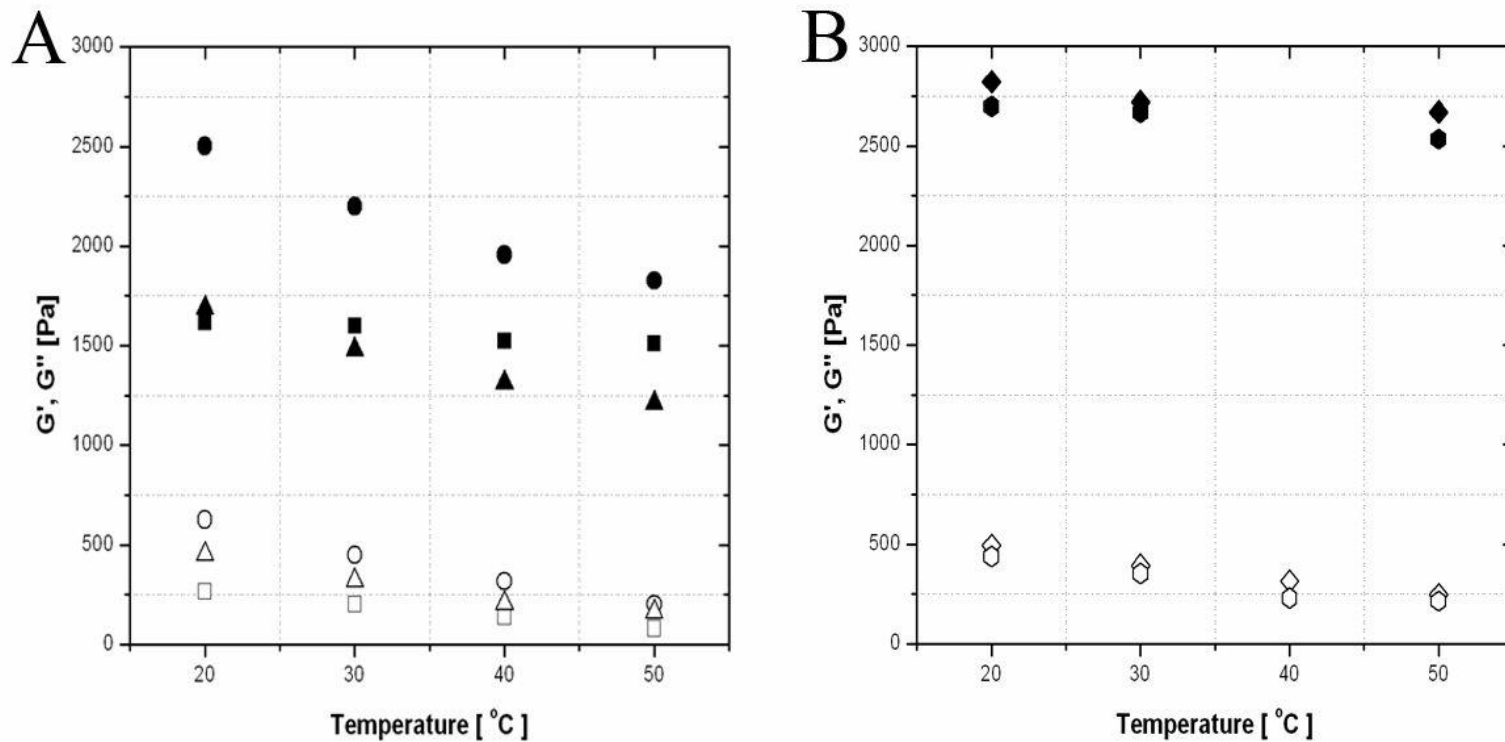


Figure 3.7: Iso-frequency comparison of mean temperature dependent rheological characterization of micro-porous pHEMA substrates. To further emphasize the effect of temperature on the viscoelastic response of micro-porous pHEMA substrates [$n=6$], G' (closed) and G'' (open) values from substrates with differing monomer to diluent (aqueous NaCl) ratios (A) and EGDMA concentrations (B) were compared at 1 Hz (6.283 rad/s). Monomer to diluent ratios of 1:4 (■), 1:8 (●), and 1:10 (▲) showed clear trends with increasing temperature. However, the effect of temperature on the viscoelastic response of substrates with 0.001875 (◆) and 0.0075 (●) EGDMA concentrations proved to be negligible

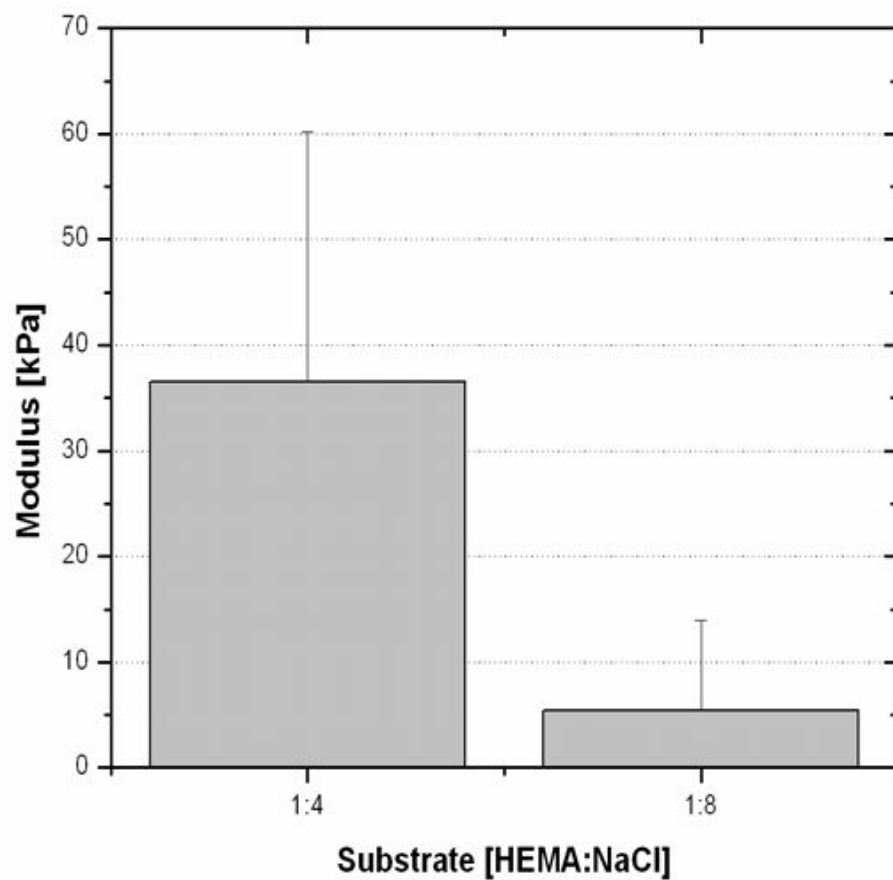


Figure 3.8: Micro-mechanical characterization of micro-porous pHEMA substrates with differing monomer to diluent ratios. Decreasing monomer to diluent ratio has a significant effect on mechanical behavior of micro-porous pHEMA substrates.

Chapter 4:

Designing PolyHEMA Substrates that Mimic the Viscoelastic Response of Soft Tissue

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Abstract

Matching the mechanical properties of a biomaterial to soft tissue is often overlooked despite the fact that it's well known that cells respond to and are capable of changing their mechanical environment. In this paper, we used NaCl and alginate beads as porogens to make a series of micro- and macro-porous pHEMA substrates [poly(2-hydroxyethyl methacrylate)] and quantified their mechanical behavior under low-magnitude shear loads over physiologically relevant frequencies. Using a stress-controlled rheometer, we performed isothermal (37°C) frequency response experiments between 0.628 and 75.4 rad/s [0.01-12Hz] at 0.1% strain. Both micro- and macro-porous pHEMA substrates were predominately elastic in nature with a narrow range of G' and G'' values that mimicked the response of human skin. The magnitude of the G' and G'' values of the macro-porous substrates were designed to closely match human skin. To determine how cell growth might alter their mechanical properties, pHEMA substrates were functionalized and human skin fibroblasts grown on them for fourteen days. As a result of cell growth, the magnitude of G' and G'' increased at low frequencies while also altering the degree of high frequency dependence, indicating that cellular interactions with the micro-pore infrastructure has a profound effect on the viscoelastic behavior of the substrates. These data could be fit to a mathematical model describing a soft solid. A quantitative understanding of the mechanical behavior of biomaterials in regimes that are physiologically relevant and how these mechanics may change after implantation may aid in the design of new materials.

1. Introduction

The development and success of biomaterials can be distilled to three primary design criteria: physical/mechanical properties; mass transport properties; and biocompatibility/biomimetic capacity [Gheduzzi et al., 2006; Kohane et al., 2008; Tibbit et al., 2009; William et al., 2009]. The majority of biomaterial development focuses on mass transport profiles and biocompatibility/bio-mimetic capacity. These parameters are often prioritized because they represent the preliminary hurdles to restoring and/or supplementing biological functionality [Stevens et al., 2005]. Essential to all biomaterials, however, is the cell-biomaterial or cell-substrate interactions which represent a primary obstacle to integration and clinical success [Stevens et al., 2005]. Few investigators recognize the importance of the mechanical behavior of substrates beyond a cursory comparative characterization and many biological processes involve mechanics. Specifically, cutaneous wound healing relies heavily on the ability of cells to detect changes in the mechanics of their environment and the ability of cells to sense and respond to their environment is well established [Wang et al., 2003; Hoffman et al., 2009], as is the role mechanical stimuli plays in affecting cellular morphology and phenotype [Cowin et al., 2000; Hoffman et al., 2009]. Unfortunately, little effort is made to synthesize biomaterials with mechanical properties that match the tissue with which it will interact and even less is understood about how the mechanics of these biomaterials might change when cells and tissues interact with these biomaterials. Furthermore, even fewer studies have attempted to garner insights from mathematical models of the viscoelastic response of biomaterials with cells.

