DEVELOPMENT AND CHARACTERIZATION OF A MODEL FOR STUDYING
MECHANOTRANSDUCTION ON OSTEOCYTE-LIKE MLO-Y4 BONE CELLS

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DEVELOPMENT AND CHARACTERIZATION OF A MODEL FOR STUDYING MECHANOTRANSDUCTION ON OSTEOCYTE-LIKE MLO-Y4 BONE CELLS

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Thesis

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ABSTRACT

The field of mechanobiology is aimed at understanding the role the mechanical environment plays in directing cell and tissue development, function, and disease. Mechanosensitive cells, such as osteocytes in bone, are capable of translating mechanical stimuli into cellular responses. This phenomenon can be widely found in many cells throughout the body, and yet little is known about the mechanisms and pathways by which this occurs. In order to investigate these mechanisms, researchers focus on developing in vitro models aimed at accurately simulating biologic samples with loads similar to those displayed in the in vivo environment. By designing their own systems, researchers can create specialized systems that meet their specific testing needs while saving money in comparison to purchasing expensive commercially available systems. Utilizing systems such as these, researchers can start to unravel the science behind mechanobiology and related diseases, and begin to improve and generate new treatments and cures.

In this study, a model for mechanically stimulating osteocyte-like MLO-Y4 cells (known for relating to mechanosensitive bone diseases) and observing cellular response was designed, fabricated, and characterized. This model consisted of an elastic substrate for cell culture and a pure uniaxial loading device designed to apply precise loads to a
biologic sample. The substrate and loading device were designed, fabricated, and characterized utilizing a series of physical and simulated tests. Once the model was completely characterized, MLO-Y4 cells were grown in the system and a series of loads were applied to correlate mechanical substrate strain to cellular metabolic and soluble activity. The resulting cellular data followed general trends found in other research verifying the effectiveness of the model for *in vitro* cellular experimentation.
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Pure uniaxial loading device. a) A pure uniaxial loading device was generated for this research. The device was designed to load cells adhered to a substrate while maintaining a b) hydrated and temperature regulated environment by suspending the sample in a heated hydration bath. c) Precautions were taken to assure that no load was applied to the samples prior to experimentation by utilizing a bar to lock the friction clamps in a set position in culture.

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CHAPTER I

BACKGROUND

In the United States, more than 53 million people have osteoporosis or are at high risk due to low bone mass [NIH, 2014]. Of the population of people living with this generally undiagnosed disease, there are 1.5 million osteoporotic fractures each year. The direct cost of expenditures for these fractures ranged from $12 to $18 billion in 2002 [Office of the Surgeon General, 2004]. This cost is anticipated to continue to increase in the United States with estimated treatment cost of $25 billion by 2025 [Cauley, 2013]. This disease not only generates a large and steadily increasing economic cost, but is also accompanied by a large impact on the quality of life for its victims. Some studies have ranked the change in quality of life for relatively healthy survivors after an osteoporotic fracture with a 20% reduction in the quality of life for spine fracture victims, and a 52% reduction in quality of life for hip fracture victims over the first 12 months post-fracture [Office of the Surgeon General, 2004; Tosteson et al., 2001].

Where osteoporosis may be the most prevalent bone disease, many others plague the population and add to these already astounding numbers (ie. osteonecrosis of the jaw, stress fractures, osteolysis, osteoarthritis, delayed fracture healing, and disuse paralysis). All of these issues share the common theme that their fate is
dependent upon the response of bone to mechanical loading. It is increasingly apparent that in order to find and improve cures and treatments for these diseases, a better understanding of the basic science behind the bone’s ability to sense and react to mechanical loading is needed. A better understanding of the fundamental science behind the role mechanics plays in bone function and pathology is critical to future prevention and eradication of metabolic bone diseases.

Current models for studying this science are ill-equipped to study the basic science of bone cells sensing and coordinating a response to mechanical stimulation, and improved models are essential for research aimed at addressing these bone related issues. The osteocyte cell type in bone is well accepted as the instigator which coordinates the overall cellular response of bone to mechanical signals by sensing the mechanical changes in its environment [Klein-Nulend et al., 2012; Burr and Allen, 2013]. For this reason, improved models created for understanding the basic sciences behind bone cell mechanics must be designed to incorporate these cells in a physiologically relevant manner.

1.1 Thesis Overview

In this work, a model for investigating the effects of mechanically loaded osteocyte-like MLO-Y4 cells was designed, fabricated, and validated. This model involved designing a substrate which allowed for the application of a range of strains to cells cultured under identical culture conditions, and a device which could accurately
load a biologic substrate and allow for measurements of cellular function post-loading. This substrate and loading device were characterized using both simulated and physical testing. To validate the success of this device with cells, a series of experiments were performed on MLO-Y4 cells. First, a range of strains were applied to cells cultured under identical culture conditions by utilizing the unique substrate, and a lactate dehydrogenase (LDH) assay was performed as a means of quantifying the cellular activity. Activity was correlated to applied substrate strain and regions of high and low cellular metabolic activity were found. Cells were then loaded at uniform strains relating to the measured high and low activity levels. Soluble activity from these cells, in the form of the two inhibitory bone formation proteins sclerostin and Dickkopf-1 (Dkk-1), was measured using standard ELISA kits. From this data, soluble activity of these osteocyte-like cells was correlated to applied strains, and the model as a whole was validated for this type of research.
CHAPTER II

INTRODUCTION

In the body, bone is known to be an important organ with the unique ability to constantly repair and remodel itself to meet current physiologic and mechanical demands. Bone uses this adaptation mechanism to maintain mechanical support, protect vital internal organs and structures, maintain mineral homeostasis, as well as many other functions [Burr and Allen, 2013]. Many of these important functions are controlled by the bone’s ability to translate mechanical signals to cellular responses. This translational ability in cells is known as mechanotransduction [Ingber, 1997]. Mechanotransduction is theorized to allow for bone cell communication for standard maintenance such as bone remodeling [Turner et al., 1998].