In this study, we made a series of micro- and macro-porous poly (2-hydroxyethyl methacrylate) [pHEMA] substrates with the intent of matching their mechanical properties to that of human skin. Despite differences in pore sizes, micro- and macro-porous substrates had similar swelling kinetics indicating that interstitial fluid flow was comparable. The elastic (G') and viscous (G'') moduli of the pHEMA substrates were measured by subjecting them to low-magnitude shear loads over a range of physiologically relevant frequencies. The introduction of macro pores caused the greatest reduction in G' and G'' and the closest approximation to human skin. However, when cells were grown on modified pHEMA substrates for 14 days, G' increased in the low frequency regime and G'' increased across the entire frequency regime when compared to unmodified substrates without cells. Additionally, cellular activity on modified substrates altered the degree of frequency dependence in the high frequency rheological response of the substrates. Lastly, we were able to fit these data to a soft-solid viscoelastic model that facilitates more quantitative comparisons between various modifications of pHEMA substrates with and without cells as well as comparisons to human skin. A quantitative understanding of the mechanical behavior of synthetic substrates before and after implantation may help to improve their performance.

2. Materials and methods

2.1. Preparation of micro- and macro-porous pHEMA substrates

To make micro-porous substrates, 1.0mL of stock solution containing 40mL of ophthalmic grade 2-hydroxyethyl methacrylate [HEMA] (Polysciences, Warrington, PA), 160mL of 0.8M NaCl, 0.450mL of ethyleneglycol dimethacrylate [EGDMA]

(Polysciences, Warrington, PA), and 0.135g of azobisisobutyronitrile [AIBN] (Aldrich Chemical, Allentown, PA) were added to a 12 well polystyrene cell culture plate (**Figure 4.1**). Once mixed, the solution was cross-linked with ultraviolet light for 60 minutes (South New England Ultra Violet Company, Branford, CT). For macro-porous substrates, dried alginate beads (as described in the Supplemental Section) were added prior to polymerization. Following polymerization, substrates were removed, cooled to room temperature and ~10mL of double de-ionized water (dH₂O) added to hydrate the sample.

2.2. Alginate bead porogen fabrication.

To make macro-porous substrates ($\geq 400\mu\text{m}$), alginate beads were used as an additional porogen. Briefly, 20mL of an 1.5% (w/v) alginic acid (Sigma Aldrich, St. Louis, MO) solution containing 0.9% (w/v) NaCl were added to a 20mL syringe and mounted in a Harvard Apparatus, Model No: 55-4140 syringe pump set at 0.1mL/min. Alginate beads were formed in a drop-wise fashion into 0.5L of 4% (w/v) BaCl₂ (Aldrich Chemical, Allentown, PA) stirred at 1100rpm. Beads were sieved and placed in fresh 4% BaCl₂ for 1.5hr at 300rpm. Finally, beads were sieved, rinsed with dH₂O for 48hrs to leach remaining BaCl₂, then dried at room temperature, and stored (**Figure 4.2**). For use as a porogen dried alginate beads were added to a 12 well plate prior to the HEMA stock solution and then polymerized using the aforementioned protocol. To leach the alginate beads, substrates were subsequently treated with ethylenediaminetetraacetic acid [EDTA] at 37°C for 24hrs changing the EDTA solution several times. After 24hrs, the substrates were gently rinsed with dH₂O, incubated at 37°C in EDTA solution for an additional 24hrs changing the EDTA solution several times, before a final dH₂O rinse.

2.3. Substrate chemical functionalization

To improve cell adhesion, pHEMA substrates were treated with 1, 1'-carbonyldiimidazole [CDI] (Sigma Aldrich) [Fukano et al, 2006]. Dried pHEMA substrates were incubated in 20mL glass vials with 30mM CDI in 1,4-dioxane at 37°C for ~2.5hrs [Fukano et al, 2006]. Following incubation, samples were rinsed several times with 1,4-dioxane and transferred to sterile phosphate buffered saline [PBS] (Mediatech, Manassas, VA) with 0.75% (w/v) gelatin type B (Sigma Aldrich), derived from bovine skin. Substrates were incubated at 37°C overnight before storage in PBS.

2.2. Cell culture

Normal human fibroblasts (NHFs) from human foreskins were cultured in NHF medium containing Dulbecco's Modified Eagle's Medium [DMEM] (Invitrogen, Grand Island, NY), 10% fetal bovine serum [FBS] (Hyclone Laboratories, Logan, UT), 1% penicillin/streptomycin [P/S] (MP Biomedicals, Irvine, CA) at 37°C in 5% CO₂. After reaching ~70% confluence, cells were trypsinized (0.05% trypsin)(Hyclone Laboratories, Logan, UT), counted and seeded (20,000 cells/well, ~5,300 cells/cm²) onto pHEMA substrates in 12 well dishes. Cell attachment and proliferation on pHEMA substrates were determined using the WST-1 cell proliferation assay. Briefly, substrates were equilibrated in NHF medium overnight and 72 hours after seeding with cells, 125µL of WST-1 reagent (Roche Diagnostics, Indianapolis, IN) was added to each well (1:10). The plate was incubated for 2hrs at 37°C in 5% CO₂ and then agitated for ~1min. The supernatant was removed and absorbance measured at 420nm with a reference of 600nm.

The student t-test was applied to determine statistically differences significant ($p < 0.001$).

2.3. Scanning Electron Microscopy (SEM)

For SEM analysis, NHF-pHEMA substrates were removed, gently rinsed and then fixed in a 1% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) solution buffered in 0.1M sodium cacodylate for 30mins. Following fixation, substrates were treated with 1% osmium tetroxide (20mins). Finally, the substrates were dehydrated in an ethanol gradient wash 5-100% in 5% increments for 10min each. The substrates were then taken through an established critical point drying protocol using liquid CO₂. The samples were sputtered coated with gold-palladium and imaged (Hitachi 2700 scanning electron microscope).

2.3. Swelling Kinetics

To measure swelling kinetics, dried (~22°C) substrates were weighed and then added to separate, 2L volumes of de-ionized water equilibrated at 37°C. Three micro- and macro-porous substrates were removed at various times over 180 minutes and weighed.

2.4. Rheological characterization

Small amplitude oscillatory measurements were performed using a controlled-stress rheometer (AR-2000N, TA Instruments, Newcastle, DE) with parallel plate geometry (8mm diameter) and specimen-dependent gaps [Holt et al. 2008] (**Figure 4.1**). Circular punch sections (8mm) of the substrates were placed, top side up, on waterproof

sandpaper (~20 by 10mm) (Norton 320 Grit, Saint-Gobain Inc, Worcester, MA) held to the bottom plate by double sided adhesive tape (Scotch 666, 3M, St. Paul, MN) for no-slip shear [Tsubouchi, et al. 2006]. The 8mm parallel plate geometry was lowered to a gap of 1600 to 2200 μm , depending on the thickness of the sample and the maximum value of the normal force [Holt et al. 2008]. Critical to this loading protocol was the maintenance of a consistent compressive normal force. Samples were uninhibited by boundary constraints ensuring strain transmission throughout the material. The maintenance of the no slip boundary condition was determined by monitoring the magnitude of shear stress and its repeatability [Holt, et al. 2008]. Protein modified and unmodified, micro- and macro-porous pHEMA substrates with and without cells were hydrated during testing with 1mL of PBS and all measurements were performed at 37°C. Oscillatory tests were conducted at strains of 0.1% over a frequency range of 0.628 to 75.398rad/s, divided into two isothermal sweeps to ease data collection; a higher range (6.283 to 75.398rad/s) and a lower range (0.628 to 6.283rad/s). Samples were loaded from high to low frequencies to maintain the optimal oscillatory torque, collecting ten data points per decade.

2.5. *Soft-solid viscoelastic model*

A standard viscoelastic solid, with relaxation modulus $[G(t)]$ and compliance $[J(t)]$, is the parallel combination of a Hookean solid (spring) and a Maxwell fluid (spring and dashpot in series). We propose the following form for a highly idealized soft solid [Pipkin et al., 1972]:

,

$$G(t) = G_e (1 - \exp(-t/\lambda))^k, \quad 0 < k \leq 1 \quad (1)$$

$$J(t) = J_e (1 - \exp(-t/\tau))^k, \quad 0 < k \leq 1 \quad (2)$$

$$G_e J_e = 1 \text{ and } G_e = G(\infty) \quad (3)$$

where G_e , J_e , λ , and τ are the equilibrium relaxation moduli, the compliance, and the relaxation and retardation times, respectively. In this form, the compliance levels off to J_e for large times, growing as $(t/\tau)^k$ for short times, and returning to standard viscoelastic solid form when $k = 1$.

With the knowledge that the complex modulus $G^*(\omega) = G' + iG''$ is $sG(s)$ in terms of $s = i\omega$, we arrive at a model that describes solids with an inherent weak internal structure that liquefies at high frequency:

$$\begin{aligned} \frac{G'(\omega)}{G_e} &= (\omega\tau)^k \{ \cos(k\pi/2) + \sin(k\pi/2)k(k+1)/(2\omega\tau) \} \Gamma(k+1) \\ \frac{G''(\omega)}{G_e} &= (\omega\tau)^k \{ \sin(k\pi/2) - \cos(k\pi/2)k(k+1)/(2\omega\tau) \} \Gamma(k+1) \end{aligned} \quad (12)$$

$$\text{where, } \Gamma(z+1) = z\Gamma(z)$$

As $k \rightarrow 1$ the above expressions become

$$G'(\omega) = G_e \quad (13)$$

$$G''(\omega) = \eta_g \omega$$

Given G' and G'' data of a given material the fit parameters are k , G_e , and τ . Note, that as $k \rightarrow 0$, $G'(\omega) = G_e$ and $G''(\omega) = G_e \omega \tau$.

3. Results and discussion

3.1. Large pores decrease viscoelasticity of pHEMA substrates

Constant strain (0.1%) was applied to micro- and macro-porous pHEMA substrates over frequencies from 0.628 to 75.40 rad/s, a physiologically relevant range (**Figure 4.3**). The addition of NaCl during polymerization of the pHEMA substrates and increasing its concentration up to 0.9M had little to no effect on either the elastic (G') or viscous (G'') moduli of these micro-porous substrates. However, the G' and G'' values of the macro-porous substrates (alginate beads as porogen) were significantly reduced by nearly a full order of magnitude and more closely matched the viscoelastic behavior of human skin (**Table 4.1**). Similar to human skin, the viscoelastic response of all the pHEMA substrates increased gradually over the frequencies tested, with the greatest increase occurring in the high frequency range ($\omega > 10$ rad/s). Moreover, the G' and G'' curves did not intersect indicating that the viscoelastic behavior of all pHEMA substrates is primarily elastic in nature as is the case for human skin.

3.2 Micro-pores dominate swelling kinetics of pHEMA substrates

Swelling experiments showed that micro- and macro-porous pHEMA substrates possessed very similar swelling kinetics (**Figure 4.4**). There was a significant degree of swelling from time $t = 0$ to 60mins during which approximately 60% of the swelling capacity of both micro- and macro-porous substrates was attained. This was supported by a similar trend in the equilibrium water content. Surprisingly, the micro-porous substrates

swelled more rapidly and possessed higher equilibrium water content when compared to the macro-porous substrates. A linear fit to the $\ln - \ln$ of the swelling factor versus time data up to 60% of equilibrium showed a similarity in the swelling exponent for the micro- and macro-porous substrates, 0.804 ± 0.254 and 0.698 ± 0.211 , respectively. The similarity in the swelling exponents suggests that fluid movement is similar for both substrates and occurs predominately via the small pores created by NaCl which is present in both micro- and macro-porous substrates.

3.3 Surface functionalization improves cellular attachment on pHEMA substrates

To enhance cell attachment, pHEMA substrates were modified with CDI and gelatin attached (**Figure 4.5**). Normal human fibroblasts (NHF) were seeded on the substrates, incubated for 72hrs and cell growth quantified using WST-1. When compared to controls, substrates with covalently linked gelatin supported increased cell growth, indicating well adhered/attached cells on the substrate surface.

3.2. Cells alter the viscoelasticity of pHEMA substrates.

To determine if cell growth influenced viscoelasticity, rheological measurements were made on micro- and macro-porous substrates on which cells had been growing for 14 days (**Figure 4.6**). In various controls where cell attachment and growth were not optimized, the pHEMA substrates had nearly identical viscoelastic behavior. These substrates included modified with CDI without gelatin and unmodified, micro- and macro-porous substrates with and without cells. Regardless of experimental group, all substrates were exposed to the same medium and medium replenishment protocol and

schedule. For pHEMA substrates optimized for cell growth (gelatin attached via CDI), the viscoelastic behavior changed in response to cell growth. In the low frequency regime ($0.628 \leq \omega \leq 10$ rad/s), these substrates with cells showed a significant increase in the elastic and viscous components of the rheological response. In the high frequency regime ($10 < \omega \leq 72.4$ rad/s), the micro-porous substrates with cells showed a decrease in elastic response, indicating a decrease in frequency dependence. In contrast, the high frequency regime for the macro-porous substrates with cells showed no changes from the controls. These observations indicate that the presence and activity of cells on the substrates affected the rheological response, particularly at higher frequencies.

In human skin at low frequencies (≤ 10 rad/s), the rate of increase for G' and G'' was gradual ($G' \sim G'' \sim \omega^{0.05}$). Micro- and macro-porous substrates with cells showed similar trends in G' and G'' ($G' \sim \omega^{0.23}, \omega^{0.42}$ and $G'' \sim \omega^{0.27}, \omega^{0.27}$, respectively). Despite an increase in magnitude of the elastic and viscous moduli, micro- and macro-porous substrates with cells at $\omega \leq 10$ rad/s, showed slower rates of increase of the elastic response while maintaining the viscous trend ($G' \sim \omega^{0.04}, \omega^{0.12}$ and $G'' \sim \omega^{0.24}, \omega^{0.25}$, respectively). At higher frequencies ($\omega > 10$ rad/s), the rate of increase in G' in control, micro- and macro-porous substrates with cells were very similar ($G' \sim \omega^{1.75}, \omega^{1.89}$, respectively), but trends in the modified micro- and macro-porous substrates with cells were dissimilar ($G' \sim \omega^{0.1}, \omega^{1.23}$, respectively). Micro-porous substrates with cells showed a significant decrease in elastic frequency dependence ($G' \sim \omega^{0.1}$), while macro-porous substrates with cells showed a rate of increase of G' similar to control macro-porous substrates without cells.

These data suggest that cellular activity reinforces the elastic components while reducing frequency dependence. Cell growth suppresses the global fluid flow within the micro-porous infrastructure, evidenced in the high frequency regime ($\omega > 10$ rad/s). The low frequency behaviors of substrates with cells were more similar to responses seen in human skin [Holt et al, 2008], other cells/soft tissues [Alcaraz et al., 2003; Nicolle et al., 2005], and F-actin gels and keratin/intermediate filament networks [Gardel et al., 2004; Ma et al., 1999, 2001; Shin et al., 2004]. Cellular activity may block the micro-pore network increasing the magnitude of the elastic and viscous response of the substrate while also disrupting the interstitial fluid flow in the substrate which causes frequency dependence. The frequency dependence in macro-porous substrates may be caused by free fluid motion/evacuation from the macro-scale pores. The degree of viscous frequency dependency in protein modified, micro- and macro-porous substrates ($G' \sim \omega^{0.5}$ and $G'' \sim \omega^{0.45}$, respectively) are similar to the untreated types ($G' \sim \omega^{0.58}$ and $G'' \sim \omega^{0.61}$, respectively), indicating the effect of changes in fluid flow behavior has little impact on the viscous component of the substrates' viscoelastic response.

3.3. *Soft-solid model for pHEMA with cells.*

The rheological data for the pHEMA substrates were fit to a mathematical model describing a soft-solid. In the high frequency regime, the response was non-linear, but over the low frequency range (≤ 10 rad/s) the fit was excellent (**Figure 4.7**). A parameter reduction analysis showed that the model could be reduced from three parameters to two,

fixing the retardation time, τ and κ at 1.15 and 0.09, respectively. The model reduced to a one parameter fit for equilibrium relaxation modulus, G_e . For micro-porous substrates with and without cells, G_e values were 2,500 and 8000, respectively. Likewise, for macro-porous substrates with and without cells, the G_e values were 3,000 and 15,000, respectively. These parameter value fits represented a 3 and 5 fold increase in equilibrium relaxation modulus due to the effects of cells on the substrates. Model parameter sensitivity showed the elastic response was most responsive to changes in the equilibrium modulus, G_e , while the viscous response was most sensitive to the equilibrium modulus and scalar exponent, G_e and k , respectively (**Figure 4.7**). Although not a traditional comparative tool, parameter sensitivity further highlighted the complexity of depicting the viscous component of the viscoelastic response.

When considering the complete frequency range (0.6283 – 75.4 rad/s), the soft-solid model fails to grasp the high frequency regime response of the NHF-pHEMA co-culture composites (data not shown). However, when focusing on the low frequency regime (≤ 10 rad/s) containing physiologically relevant frequencies, the model replicates the elastic behavior of the substrates, with and without cells, well (**Figure 4.6**) but fails to reproduce the viscous response. This was supported by the mean percent difference of experimental data to model prediction for G' and G'' (**Table 4.2**) and lesser so by the correlation coefficient. The correlation coefficient, a trend comparison index between -1 (reciprocally correlated) and 1 (perfectly correlated), showed that the mean correlation coefficients for G' and G'' for the micro- and macro-porous substrates were 0.921 and 0.917, respectively (**Table 4.2**). Together, the mean percent difference and correlation

coefficient highlights the capacity of the model to replicate the trends of the rheological behavior but fails to capture the complexity of the viscous response.

3.4 Scanning Electron Microscopy provides insights into cellular activity on pHEMA substrate surface

Substrate surface imaging using critical point drying SEM analysis of untreated and protein modified micro- and macro-porous substrates yielded important information about the degree of the biological response of NHFs to the substrates. Untreated substrates showed very little cellular activity and attachment (**Figure 4.8**). This is consistent with previous groups' work which asserts pHEMA is protein non-adherent [Fukano et al, 2006]. After 14 days of medium and serum exposure some level of protein attachment would occur, leading to the observed sporadic attachment. Protein modified micro- and macro-porous substrates showed more cellular and protein attachment than their untreated counterparts (**Figure 4.8**). Regular cellular borders are difficult to determine, as is the maturity and composition of the protein layer. However, these cell-substrate images are similar to those previously reported by groups investigating 3T3 mouse fibroblast growth on fibroin/recombinant human-like collagen scaffold [Hu et. al 2008] and NHFs on biodegradable collagen scaffolds [George et al 2008]. Although environment or vapor-pressurization scanning electron microscopy techniques [ESEM and VPSEM, respectively] and immuno-histochemistry would provide improved resolution and confirm the maturity and bioactive capacity of the deposited matrix. SEM analysis demonstrated the magnitude of cellular activity and established a sound basis for substrate with cell comparison.

4. Conclusions

Understanding the mechanics of cell-substrate interactions is critical to the success of biomaterials for many applications, especially in soft tissue. The viscoelasticity of both micro- and macro- porous substrates were predominately elastic in nature and the viscoelasticity of macro-porous substrates were the most similar to skin. At low frequencies ($\omega \leq 10$ rad/s), micro- and macro-porous substrates with and without cells showed very similar magnitudes and trends in G' and G'' ($G' \sim G'' \sim \omega^{0.25}$ and $G' \sim G'' \sim \omega^{0.35}$, respectively). The trends were similar to those seen in skin ($G' \sim G'' \sim \omega^{0.5-1.0}$) despite differences in magnitude. At high frequencies ($10 < \omega \leq 72.4$ rad/s), micro- and macro-porous substrates with cells had very distinct and dissimilar behaviors. Micro-porous substrates with cells were largely frequency independent ($G' \sim \omega^{0.7}$). Macro-porous substrates with cells maintained their frequency dependence at $\omega > 10$ rad/s, mimicking the rate of increase of their untreated counterparts ($G' \sim \omega^{1.23}$ and $G' \sim \omega^{1.89}$, respectively). Micro- and macro-porous substrates with cells showed similar viscous component responses ($G'' \sim \omega^{0.5}$ and $G'' \sim \omega^{0.45}$, respectively). These data suggest that cellular activity affects interstitial fluid flow and reinforces the elastic components of the substrates while reducing frequency dependence. Lastly, we adapted a soft-solid model and found it useful to model the viscoelastic behavior of these substrates. .