2.1 Bone Remodeling

Bone modeling is defined as either the formation of bone by osteoblasts or resorption of bone by osteoclasts on a given surface and largely occurs in the development of bone. Bone remodeling is then defined as the process by which osteoblast and osteoclast activity occurs sequentially in a coupled manner on a given bone surface [Burr and Allen, 2013]. This involves continuous removal of old bone by

4
osteoclasts, and replacement with new proteins and minerals by osteoblasts to form new bone matrix [Clarke, 2008]. This remodeling cycle begins with activation of the cycle, figure 2.1a. Activation of the remodeling cycle has been long been theorized to be caused by microdamage to the bone [Frost, 1964]. During this step of the cycle, recruitment of osteoclasts to the bone surface occurs.

Following recruitment of the osteoclasts, bone lining cells retract from the bone surface and allow for osteoclast attachment and resorption of the bone, figure 2.1b. Upon completion of the resorption step of the remodeling process, reversal occurs where osteoclast resorption ceases and osteoblast formation is initiated, figure 2.1c. Next, the formation cycle then begins where osteoblast cells form bone by laying down new bone matrix, figure 2.1d. Of these osteoblasts, most die through the process of apoptosis while the remainder are either embedded into the bone matrix to eventually become osteocytes, or become inactive bone lining cells. The final step of this remodeling cycle, quiescence, consists of osteoblasts reverting to their inactive form, bone lining cells, which cover the surface [Burr and Allen, 2013], figure 2.1e.
Figure 2.1 - Bone remodeling process. The bone remodeling process is shown here is 5 steps. These steps are a) activation where microdamage occurs in the bone triggering the remodeling cycle, b) resorption where osteoclasts begin resorbing the damaged bone, c) reversal where osteoclast resorption ends and osteoblast formation is initiated, d) formation where osteoblasts form new bone, and e) quiescence where the cycle is completed and the bone surface is covered again with bone lining cells [Burr and Allen, 2013].

In addition to repairing damaged bone, remodeling also allows for adaptation of the bone to changes in its mechanical environment. For example, bone formation occurs when localized strains exceed a certain threshold, and bone resorption occurs when strain falls below a given level [Frost, 1964; Haapasalo et al., 2000; Leblanc et al., 1990].
Both of these situations occur in concert in order to adjust the strain experienced by the bone back to physiologically acceptable ranges. These mechanisms as a whole allow for bone to remain in a constant state of repair and mechanical adaptation [Burr and Allen, 2013].

If the bone did not remodel on a very consistent basis, many issues would occur that could be detrimental. Since daily activities such as repeated loading on the bone can cause microscopic damage, if not repaired this damage can increase to a point of extreme bone fragility and eventual fracture [Burr and Allen, 2013]. This damage can parallel itself with loss of bone mass with advancing age further increasing fracture risk [Raisz, 2005]. Repair of these damaged areas in bone is important in maintaining bone strength and preventing morbidity fractures [Burr and Allen, 2013].

2.2 The Role of the Osteocyte

Understanding the basics of bone remodeling makes the function of the osteoblast and osteoclast cell types obvious, however the role of the third major bone cell type, the osteocyte, is less obvious. Among the three types of cells in the bone, the osteocyte is the most abundant [Burr and Allen, 2013]. Evidence increasingly supports the dogma that these cells respond to mechanical and hormonal cues to coordinate the response of osteoblasts and osteoclasts in bone remodeling [Ikeda, 2008; Robling et al., 2008]. Some indicators supporting the osteocyte’s role in sensing these changes include their high population and distribution across the whole bone volume in contrast to the
low population and limited, variable location of the other two cell types. The fact that the osteocyte is known to have a relatively long life, potentially decades, in comparison to other cells also alludes to this belief [Burr and Allen, 2013]. Osteocytes are located in the lacunae of the bone where they are able to ideally sense variations in mechanical signals such as fluid shear or strain in the bone. The lacunae then allow for the transportation of proteins secreted by osteocytes for the purpose of cellular interactions [Nicolella et al., 2006]. Finally, the osteocyte is not an effector cell in bone and lacks any real ability to add or remove bone matrix [Burr and Allen, 2013].

Many researchers have been able to show the importance of the osteocyte in bone remodeling [Ikeda, 2008; Heino et al., 2004; Robling et al., 2008; Turner and Pavalko, 1998]. Research has shown osteocyte specific protein changes in regions of new bone mass development as a result of mechanical loading in vivo [Moustafa et al., 2011]. In another study osteocyte networks in mice were completely ablated. These mice were unable to lose bone mass from a decrease in mechanical loading despite having normally functioning osteoblasts and osteoclasts [Ikeda, 2008]. Additional studies have shown that conditioned medium from the osteocyte MLO-Y4 line supports osteoblast differentiation, as well as mesenchymal stem cell differentiation [Heino et al., 2004]. Collectively, these observations support the theory that the osteocytes in bone sense mechanical load and relay this information to osteoblasts and osteoclasts to coordinate bone remodeling. However, the cellular mechanisms by which these cells communicate mechanical change remain unclear.
2.3 Wnt Signaling Pathway

In order to further investigate the cellular interactions of these cells during bone remodeling, it is important to understand the signaling pathway(s) that affect remodeling. One recently found signaling pathway known to relate to bone mechanotransduction and metabolism is the canonical Wnt signaling pathway, figure 2.2. With the use of lipoprotein receptor-related protein 5 and 6 (LRP5 and LPR6) and a heptahelical frizzled (FZD) receptor, Wnt is known to help promote survival of the transcription factor β-catenin [Richards et al., 2012]. β-catenin has target genes that are associated with enhanced osteogenesis and reduced resorption of bone [Burr and Allen, 2013]. Two documented antagonist for this pathway have been identified as sclerostin and Dickkopf 1 (Dkk-1) [Li et al., 2005]. It is believed that both of these proteins bind to LRP5/6 and inhibit the binding of Wnt, which leads to inhibition of osteoblast differentiation and bone formation [Raisz, 2005; Bonewald and Johnson, 2008; Baron and Kneissel, 2013], figure 2.2. Osteocytes specifically are known to express high levels of these inhibitory bone formation proteins. Protein levels are thought to be altered with changes in strain levels, and these proteins act as a means of communication between cells to initiate the bone remodeling process [Bonewald and Johnson, 2008].
Figure 2.2 – Wnt signaling pathway. This pathway is shown in the a) inhibited form where sclerostin (SOST) and Dkk-1 (DKK1) bind to LRP5 and LRP6 to prevent the binding of Wnt. This reduces survival of the β-catenin and leads to inhibition of bone formation and osteoblast differentiation. b) In the activated state, Wnt binds to LRP5 and LRP6 which promotes survival of β-catenin. This allows for activation of genes that lead to enhanced bone formation and reduced resorption of bone [Richards et al., 2012].

2.4 Current Models

In order to investigate and understand the complex mechanics that regulate bone mechanotransduction, researchers have developed many methods to load and
then measure bone response. These studies can be grouped into two the major categories, *in vivo* and *in vitro* models.

A variety of *in vivo* models have investigated the impact of mechanical stimuli on bone remodeling [Turner et al., 1995; Rubin and Lanyon, 1984; Moustafa et al., 2011]. These models were able to show the effects of external strains on living bone; yet they were unable to elucidate the specific cellular response created by strains in bone remodeling. Where understanding the overall reaction of bone to loading in an *in vivo* system can help us to understand the general response, useful cellular results typically need to be teased out of the large amount of data produced. With all of the other complex systems of an organism also influencing the results, it is practice to reduce the noise from systems outside the scope of the project by performing these experiments in a relevant *in vitro* environment.

*In vitro* experimentation allows for reducing uncontrolled factors so that specific cellular responses can be more easily controlled for and observed [Vignais and Viganis, 2010]. For this reason, *in vitro* experimentation is needed in order to understand the true cellular mechanics behind bone remodeling and bone cell interactions. One major consideration in this type of research comes with using appropriate loading patterns which mimic native loads experienced by the cells *in vivo*. 
Due to their location, osteocytes are known to be exposed to many types of mechanical forces during standard loading including fluid shear, strain, and compression. Due to the complexity of native loading patterns, the key type of load to elicit a cellular response in bone remodeling remains a complex subject. Of these loads, fluid shear has historically been accepted as the most influential load for eliciting a mechanotransduction response in these cells [Tzima et al., 2005; Pahakis et al., 2007].

Fluid shear models for applying load to bone cells are well established; however researchers must constantly acknowledge weaknesses and limitations of these systems. One issue with this model includes the calculations used to derive shear stress in the chamber. These calculations are made sans the cells and fail to consider how the flow at a cellular level might be affected by cell size, adhesion, density, and membrane viscoelasticity. Developing flow around the slits is also not acknowledged, so what ends up being derived is an equation for the average shear stress in the empty chamber of known dimensions [Tzima et al., 2005; Pahakis et al., 2007]. Finally, these systems hold millions of cells producing an averaged response of the cells. In order to truly understand the cellular response of these cells to loads, smaller populations of cells need to be tested at more well established loads. For this reason, improved systems need to be developed for more advanced bone research.

In order to create an improved loading model for bone cells, a physiologically relevant loading pattern must be utilized. A model developed by Han et al. describes
how, due to cellular tethering to the lacunae-canalicular walls, cells deflect and experience tensile strain from the fluid movement. This can cause a cellular strain amplification of up to 100 times that of the measured bone strains [Han et al., 2004]. Utilizing digital micrograph correlation strain measurements, researchers were able to show local strain on the lacunar wall several orders of magnitude higher than what is displayed on the surface of the bone [Nicolella et al., 2006]. Both of these studies allude to the possibility of direct strains displayed on the cell much higher than previously suggested. For these reasons, the effects of direct strain on these cells may be under-exaggerated in comparison to what was previously assumed.

2.6 Current Work

In order to further understand the science behind bone cell interactions and response to mechanical load, a model for loading bone cells was created in this work. This model was designed and fabricated in order to load bone cells at physiologically relevant conditions. This model consisted of a profile for cellular growth and adhesion, and a pure uniaxial loading device designed for the specific cellular loading requirements outlined in this study. For validation of this model for bone cell mechanotransduction studies, a series of experiments were performed on the cells. First, in order to understand the ranges where these cells start to respond to mechanical strain, strain was applied to cells adhered to the polydimethylsiloxane (PDMS) substrates designed with a range of thicknesses which introduced the cells to a strain
gradient under identical culture conditions. The activity levels of these cells were quantified utilizing a LDH assay, an indicator of cellular energy conversion, and strain regions correlating to high and low levels of activity were found. Cells on uniform thickness PDMS substrates were then exposed to these high and low activity level strains, and soluble activity of the cells was measured using standard ELISA kits. This allowed for correlation of the applied substrate strain to soluble activity and showed soluble protein levels changing in agreement with other current research. This validated the model’s success for this type of research.

2.7 Specific Aims

Aim 1 – Development of a Multi-Strain Profile for Cellular Mechanotransduction Testing. Designed a substrate that allows for application of a range of substrate strains to adhered cells cultured under identical culture conditions

Aim 2 – Design, Fabrication, and Characterization of a Pure Uniaxial Microloading System for Biologic Testing. A loading device was designed for pure uniaxial mechanical stimulation of cells adhered to a substrate while maintaining physiologic conditions.