The frequency dependent rheological behavior of cells cultured on soft substrates is an area of increasing interest. At the cellular level, several studies have used tensegrity and soft glassy rheology to describe the cytoskeletal remodeling-mediated viscoelastic

rheological behavior [Fabry et al. 2001, Mandadapu et al. 2008, Stamenović 2008, Kollmannsberger et al. 2009] with differing conclusions. The soft-solid viscoelastic model of this paper is capable of reproducing and replicating dynamic shear behavior and trends in the frequency response of skin as well as porous synthetic substrates and may be useful for understanding cellular responses as well as the design of new soft tissue analogs. With regards to whole skin, this model may form the basis for a more advanced finite element model capable of describing the load conditions around percutaneous devices. Future models will need to account for the more pronounced biphasic and frequency dependent nature of the porous substrates with cells in order to achieve an improved understanding of the differences between these synthetic substrates and normal skin. Future models will need to include additional, frequency regime specific, time constants capable of accurately describing the physiological relevant frequency range.

The aim of this study was to fabricate synthetic porous substrates that approximate the mechanical behavior of soft tissue such as skin and to determine the mechanical changes that occur when cells are grown on these substrates. Our rheological measurements show that pHEMA substrates are predominately elastic in nature with a narrow range of G' and G'' values that mimic the response of human skin. Substrates with macro-pores more closely matched human skin and cell growth on all substrates preferentially enhanced the elastic response. A quantitative understanding of the mechanics of synthetic materials prior to implantation, as well as how these mechanics change after implantation due to cell growth may aid in the design of new materials that retain their mechanical match

with soft tissue after implantation. Such materials would be expected to dissipate stress discontinuities between materials and tissues, minimizing adverse cell responses and aid in soft tissue integration.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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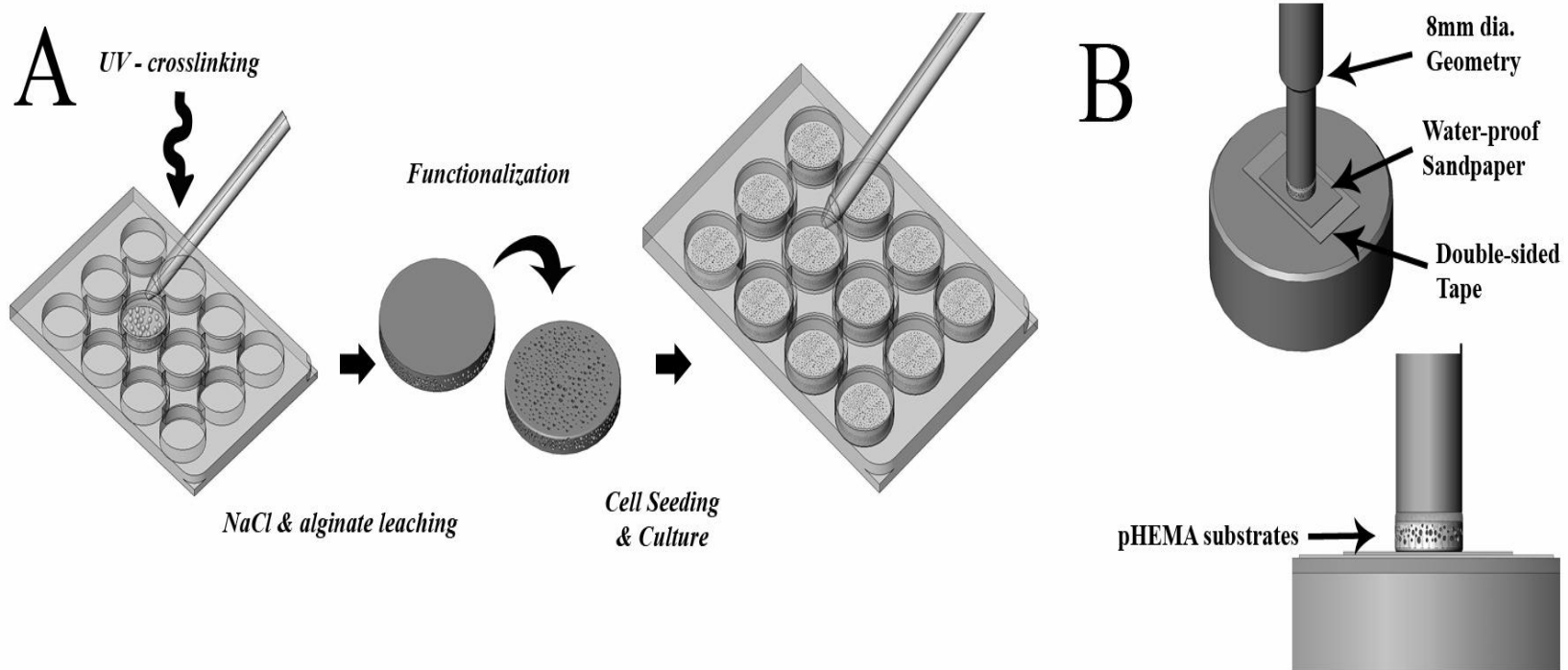


Figure 4.1: Preparation and rheological measurements of porous pHEMA substrates. (A) Micro- and macro- porous pHEMA substrates were polymerized by UV-cross linking. Micro pores and macro pores were created using NaCl and NaCl plus alginate beads, respectively. For cell growth, substrates were modified with CDI enabling gelatin attachment. (B) Micro- and macro-porous substrates, on which were cultured normal human fibroblasts, were subjected to an isothermal (37°C) oscillatory strain of 0.1% between 0.6283 and 75.398 rad/s, a physiologically relevant frequency range. The resulting G' and G'' values at various frequencies were measured

A

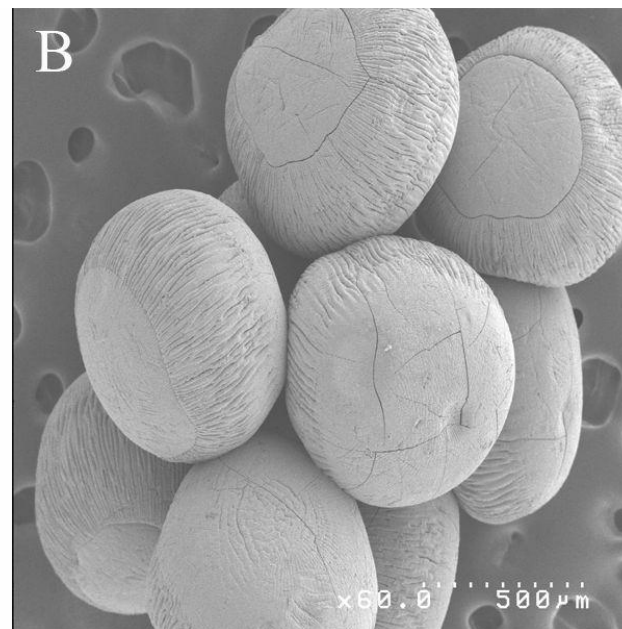
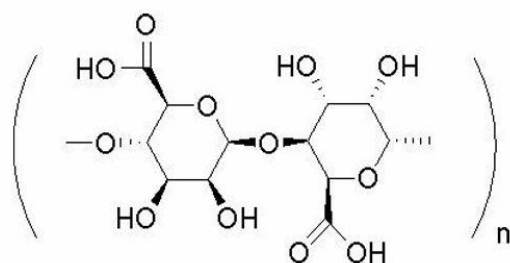


Figure 4.2.: Alginate bead porogen preparation. (A) Cross-linked alginate was used to create macro-scale pores in pHEMA substrates. Alginate beads were cross-linked in a drop-wise fashion by exposure to 4% (w/v) BaCl₂ stirred at 300 rpm. (B) From scanning electron micrographs, alginate beads were estimated to be between 200 – 400 µm in diameter