Aim 3 – Soluble Protein Activity of Osteocyte Cells Stimulated by Pure Uniaxial Loading. Validated that the model could successfully stimulate cells and allowed for critical cellular measurements. This was done by first stimulating cells and finding critical strains
where cellular activity levels peaked. Then these strain values were used to find changes in soluble activity of critical inhibitory bone formation proteins.
CHAPTER III

DEVELOPMENT OF A MULTI-STRAIN PROFILE FOR CELLULAR MECHANOTRANSDUCTION TESTING

3.1 Introduction

Mechanotransduction is a field of study that focuses on understanding the translation of mechanical stimuli to cellular responses [Ingber, 1997]. This field is important to biomedical research due to the fact that tissues develop in environments with constant physical forces surrounding them such as gravity, movement, and pressure [Ingber, 1997]. These forces can help to shape and control the function of these tissues during cellular activities such as differentiation, migration, and signal transmission [Katsumi et al., 2004]. Many cells are known to be mechanosensitive in this sense including cartilage [Ramage et al., 2009], smooth muscle [Hisada et al., 1993], endothelial [Hoyer et al., 1996], and bone [Orr et al., 2006]. Where some cellular mechanisms related to mechanotransduction have been discovered [Orr et al., 2006], many have yet to be elucidated due to the staggering complexity of the cellular interactions. Many of these complexities come from the in vivo environment in which these cells develop. For this reason in vitro experimentation on these cells and tissues
can help to isolate specific cellular responses under quantifiable mechanical stimuli [Brown, 2000].

Creating in vitro mechanotransduction models enables simulation of cellular responses to mechanical stimuli in a controlled, physiologically relevant, and accurate manner. Many variations of these systems exist with each having their own advantages and disadvantages depending on the need of any specific experiment. These include systems for applying longitudinal stretch, out-of-plane circular substrate distention, in-plane substrate distention, and fluid shear [Brown, 2000]. In many of these systems cells are grown on a substrate that is loaded into the system where average cellular response to stimuli can then be measured. For this reason it is important to consider a substrate’s mechanical nature in order to assess if it will be ideal for specific experimental parameters. For example, with a fluid shear model cells are generally seeded onto a rigid glass slide that forms the base of a parallel plate chamber [Tzima et al., 2005; Pahakis et al., 2007], while in-plane substrate distention utilizes a more flexible, generally silicone, substrate for seeding the cells. This difference in material is due to the distinct difference of how the mechanical stimuli are applied [Stotoudeh et al., 1998].

In addition, current in vitro mechanotransduction systems may not accurately represent net cell responses. For example, typical loading of bone and endothelial cells is performed using fluid shear models where fluid flow occurs over a monolayer of millions of cells [Bacabac et al., 2004; Young et al., 2001]. This type of cellular response characterization averages the response from many cells instead of the exact response of
a cell to a known strain. In order to better understand this cellular response to strain, models which use fewer cells are needed in order to find a specific response instead of an average. For these reasons a system is needed to apply precise, known mechanical stimuli to small cell populations in order to quantify the cellular response in a more exact and meaningful manner. The first step is creating a model to mimic a more precise cellular response.

In bone research there is much interest in understanding the relation between mechanical stimuli and cellular response. This is due to the known reaction that bone has to mechanical load. This relationship is outlined by Wolff’s Law and known as mechanical adaptation where an increase in mechanical loading on the bone leads to an increase of bone mass and strength while a decrease in loading on bone leads to a decrease in bone mass and strength [Burger, 1993]. In vivo studies have replicated this reaction in both human and animal models [Szefek et al., 2010; Maïmoun and Sultan, 2011]. Even though the general idea that bone cells react to mechanical stimuli is well established, many of the underlying cellular mechanisms that allow for this mechanism to occur remain unclear.

In order to understand the strain where these mechanosensitive cells react we need to first observe the cells’ reactions to different strain gradients. This will then allow for the ability to identify the more specific strains in which mechanotransduction is measureable. For this reason a unique profile was formed from elastomeric polydimethylsiloxane (PDMS). This material was chosen due to its optical transparency, ease of fabrication, microscale features, and elastic nature. This substrate has been
shown to support cellular growth, specifically with osteocytes [York et al., 2012], and with a variety of cross-sectional thicknesses, allows for a range of strains to be applied to these cells under identical culture and loading conditions. With a well characterized substrate such as this the ability to view cellular response initiation becomes possible. This study analyzed two design profiles and characterized the strains displayed on each profile with respect to the overall applied strain.

3.2 Methods

This study focused on characterizing two profiles designed to create a range of strains under small overall displacements. Numerical (Finite Element Analysis (FEA)) and experimental testing were performed to characterize the response of varying profile thickness as a function of overall strain.

3.2.1 Profile Design

The two profiles created for this testing were designed to be loaded utilizing an uniaxial load and created strain ranges over the substrate. Both of the designs were the same length and width with the side profile being the only difference between the designs, figure 3.1.
Figure 3.1 - Side profile view of the two designs with steps on Profile A ranging from 1.778 – 3.81mm and steps on Profile B ranging from 0.635 – 2.667mm. Profile width was set at 12.70mm with a step length of 2.54mm.

Profile A was designed with steps which vary from a larger cross-sectional area that decreased linearly down to the smallest cross-sectional area. Profile B was designed in order to keep the uniaxial load along the centerline of the profile. This profile was intended to produce a more uniform strain along the profile. This design incorporated staggered step sizes where the middle step had the smallest cross-sectional area followed by the step directly to the right of the middle step and then followed by the one to the left. The steps increased in size in this manner until they reached the clamp area. The plus and minus sign on the side of the profile indicates which side had the larger of the staggered steps. In order to relate the data to the step cross-sectional area the steps were numbered with 1 correlating to the step with the largest cross-sectional area and 9 correlating to the step with the smallest cross-sectional area, figure 3.2.
3.2.2 Fabrication

The molds to create the profile designs were made using Solidworks 2013 and saved as stl files. The stl files were sent to Fineline Prototyping (Wilmington, NC) for stereolithography using Phototherm, a high temperature resistant material. Once the top and bottom molds were made they were assembled together and clamped in place. Then a silicone elastomer, PDMS (Dow Corning, Midland, MI), mixed 10:1 with a cross-linking agent was mixed, degassed and poured into the assembled mold and cured at 80°C for 12 h in a convection oven. Following curing the molds were disassembled and the cured PDMS parts were taken out of the molds and washed with acetone and isopropyl alcohol and air dried.
3.2.3 Finite Element Analysis (FEA)

Models for both profile designs were generated in Solidworks 2013. Using this same software and the material properties from mixtures gained from a previous study [Shah et al., 2013] FEA simulations were performed for both profiles. Table 3.1 displays the properties used to define the PDMS material for the simulations.

Table 3.1 - PDMS material strength properties obtained from previous study [Shah et al., 2013] were used for FEA simulations.

<table>
<thead>
<tr>
<th>PDMS Material Properties</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastic Modulus (MPa)</td>
<td>14.47</td>
</tr>
<tr>
<td>Poisson’s Ratio</td>
<td>0.49</td>
</tr>
<tr>
<td>Mass Density (kg/m³)</td>
<td>0.97</td>
</tr>
<tr>
<td>Ultimate Tensile Strength (MPa)</td>
<td>2.59</td>
</tr>
</tbody>
</table>

The models for both profiles were designed with a 22.86 mm gauge length so that all of the steps on the profiles were exposed and experienced the strain. Restrictions were placed on either end of the profile to simulate the friction clamps holding the sample in place. These restrictions consisted of one end having fixed nodes for both of the surfaces that were in contact with the clamp figure 3.3a, and the opposite end had rollers on both of the surfaces with which the clamp was in contact figure 3.3b. This allowed for both of the simulated clamps to remain on axis during loading simulating the physical tests in the loading system. A remote displacement was used to simulate the applied strain to the profile. This remote displacement was applied
to the whole surface where the friction clamp contact was simulated with rollers figure 3.3c. This location was chosen to simulate a no slip condition in the boundaries of the friction clamp at any time during testing. Remote displacements of 0.009 and 0.0225mm, 1 and 2.5% strain respectively, were applied to the models. The node and element properties for the models are displayed in table 3.2.

Figure 3.3 - FEA model restrictions a) fixed points on model, b) roller points on model, c) applied remote displacement. Remote displacements were set for 1.0 and 2.5% overall strain in order to simulate strains thought to be experienced by bone cells in vivo.

Table 3.2 - Node and element properties in the FEA model. Models were set with the maximum resolution for the FEA mesh.

<table>
<thead>
<tr>
<th></th>
<th>Degrees of Freedom</th>
<th>Elements</th>
<th>Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profile A</td>
<td>361,995</td>
<td>119,444</td>
<td>86,045</td>
</tr>
<tr>
<td>Profile B</td>
<td>248,247</td>
<td>57,740</td>
<td>85,767</td>
</tr>
</tbody>
</table>
3.2.4 Physical Test

Physical tests were performed with the stepped profiles in order to quantify the strains displayed on each step with relation to overall strain. A gauge length of 22.86 mm was used so that all of the steps on the profiles were uncovered by the friction clamps holding them into the system. Strain rates of 2.08 mm/sec were used to apply the strains. Samples were loaded on a small-scale testing machine via poly-carbonate friction clamps built in-house [Saunders and Donahue, 2004]. These samples were loaded to 2.5% strain with strain measurements taken for each step at overall strain increments of 0.5%.

To measure the strains during loading a non-contact method was utilized. This method used a camera (8 megapixels at 30 fps) mounted perpendicular to the stepped profile during loading. Video of the experiments was recorded and frames from the video were extracted at desired overall strains using Microsoft Movie Maker 2012. Using ImageJ software (NIHImage, Bethesda, MD, USA), strains were calculated by measuring the change in length of each step 5 separate times and comparing those values to the original length of the step. This measurement resulted in the strain per step while the samples had an overall applied strain. This testing was performed on 4 samples per profile.
3.3 Results

Results were obtained for both the experimental as well as the physical tests and displayed separately.

3.3.1 Finite Element Analysis (FEA)

Data collected from these tests included both the qualitative images of the strain fields and deformations during loading, as well as the average strains per step during loading for all loading conditions. Figure 3.4 displays the side profile of the samples under a 2.5% overall strain with strain gradients normalized to each other between 0-11% overall strain.

Figure 3.4 - Side view of profiles during FEA loading. The color gradient relating to the strains (displayed on the right) was set from 0.0% strain at the low end of the scale (blue) to 11.0% strain at the high end (red).
The gauge length was set for the entire area not covered by the friction clamps during loading. In figure 3.5 a top projected view of the profiles is displayed with strain gradients normalized to each other between 0.0-11.0% overall strain. The average strain on each step was measured for each overall testing strain. This data is presented in figure 3.6a and 3.6b.

Figure 3.5 - Top view of profiles during FEA loading at 2.5% overall strain. Color spectrum of strains set the same for both designs ranging from 0.0-11.0% strain.
Figure 3.6 - Resulting average strain per step during overall strains of 1.0 and 2.5% for a) Profile A and b) Profile B. Both profiles displayed increasing strain with decreasing cross-sectional step thickness.

3.3.2 Physical Test

From the frames extracted from the video taken of the loaded profiles, strains from each step were measured using distance measurements made in ImageJ. These strains were recorded for all samples and averaged. In order to view the data in a more appropriate manner the data was grouped into bins that were defined by the thickness of the cross-sectional area. The bins consisting of the 3 thinnest steps, the 3 thickest steps, and the steps ranging in the middle thicknesses, table 3.3.
Table 3.3 - Bin contents. Thickest bin contained the 3 steps with the thickest cross-sections (steps 1-3), the middle bin contained the 3 steps with the middle thickness cross-sections (4-6), and the thinnest bin contained the 3 steps with the thinnest cross-sections (7-9).