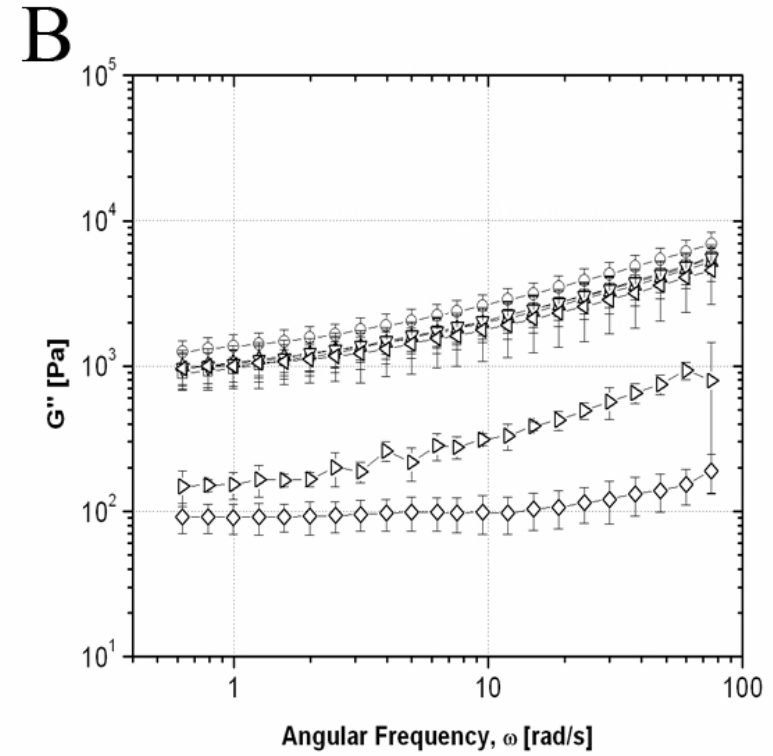
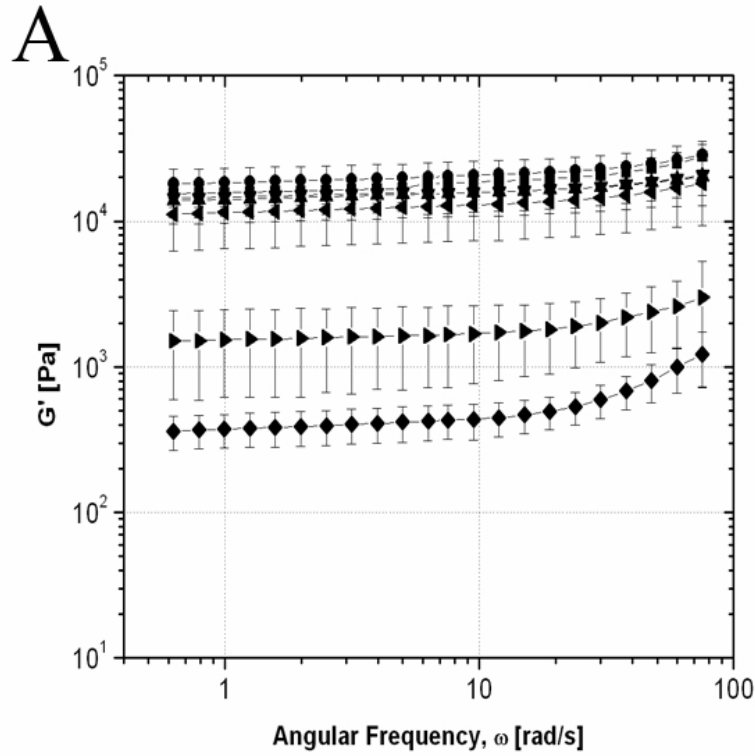


Figure 4.3: Rheological characterization of substrates mimicking the mechanical behavior of human skin. pHEMA substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% and the resulting G' (A) and G'' (B) values were measured. Micro-porous substrates had various concentrations of NaCl: No NaCl (■, □); 0.6M (●, ○); 0.7M (▲, △); 0.8M (▼, ▽); and 0.9M (◀, ▷). Macro porous substrates had 0.8M NaCl plus 1.5% (w/v) dried alginate beads (▶, ◇). Human skin is also shown for reference (◆, ◇)

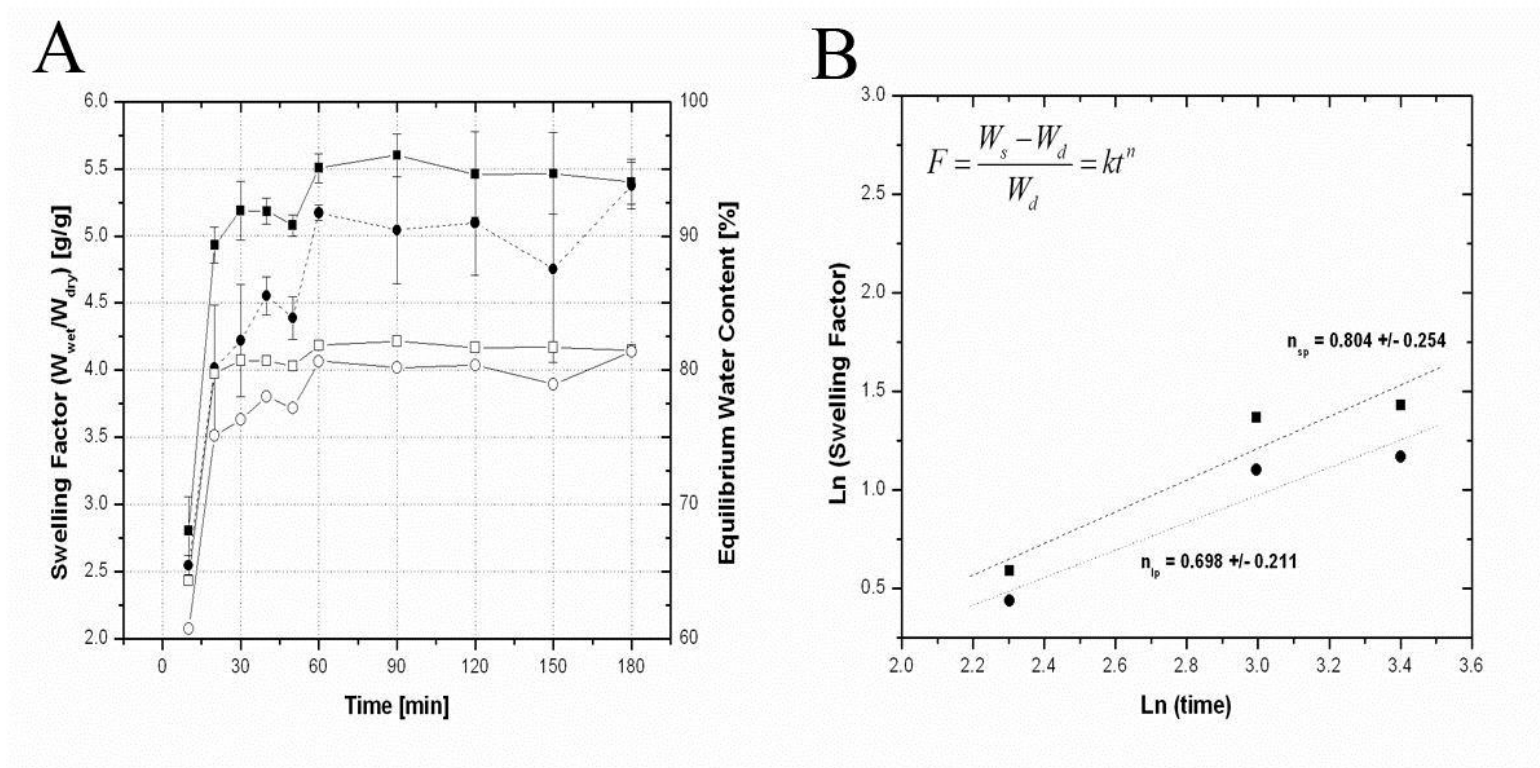


Figure 4.4: Kinetics of swelling and water uptake of pHEMA substrates. Micro-porous (■) and macro-porous (●) substrates were dried, weighed and then added to de-ionized water equilibrated at 37°C over 180mins. At specific time intervals, substrates were removed and weighed. (A) The resulting weights were used to calculate the swelling factor (closed symbols) and equilibrium water content (open symbols). (B) Linear curve fitting to a reduced ln-ln swelling factor v. time plot revealed information about the swelling kinetics of the micro- and macro-porous substrates

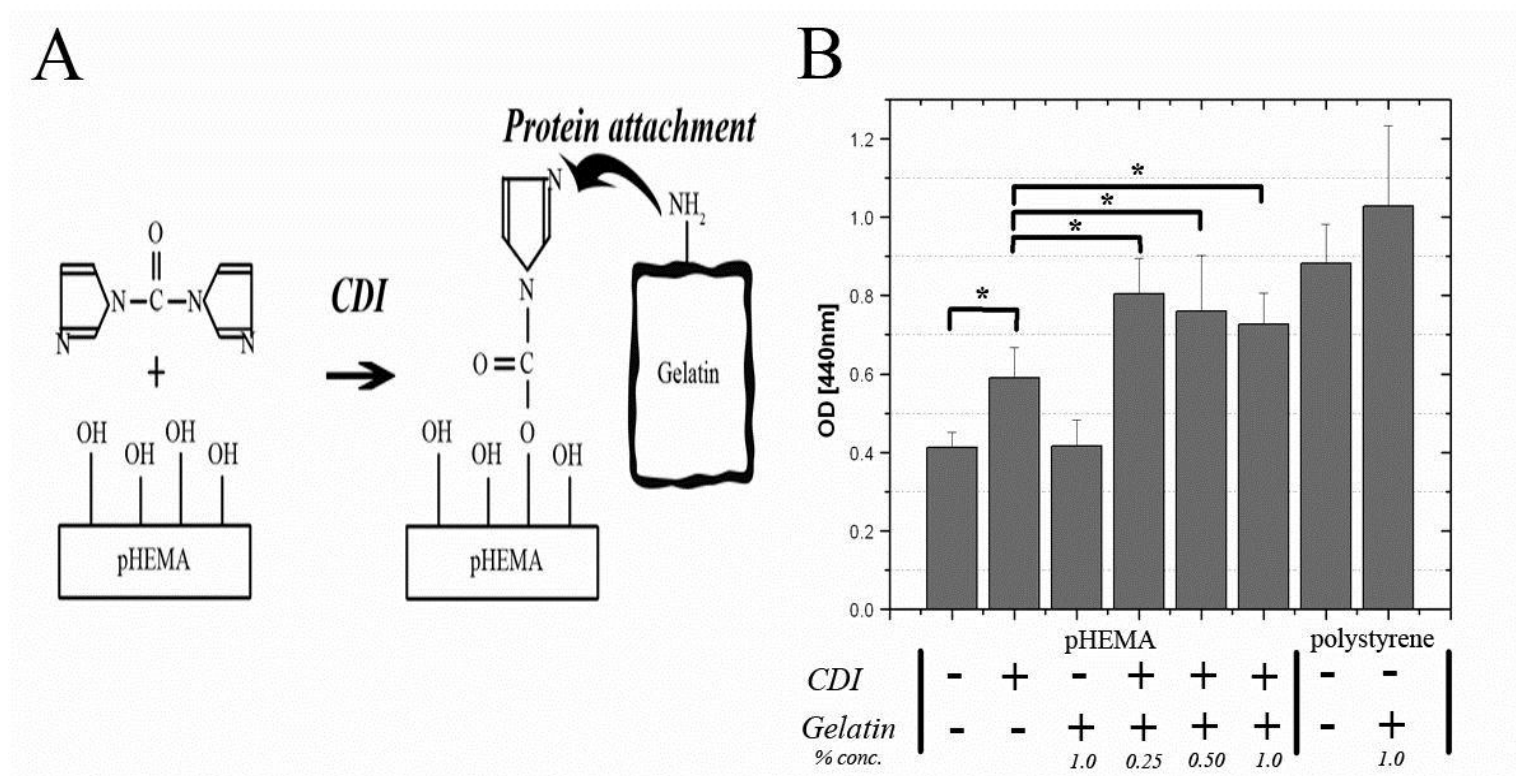


Figure 4.5: Functionalization and cell growth on pHEMA substrates. **(A)** pHEMA substrates were functionalized via 1,1'-carbonyldiimidazole-mediated hydroxyl group activation and gelatin attached. **(B)** Human fibroblasts were seeded on various control and modified pHEMA substrates and cell proliferation was measured using the WST-1 assay 72 hours after seeding cells. Cell growth was significantly increased when pHEMA were treated with both CDI and gelatin