<table>
<thead>
<tr>
<th>Thickest Bin</th>
<th>Middle Bin</th>
<th>Thinnest Bin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steps 1, 2,</td>
<td>Steps 4, 5,</td>
<td></td>
</tr>
<tr>
<td>and 3</td>
<td>and 6</td>
<td>Steps 7, 8,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and 9</td>
</tr>
</tbody>
</table>

The data was plotted and regression lines were fit for each bin data set, figure 3.7a and 7b. The thick and thin bin data were then compared at both the low level (0.5%) and the high level (2.5%) strains for both profiles using a standard two-tailed t-test, figure 3.8. To understand how the PDMS responds to higher strains several samples of uniform thickness were loaded out to failure and strain was measured as a function of completion to sample failure, figure 3.9.
Figure 3.7 - Average measured physical strains for different thicknesses on a) Profile A and b) Profile B. The physical strain results were placed into the same 3 bins as the FEA results with blue corresponding to the thinnest bin, the orange corresponding to the middle bin, and the gray corresponding to the thickest bin.
Figure 3.8 – Comparison of strains between the thick and thin bins on both profiles.

Comparisons were made using standard two-tailed t-tests. Results showed significant differences at a 95% confidence interval in the strains displayed in the thick and thin bins for a) Profile A at 0.5% overall strain (P = 0.0012), c) Profile B at 0.5% overall strain (P = 0.0011), and d) Profile B at 2.5% overall strain (P = 0.0395).
Figure 3.9 - Uniform thickness PDMS samples loaded to failure. It was observed that the 11 samples tested produced linear strain results at up to 25% completion of the loading to failure.

3.4 Discussion

One notable difference between the two profile designs existed in the side profile view under strain from the FEA model, figure 3.4. Here it was observed that while Profile B had no bending off axis from the load, Profile A had a very distinct bend as the strain was applied. This bend did not significantly affect the average strains on the steps which increased with decreasing cross-sectional step area. The bending is noted due to the possibility of introducing the cells on these steps to not only tensile forces from the strain, but to compressive forces from bending.

From the physical testing it can be noted that Profile A reacted under load counterintuitive to what was anticipated. The general assumption for strain was that as cross-sectional step area increased the strain would decrease under identical loading
conditions. And conversely as cross-sectional step area decreased the strain would increase under identical loading conditions. On Profile A in figure 3.7a it was observed that during the physical testing of this profile the strain increased with an increase in cross-sectional step area. It was also observed that at the higher overall loads (2.5% overall strain) there was no significant difference in the strains displayed by the steps in the thin and thick bins for Profile A, figure 3.8b. This was not observed in Profile B, showing that at these low strains the geometry of Profile A exhibited a less desirable strain range. At higher overall strains it is believed that this is not an issue due to PDMS having a more linear nature at higher strains, figure 3.9. This abnormal strain behavior may be corrected by altering the manner in which the profile is clamped into the system. One example of this could be to alter the clamp locations such that its contact is with the first and last step. However for the purpose of cell stimulation, it is critical to characterize the mechanical performance at the low strain ranges (<5%).

Due to the use of low strains to create a more physiological relevant strain range in our applications, Profile B performed in a more desirable manner. It is important to note that where the profiles did not display a simple linear curve that correlated step cross-sectional area to average step strain, such as the low strain ranges, mechanical characterization was possible. The goal of this work was to investigate various profile geometries to select a substrate that would allow for a range of strains to be reproducibly applied to seeded cells in a physiologic range. With this goal, Profile B was superior to Profile A.
CHAPTER IV

DESIGN, FABRICATION, AND CHARACTERIZATION OF A PURE UNIAXIAL MICROLOADING SYSTEM FOR BIOLOGIC TESTING

4.1 Introduction

The body is continually in a state of loading. As such, cells at the microscopic scale reside in highly dynamic environments and are subjected to a variety of loading modes [Ingber, 1997]. The loads are believed to be critical to cell development, function, and survival. For example, cell differentiation, migration, and signal transduction are influenced by mechanical loading [Katsumi et al., 2004; Riehl et al., 2012]. Mechanical forces are particularly critical to bone cells, and it is known that mechanical forces at least in part, regulate bone remodeling [Burr and Allen, 2013; Li et al., 2013; Van Oers et al, 2015]. Given that the mechanisms and pathways by which bone cells coordinate activity in response to normal loading have yet to be elucidated, research aimed at understanding the role of load in bone disease (eg, osteoporosis), overload and disuse must first focus on understanding the role physiologic loading plays in bone adaptation. In vivo these mechanisms can be difficult to test and observe. In vitro systems offer the opportunity to investigate these mechanisms and pathways of interest in an isolated, simplified environment [Brown, 2000; Davis et al., 2015].
To mechanically load cells in a biomimetically appropriate environment, specialized platforms are often fabricated in-house [Riehl et al., 2012; Li et al., 2013; Keyes et al., 2011; Huang et al., 2013; Kim et al., 2012; Delaine-Smith et al., 2015; Wang et al., 2013; Iwadate and Yumura, 2009; Huang et al., 2015; Saunders and Donahue, 2004]. Commercial platforms can be cost prohibitive and customization is highly dependent upon user expertise [Wang et al., 2013; Iwadate and Yumura, 2009; Huang et al., 2015]. Most commercial loading machines work off the principle of a fixed platform with one mover on a central axis. During testing, specimens are clamped in fixtures such that one end of the specimen is fixed to the stationary base of the platform and one end is attached to the mover. As such, tension/compression testing in a commercial machine is not pure, or equal and opposite. Since the exact mechanical loading patterns experienced by cells are difficult to replicate, creating the most uniform loading possible, such as in pure loading, is important. This will begin to allow us to correlate cellular activity to specific loading patterns. Ultimately, the goal would be to adequately simulate and verify complex in vivo loading scenarios. The goal of this study was to design, fabricate, and characterize performance of a microactuated pure uniaxial loading platform for straining bone cells seeded on polydimethylsiloxane (PDMS). However, given that the platform was intended as a generic system, it could be used to test a variety of small specimens in pure tension/compression. As such, once fabricated device performance was initially characterized/validated with suture testing.
4.2 Methods