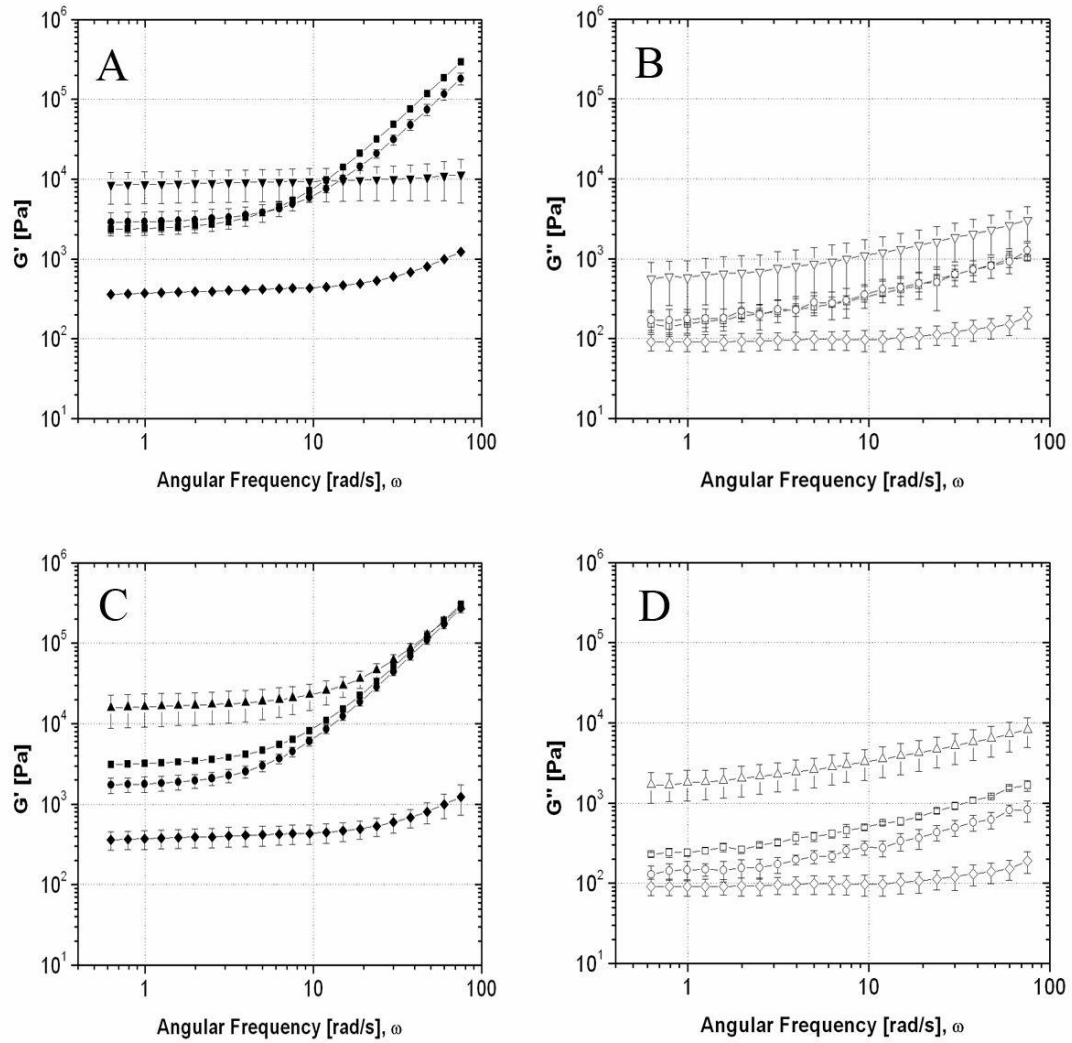


Figure 4.6: Viscoelastic response of micro- and macro- porous substrates with and without NHFs. Cells were grown on micro- (A, B) and macro-porous (C, D) substrates for 14days, after which substrates were subjected to an isothermal (37°C) oscillatory strain of 0.1% between 0.628 and 75.40 rad/s and G' (A, C) and G'' (B, D) were measured. Untreated substrates, with (●,○) and without (■,□) cells and substrates treated CDI and gelatin with cells (▲,△) were measured and compared to human skin (◆,◇). Note, cellular activity on modified substrates increased the magnitude of G' and G'' at low frequencies while also altering the degree of high frequency dependence, indicating that cellular interactions with the micro-pore infrastructure has a profound effect on the viscoelastic behavior of the substrate

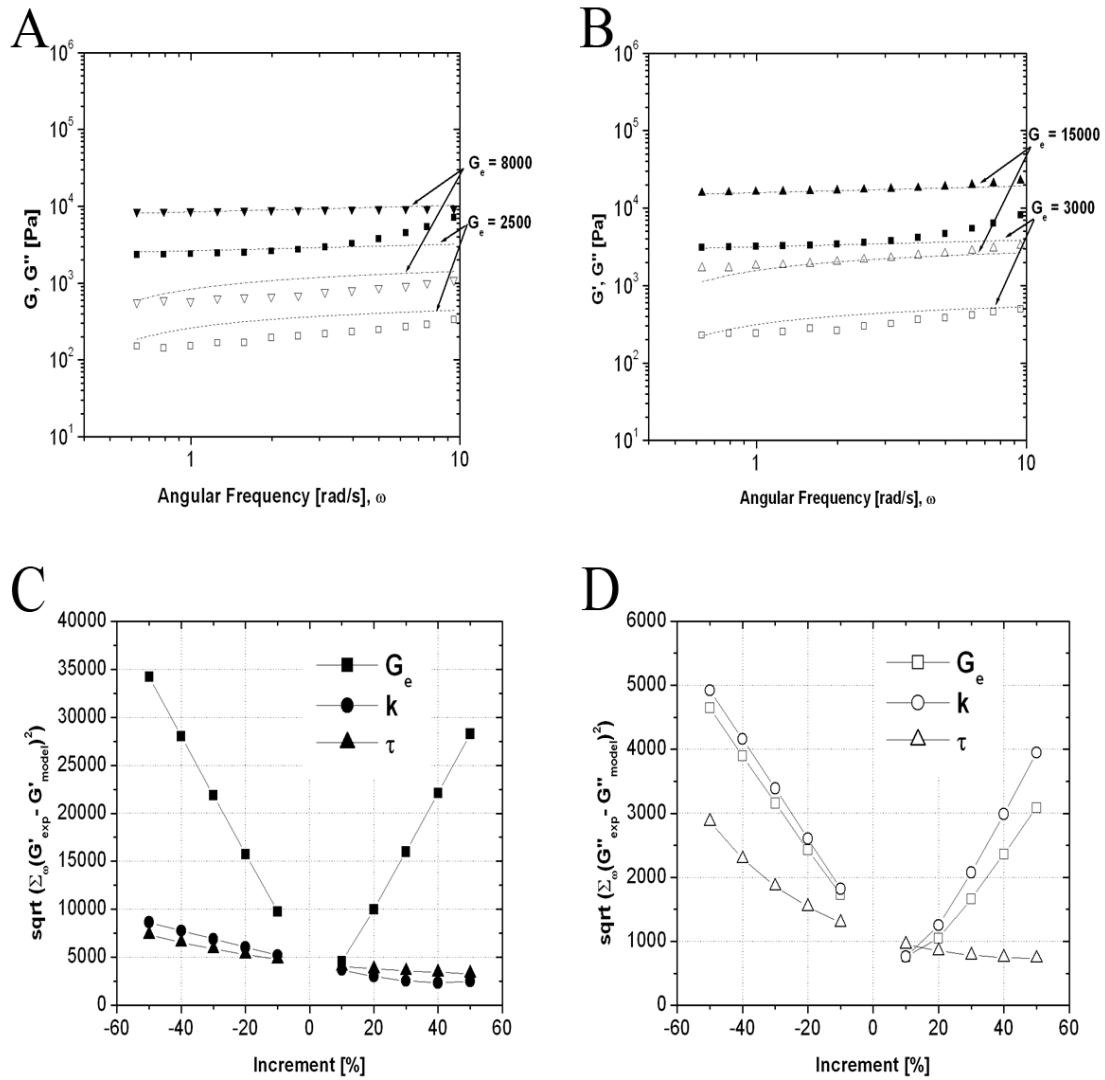


Figure 4.7: Adapted soft-solid model – A soft-solid viscoelastic model was applied to the rheological data on control, micro-porous and macro-porous substrates without cells (■, □) and modified micro-porous and macro-porous substrates on which normal human fibroblasts were cultured (▼, ▽ and ▲, △, respectively) (A, B). [open symbols denote G'' values] The soft-solid model simulates viscoelastic solids with an internal structure that liquefies at higher frequencies. The resulting G' and G'' values predicted by the model (dashed lines) were compared qualitatively to experimental data. A model sensitivity analysis showed G' (C) response of the Caswell model was essentially a one parameter fit in G_e while G'' (D) required a more complex three parameter fit.

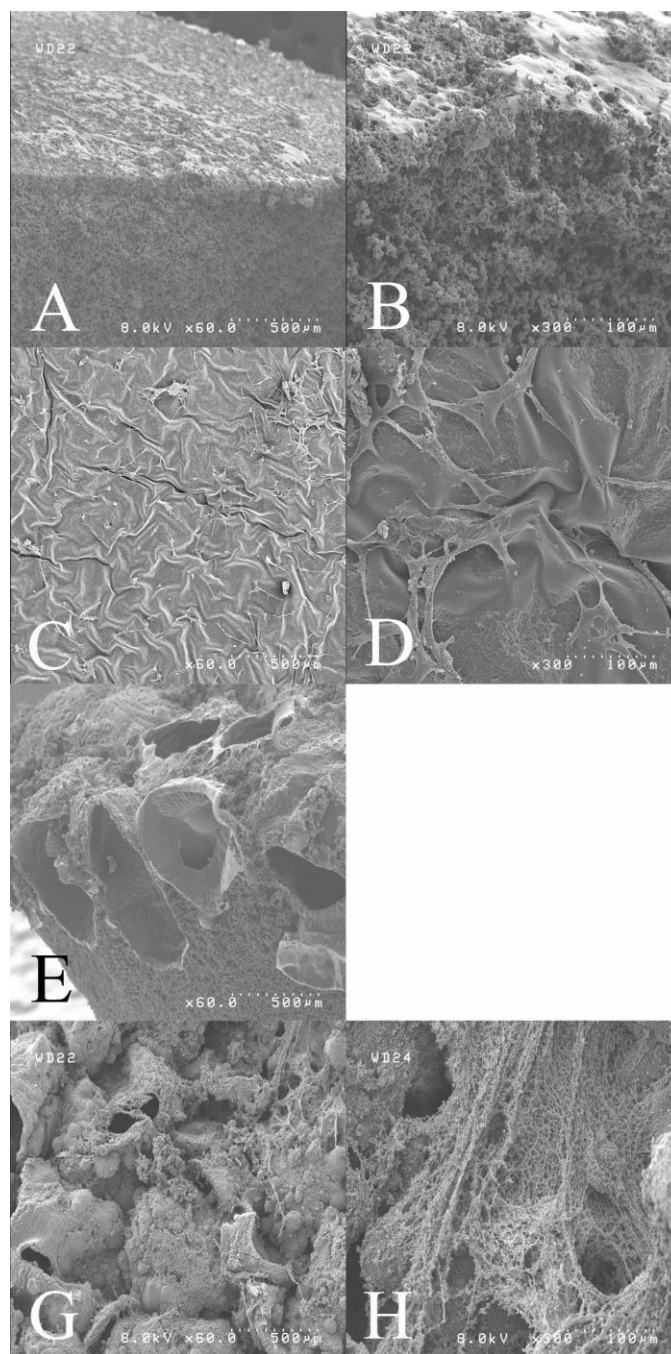


Figure 4.8: Scanning electron microscope [SEM] analysis of poly(2-hydroxyethyl methacrylate) [pHEMA] substrates – SEM analysis of freeze-dried micro-porous (A-C) and macro-porous (D-F) pHEMA substrates shows a dense, interconnected pore network, characteristic of hydrogels and ideal for biomaterial related, soft-tissue applications. Panels A, B, and C show micro- and macro-porous substrates without cells. Panel F is omitted because closer magnification reflects substrate topography already seen in Panels A and B.

	<i>Micro-pore [untreated]</i>		<i>Micro-pore [protein modified]</i>		<i>Macro-pore [untreated]</i>		<i>Macro-pore [protein modified]</i>		<i>Human Skin</i>	
	<i>G'</i>	<i>G''</i>	<i>G'</i>	<i>G''</i>	<i>G'</i>	<i>G''</i>	<i>G'</i>	<i>G''</i>	<i>G'</i>	<i>G''</i>
Mean Values $[10^3]$										
<i>Low Frequency</i> (≤ 10 rad/s)	2.88 – 5.90	0.171 – 0.356	8.47 – 9.40	0.558 – 1.10	1.73 – 6.07	0.129 – 0.281	15.70 – 22.80	1.69 – 3.27	0.361 – 0.435	0.092 – 0.098
<i>High Frequency</i> (> 10 rad/s)	7.57 – 182.0	0.419 – 1.270	9.47 – 11.40	1.20 – 3.05	8.57 – 271. 0	0.274 – 0.823	25.50 – 286.0	3.54 – 8.23	0.448 – 1.27	0.097 – 0.190
Power Law										
<i>Low Frequency</i> (≤ 10 rad/s)	0.23	0.27	0.04	0.24	0.42	0.27	0.12	0.25	0.69	0.33
<i>High Frequency</i> (> 10 rad/s)	1.75	0.58	0.10	0.50	1.89	0.61	1.23	0.45	0.54	0.04

Table 4.1: Quantitative comparison of untreated and gelatin modified, micro- and macro-porous substrates and human skin from isothermal (37°C), constant oscillatory strain (0.1%) experiments between 0.628 and 75.40 rad/s. Power law curves ($G' \sim G'' \sim \omega^x$) were fit to G' and G'' values within the low (≤ 10 rad/s) and high (> 10 rad/s) frequency regimes for comparison of the rate of increase of frequency dependent viscoelastic behavior. All pHEMA substrates are with normal human fibroblast cells

	<i>Parameters</i>	<i>Micro-pore [untreated]</i>	<i>Micro-pore [protein modified]</i>	<i>Macro-pore [untreated]</i>	<i>Macro-pore [protein modified]</i>
Model Parameters					
	Equilibrium Relaxation Modulus, G_e	2500	8000	3000	15000
	Retardation Time, τ	1.15	1.15	1.15	1.15
	Kappa, κ	0.09	0.09	0.09	0.09
Average Percent Difference [%] (Experimental Data v. Model) [Model Accuracy]	Elastic [Storage] Modulus, G'	16.5	3.94	14.2	3.94
	Viscous [Loss] Modulus, G''	61.3	44.3	26.6	10.1
Average Percent Difference [%] (Experimental Data v. Model) [Trend Matching]	Elastic [Storage] Modulus, G'	0.869	0.996	0.875	0.945
	Viscous [Loss] Modulus, G''	0.916	0.898	0.915	0.937

Table 4.2: Qualitative comparison of experimental data from isothermal (37°C), constant oscillatory strain (0.1%) experiments between 0.628 and 10 rad/s on untreated, micro- and macro-porous substrates without cells and protein modified, micro- and macro-porous substrates with normal human fibroblasts to an adapted soft-solid model. Experimental data and model predictions were compared using percent difference (accuracy) and correlation coefficient (trend-matching). All substrates are with NHF cells

Conclusion

The objective of this dissertation was to investigate and develop a synthetic biomaterial construct intended to address the challenges of the soft tissue to device interface of percutaneous devices, motivated chiefly by matching the mechanical behavior of the material to human skin. In order to accomplish this objective the study was divided into three main components: 1) the physiologically relevant viscoelastic characterization of human skin and dermis; 2) the rheology-based design and optimization of a cross-linked, biocompatible and biomimetic polymer-based biomaterial; and 3) the biological and mechanical interrogation of that polymer biomaterial when exposed to human dermal cells through in vitro co-culture. The dissertation expanded upon how mechanical characterization based approaches can be further utilized in the design of soft-tissue related biomaterials, as well as providing insight into the unique cellular response to biomaterials intended to mimic the viscoelastic responses of in situ tissues.

In Chapter 2, human skin and dermis-only samples from neonatal foreskins were exposed to low magnitude, physiologically relevant frequencies eliciting a measurable viscoelastic response. The viscoelastic characterization of human skin and dermis provided the baseline viscoelastic response of the two tissues. Furthermore, the viscoelastic characterization data further elucidated the role of the epidermis, albeit by subtractive reasoning. The comparison of the viscoelastic responses of skin and the dermis confirmed the epidermis provided the majority of elastic rigidity of the skin, while the relatively more viscous dermis served as the mechanical foundation of the skin. These roles were

supported by the assessment, analysis, and comparison of the elastic, G' and viscous, G'' moduli from the frequency response experiments and the creep compliance and recovery responses from the step-stress response experiments. From a rheological perspective, the data indicated that human skin behaved like a “solid-like fluid”, with relatively frequency independent behavior at low frequencies, $\omega \leq 10 \text{ rad/s}$, and moderate frequency dependence at higher frequencies, $\omega > 10 \text{ rad/s}$. Further, the dominance of elastic component throughout the physiologically relevant frequency range and the narrow range of G' and G'' values, highlighted the opportunity for the development of a synthetic biomaterial for the skin-related biomedical applications. The intrinsic specificity of the physiologically relevant viscoelastic characterization of human skin would be used as the basis for a rheology based biomaterial development process.

Also within Chapter 2, to improve the computational strength of the study and supplement any subsequent comparative study of material properties, a simple Kelvin Voigt viscoelastic model was also applied to the viscoelastic characterization data. The viscoelastic model highlighted the complexity of the system and specifically the inherent difficulty in accurately capturing the viscous response of the tissue. The need for a more advanced model was made evident.

In Chapter 3, a micro-porous poly(2-hydroxyethyl methacrylate) [pHEMA] substrate intended to mimic the viscoelastic behavior of human skin was developed utilizing a physiologically relevant rheological characterization approach. PHEMA is a highly

adaptable, hydrophilic polymer that has been utilized in many soft-tissue related biomaterial applications. Using phase separation, previous work has shown that when aqueous sodium chloride [NaCl] is added to 2-hydroxyethyl methacrylate [HEMA] with cross-linker and photo polymerized, a highly porous polymer network is created. Utilizing similar rheological characterization experiments from the study of human skin presented previously, substrates were polymerized with different molarities of NaCl, monomer to diluent ratios [HEMA : NaCl], and ethylene glycol dimethacrylate [EGDMA] cross-linker concentrations to develop substrates intended to mimic the viscoelastic trends of human skin. All substrate types showed relatively stable, linear viscoelastic behavior across a wide range of physiologically relevant shear strains and stresses. The resultant rheological data showed monomer to diluent ratio had the greatest effect on the viscoelastic response of the substrates. Changes in cross-linker concentration had very little effect on the viscoelastic response of the substrates. Previous studies have shown differences in cross-linker have a significant impact on the mechanical behavior of pHEMA substrates, indicating that in order to see a cross-linker mediated shift in rheological behavior a different cross-linking agent may need to be selected [Mabilleau et al. 2006]. Although, the rheological behavior of micro-porous pHEMA substrates in response to physiologically relevant frequencies did not reproduce the magnitudes of the response of human skin exactly, the substrates did show very similar frequency-independent and -dependent behavior in G' and G'' . Ultimately, the rheological data indicated that an additional means of altering the mechanical behavior of the substrates must be identified, and the inclusion of an additional porogen was advanced. Additionally, the rheological characterization involved an examination of the

effect of temperatures on the viscoelastic behavior of the various micro-porous pHEMA substrates. Temperature was shown to affect the viscoelastic capacity of the substrates but not around the ideal physiologic temperature ($\sim 37^{\circ}\text{C}$), optimistically having little impact on the efficacy of the biomaterial in its anticipated application. The resultant micro-porous pHEMA formulation was advanced to experimentation focused on investigating the biological and mechanical response of cells to the substrates.