A pure uniaxial platform was designed. The device centered around 2 commercial microactuators, 2 commercial load cells, and 1 rail/carriage block system. The microactuators (Zaber Technologies) were selected to provide the necessary resolution and smooth motion. To accommodate a variety of potential uses, 2 sets of microactuators were purchased with either a 30mm (NA11B30) or 60mm (NA11B60) travel range. Both models had a 67 N force capacity, 58 N peak thrust, 0.9302 μm /s speed resolution, and either a 25 μm (NA11B30) or a 36 μm (NA11B60) unidirectional accuracy. The microactuators utilized a precision lead screw drive mechanism, stepper motor, and a 24 V controller (A-MCA, Zaber Technologies). Canister load cells (44.5 N, Honeywell, Sensotec) were selected for their small profile, robustness, and user familiarity. The rail/carriage block system (9184T31, McMaster Carr) was selected as a convenient and cost effective way to ensure pure uniaxial loading on center. The maintenance-free system uses two ball bearing carriages that ride on a 15 mm wide track, has a 7800 N dynamic load capacity, and is corrosion resistant (400 series stainless steel). The commercial components were purchased and the platform was designed to accommodate them.

The goal of the platform was to apply equal, uniaxial strains (linear displacement) to small-scale specimens in an accurate and reproducible manner. This included ensuring that pre-load was not applied during manipulation of the samples pre-testing. In addition the platform was designed to accommodate synthetic (fibers)
and biologic (tissues) materials. For the latter, an environmental chamber and fluid reservoir were fabricated. A horizontal loading approach was selected for convenient submersion of the samples during testing given that a major focus of the device was cell stimulation research.

4.2.1 Device Main Wall

The dimensions of the commercial components dictated the design and scale of the platform. All fabricated components were machined from stock aluminum 6061-T6. The basic design consisted of a base plate and 2 side (left and right) plates, figure 4.1a-b. A track was machined into the base plate to accommodate the thickness of the side plates and maintain alignment, figure 4.2a-c. Clearance holes were drilled through the bottom of the base plate in the track to secure each side plate via holes that were drilled and tapped. A track was machined into the front face of each of the side plates to mount the rail of the commercial rail/carriage system. In addition, a series of drilled and tapped holes on the front face flanked the track to accommodate the actuator mount, figure 4.2b. These holes allowed for placement adjustability of the actuator mount and spanned the entire length of each side plate. This allowed objects ranging in gauge length from 1 mm to several cms to be tested on the same platform. The commercial rail was attached to the track using the mounting (clearance) holes in the rail and drilling and tapping holes in the side plate track. The rail was mounted stationary between the side plates. The 2 side plate approach in comparison to 1 large side plate was selected.
to provide clearance in the event that an overhead camera was required in future testing. The rail/carriage system was purchased as a unit and consisted of 1 rail and 2 carriages. Each carriage mounted one actuator to the rail.

Figure 4.1 – Pure uniaxial loading device and friction clamps. a,b) A pure uniaxial loading platform was designed around commercial components and fabricated from aluminum 6061-T6. c-e) Friction clamps with polycarbonate brace to eliminate unintended loading effects during manipulation of the sample before experimentation.
Figure 4.2 – Device main wall and rear actuator mount. a) Main wall of the device includes b) a slot for alignment of the guide rail and actuator mounts. c) Left (shown) and right side plates connect to the base plate using holes on the underside of the base plate and a track for alignment. d-f) Rear actuator mount for attaching actuators to the side plate machined from aluminum angle stock. e) A key was used to align the mounts to the side plate. f) Slots allow for simple adjustment of the actuator position while remaining in-line with the guide rail.
4.2.2 Friction Clamps and Brace

Although the platform can be used for pure compression testing, it was designed for tensile loading of soft tissues, synthetic fibers, and cell-seeded elastomers. To this end, serrated friction grips were fabricated for tensile testing specimens; compression platens were not fabricated. The serrated grips, figure 4.1c-e, consisted of a left and right fixture with each fixture comprised of a top and bottom piece. The fixtures were machined from Plexiglas and the serrations were milled on the face using a double angle cutter (90°). Given that a major purpose of the platform was to subject small tissues and bone cells seeded on PDMS to precise strain, there was concern that the manipulation loads applied to the specimens in setting up the test could exceed those applied during the test. To avoid unintended pre-loads, a polycarbonate brace was developed to mount between the right and left fixtures. For cell work, the PDMS substrates were clamped in the fixtures and the cells were then seeded on the substrate. Stainless steel screws were used and 5 sets of the fixtures and braces were fabricated to accommodate preparation of multiple samples at the same time. Fixtures were able to be sterilized with alcohol and UV light before each use.

4.2.3 Actuator Mounts

Each actuator used two mounts, a rear mount and a front mount. The stationary rear mount, previously described, connected the actuator body to the side plate via the
track, figure 4.2. The front mount attached the tip of the actuator to the carriage block via connectors that accommodated the load cells, figure 4.3a-c. The front and rear mounts were machined from 6061-T6 aluminum 90° angle stock. To ensure reproducible alignment of the angle stock in the track (rear mount), a key was made on the underside of the mount by adding a rectangular plate to the base, figure 4.2e. The plate rode in a track machined to maintain alignment. This was done to maintain alignment, ensure in-line linear movement of the actuators, and minimize user variability in setup. The slotted rear mount enabled the microactuator to be attached anywhere along the side plate.
Figure 4.3 – Front actuator mount and slide arm. a-c) Connectors for attaching load cells to the actuator and front actuator mount. b) Nuts were fabricated due to the small working space. d) Adjustable slotted plate fixture for elevating or suspending samples into a heated media bath during testing. e) The holder had a track machined to provide a recess to hold the Plexiglas friction clamps.
In addition to the front actuator mount providing actuator support and alignment, it also held a mounted plate with a vertical track, figure 4.3d-e. The vertical track provided alignment and held the slotted plate to which the specimen fixtures attached. The vertical slot in the plate enabled adjustable clamp height, figure 4.3d. This was done to enable testing of dry and wet specimens. At the lowest point on the track, the specimen can be completely submerged in a bath (cell medium, saline) and environmental chamber controlling for temperature, pH, and humidity. Once setup was complete, the plate was locked in position for the duration of the test. Feeler gauges and gauge blocks were used in combination to set height on the right and left fixtures.