In Chapter 4, unmodified and protein modified, micro-porous and macro-porous pHEMA substrates were co-cultured with dermal human fibroblasts for 14 days, after which substrates with cells and without were mechanically characterized for a comparative understanding of how cellular activity effected the mechanical behavior of substrates intended to mimic human skin. The physiologically relevant rheological examination of protein modified and unmodified micro- and macro-porous pHEMA substrates showed cellular activity effected the frequency dependent rheological behavior of protein modified, micro-porous substrates the most when compared to other groups. All protein modified substrates with cells showed an order of magnitude increase in G' and G'' . When compared to other micro-porous and macro-porous substrate groups, protein modified, micro-porous substrates showed significant decrease in high frequency, $\omega > 10 \text{ rad/s}$, regime frequency dependence. Although protein modified, macro-porous substrates showed similar increases in magnitude of G' and G'' values across the entire frequency range, there was no change (i.e. no reduction) in frequency dependent rheological behavior. The shared increase in magnitude of the G' and G'' but difference in frequency dependence between protein modified, micro- and macro-porous substrates

indicated a cell-substrate interaction mediated effect in rheological behavior. Substrates with protein modification promoted the most cellular activity. Micro-porous substrates contained features/pores most similar in scale to the co-culturing cells and their products, while the large pores of the macro-porous substrates were at least 1° of magnitude greater than the cells. Both substrates contained micro-pores and swelling kinetics studies verified the dominance of the micro-pores in diffusive fluid flow within the two types of substrates. Fluid flow is often implicated in high frequency rheological dependent behavior. Thusly, one proposed cause for the difference in high frequency behavior was the disruption/blockage of interstitial fluid flow within the protein modified, micro-porous pHEMA substrate caused by cells and cellular activity on the surface. Further investigation is required to verify that theory however. Similar to the rheological findings from Chapter 2, the rheological behavior of protein modified, micro- and macro-porous with cells did not replicate the magnitudes of the rheological behavior of skin but did reproduce the trends in frequency-related behavior.

Also within Chapter 4, to improve the understanding of the low frequency rheological behavior of protein modified, micro-porous pHEMA substrates for further quantification of substrate mechanical properties for future comparative studies, a “soft-solid” viscoelastic model was applied. The “soft-solid” model was developed for the investigation of solid materials whose internal structure liquefies at high frequency. The specificity and added complexity of the soft-solid model was intended to address the inadequacies of the less simple model utilized in Chapter 2. The model was shown to replicate the elastic component of the low frequency response but failed to accurately

depict the viscous component. Similar to the rheological study in general, the trends of the frequency dependence of the elastic, and to a lesser degree the viscous, component of the substrate was reproduced.

The rheology-based approach utilized for the development of the micro- and macro-porous substrates examined throughout this dissertation addressed two key design considerations presented and discussed in the literature. Firstly, underlying deep tissue has been shown to inhibit or reduce epidermal regression in vivo. Unlike the use of more conventional, device oriented subcutaneous anchors, designing substrates that mechanically match the viscoelastic response of human skin is intended to take advantage of the in situ tissue's response to load without the addition of additional device components. The burden of dissipating loads away from the device is minimized if the device itself is designed to respond to load application in a fashion similar to in situ tissue. Micro-porous and macro-porous, pHEMA substrates are designed to work in concert with the surrounding tissue. Secondly, designs with increased surface area have been shown to maintain cellular adhesion and tissue integration longer when compared to similar substrates without enhanced surface area modifications. The interconnectivity and porosity gradient within the micro- and macro-porous substrates influences the mechanical behavior of the substrates but also increases the potential tissue-substrate contact surface area; improving the attachment and reattachment capacity of the substrates.

Despite the comprehensive nature of the rheological approach to skin-related biomaterial design presented in this dissertation there are six areas that would need to be addressed to advance the study. Those six areas are: 1) rheological characterization of human skin for different age groups and anatomical locations; 2) the further interrogation of the role of porosity and interstitial fluid flow; 3) the investigation of different cross-linking agents, co-polymers, or bio-/metal-related additives; 4) the development of an alternative means of substrate surface functionalization; 5) the inclusion of micro-patterning to resist tissue-substrate interface shear; and 6) the inclusion of micro- and macro-porous pHEMA substrates in an in vivo animal model.

The characterization of human skin for different age groups and anatomical locations would vastly improve the clinical efficacy of a skin-related biomaterial intended to reduce the stress discontinuities present at the skin to device interface. Skin elasticity and viscoelasticity is dependent on the adaptation and modification of the dermal collagen lattice, a process that relies heavily on density and composition of cross-linking agents (e.g. elastin) within the tissue. These qualities have been shown to be age and location dependent. The human skin characterized here is from neonatal foreskins, a combination of age and location that is potentially very different from the anatomical locations in which many percutaneous devices are implanted in adults. The clinical success of any soft-tissue related biomaterial relies heavily on the biocompatibility and biomimetic capacity of the substrate. In traditional biomaterial design that process would involve determining the biochemical landscape of the in situ tissue so the biomaterial can be tailored to match. In the development of a biomaterial intended to reduce stress

concentrations mediated by discontinuities at the soft-tissue to device interface, an accurate mechanical landscape of the in situ tissue must be obtained which would necessitate the inclusion of other age groups and differing anatomical locations.

With mechanics in mind, improving the understanding of the role of interstitial fluid flow in the rheological behavior of micro- and macro-porous pHEMA substrates will advance the optimization of the substrates by deconstructing the role of porosity in the frequency dependent rheological response of the substrates. The application of biphasic and triphasic models in the dynamic mechanical characterization of biomaterials, linking the contributions of compression, oscillatory load application, viscoelasticity, porosity, and permeability, has been invaluable for the advancement of the study of human cartilage tissue and cartilage substitutes. Similar to soft-tissue to device interface biomaterials, the development and success of cartilage scaffolds and substitutes relies heavily on mechanics and fluid dynamics. Consequently, the anticipated benefit to skin-related biomaterials intended to match tissue at the skin to device interface would be similarly positive.

Combining discussion points (3) and (4), the investigation of the effect of different cross-linking agents, co-polymers, or bio-/metal-related additives for micro- and macro-porous substrates has the potential to improve the fluid dynamics of the substrates, as well as offer other means for surface functionalization for improved biocompatibility. The choice of different cross-linking agents has been shown to affect the swelling kinetics and

mechanics of phase separation pHEMA substrates. The length of the cross-linking agent has been shown to have an effect on the mechanical behavior of the substrates but will also affect the bound water content of the substrate, altering the biocompatibility of the substrate. Additionally, a different cross-linking agents, co-polymers, or bio-/metal-related additives may allow for alternative surface functionalization techniques to be introduced to the system. The current technique involves chemicals with known cytotoxic and carcinogenic effects and if not properly removed may cause serious damage to host tissue. Therefore, these additional components may allow micro- and macro-porous pHEMA substrates to improve their bio-integrative capacity.

Micro-pattern designs that are oriented parallel to the skin surface show improved tissue-substrate interaction *in vivo* when compared to micro-patterns placed perpendicular to the skin surface. Regardless of orientation, micro-patterns increase surface area, however parallel orientations shorten and disrupt shear planes at the soft-tissue to device interface. The disruption of shear wave propagation at the tissue-substrate interface is a possible mechanism for the improved performance. Although not discussed here, preliminary polymerization studies have been conducted with the micro-porous pHEMA to cast the substrates in molds that replicate the shear-resistant, Rete ridges of the skin-fascia interface. Inclusion of micro-patterns on mechanically matched micro- and macro-porous substrates would no doubt improve tissue response at the soft-tissue to device interface, particularly *in vivo*.

The inclusion of micro- and macro-porous pHEMA substrates in vivo is essential to the development of the biomaterials. The inherent complexity and dynamism of biological tissue requires that any material intended to mimic either the physiologic or mechanical response of in situ soft-tissue must be tested in vivo. The work presented here has shown the effect of in vitro cell culture on the rheological behavior of micro- and macro-porous substrates. Previous groups working with pHEMA-based substrates in murine models have obtained invaluable insight from the in vivo interactions with their substrates and the substrates developed here would benefit similarly. The contact afforded biomaterial substrates in vivo allows the substrate to interact and incorporate with a dynamic extracellular matrix. In vivo tissue-substrate interactions with the host immune and nervous systems, vasculature, and connective tissue have been invaluable to the development of more complex biomaterials. Furthermore, these interactions may also help further dissect the role of mechanics in the wound healing and foreign body response of soft-tissues and specifically skin.

The use of a rheology-based mechanical characterization in the development of novel biomaterials is an approach not unique to this study; however the emphasis of mechanics and more accurately the mechanical matching of a biomaterial to a soft-tissue with a particular application is unique. As the fields of biophysics and molecular biology advance, so too will the understanding of the role of mechanics in physiologic processes. Consequently, this study will be one of many guide-posts to future scientific endeavors focusing on not only the challenges of the soft-tissue to device interface of percutaneous

medical devices, but to research aimed at addressing the biological and technological obstacles of other soft-tissue related clinical and biomedical applications.