4.2.4 Load Cell Attachments

To accommodate the thread sizes of the commercial load cells and enable attachment to the actuators, round aluminum stock was drilled and tapped on end to accept either the threaded actuator tip or the load cell connector, figure 4.3c. To anchor the tip of the microactuator to the carriage block one of the round connectors was turned down to provide the clearance and alignment to the front actuator mount. A crude nut with flats was machined to anchor the assembly given the confined space, figure 4.3b-c.
4.2.5 Actuators

Once assembled, the platform was run by controlling microactuator motion. The microactuators were controlled using the computer software Zaber Counsel. The proprietary software utilized code written in C Sharp, Visual Basic, or Java. Codes were written in order to move specified distances based on desired strain.

Microactuator performance was characterized for travel distance, speed, and cyclic temperature. To verify travel accuracy, an arbitrary distance was input and physical measurements of actuator tip displacement were made using feeler gauges with distances ranging between 254-2540 μm. These distances were chosen to represent the desired 1-10% strain range used in our cell work. Speed tests were performed using arbitrary speeds and timing actuator motion. Input speeds ranged from 1-28000 μm/s with actuator travel ranging from 10-56000 μm. Speeds and distances were selected to span the range of actuator performance. For each speed test actuators were extended and retracted in one full cycle. The actuator travel time was measured and compared to the calculated travel time from the inputted speed and distance. To verify overheating was not a concern, each actuator was cycled for 1 hr at maximum speed and distance with temperature recorded at 5 minute intervals. All tests were repeated a minimum of 3 times.
4.2.6 Device Characterization Testing

To verify accurate and reliable performance of the device, suture break testing was conducted using the new platform and the results compared to identical tests conducted in a uniaxial loading machine developed in-house and previously validated [Saunders and Donahue, 2004]. Suture was utilized to establish proof-of-concept for this device due to its simple and consistent geometry. For this test, 10 Ethicon 2-0 silk suture samples were loaded to failure in each loading device. Samples were knotted with 3 knots tied on top of each other to create a stress riser and concentrate failure to the knot and away from the fixtures. To minimize any fixture variability, the fixtures fabricated (figure 4.1c-e) were used in both loading devices. A gauge length of 25.4 mm was used (knot placed centrally) with a loading rate of 1.22 mm/sec. Given that the in-house machine previously developed utilized one mover and a fixed base, the pure uniaxial platform was first tested locking in one actuator to simulate the fixed base. The load was measured using a 44.5 N load cell. The failure performance of the two sets of sutures was compared statistically for stiffness, failure load, and displacement at failure using two-tailed Mann-Whitney t-tests.

For pure uniaxial testing, 10 sutures were loaded to failure. Both actuators were used at ½ speed of 0.61 mm/s to generate a pure load on the sample at the same rate used in the previous tests. Data was collected using two 44.5 N load cells. Samples were knotted in the same manner and the same 3 parameters were measured.
4.3 Results

Microactuator distance and speed performance tests were analyzed using linear regression. In both cases, an $r^2$ value > 0.999 was obtained when an intercept of 0 was set. The results were also compared to the input values to find deviation, table 4.1. Results for the distance tests remained under 4% deviation from the input, and results for speed tests remained under 10% deviation from the inputs. The temperature tests determined that the actuator temperatures never exceeded 40°C over the course of the 1 hr tests with an observed maximum temperature of 39.9°C.

Table 4.1 - Average actuator distance and speed accuracy test results. Standard deviations calculated from result’s deviation from input.

<table>
<thead>
<tr>
<th>Distance Accuracy</th>
<th>30mm Actuators</th>
<th>60mm Actuators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input Distance (µm)</td>
<td>Measured Distance (µm)</td>
<td>Deviation from Input (%)</td>
</tr>
<tr>
<td>254</td>
<td>263.2</td>
<td>3.6</td>
</tr>
<tr>
<td>635</td>
<td>641.4</td>
<td>1.0</td>
</tr>
<tr>
<td>1270</td>
<td>1260.7</td>
<td>0.7</td>
</tr>
<tr>
<td>2540</td>
<td>2536.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Speed Accuracy</th>
<th>30mm Actuators</th>
<th>60mm Actuators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input Distance (µm)</td>
<td>Input Speed (µm/s)</td>
<td>Calculated Time (s)</td>
</tr>
<tr>
<td>1,000</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>20,000</td>
<td>10,000</td>
<td>40</td>
</tr>
<tr>
<td>30,000</td>
<td>15,000</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>1,000</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>56,000</td>
<td>14,000</td>
<td>8</td>
</tr>
</tbody>
</table>
For suture break comparisons, a representative load-displacement curve from samples broken using each loading platform is provided, figure 4.4a. Each sample tested failed at the knot located in the center of the gauge length. Stiffness, maximum load, and displacement at failure were used to compare results between devices. These measurements showed no statistically significant differences at a 95% confidence interval between the samples as a function of loading machine, figure 4.4b-d. Once it was determined that the pure uniaxial platform when operating as a fixed end platform performed similarly to a fixed end loading platform, the pure uniaxial performance was evaluated. As shown in figure 4.4e, identical load-displacement curves were generated for the two actuators during application of a pure load. In addition, failure loads, stiffness, and displacements were similar to those found in the previous tests. Statistics were not conducted between the fixed axis end and pure uniaxial tests as the comparisons were not appropriate.
Figure 4.4 – Device characterization data. a) Representative data from the suture break test showed b-d) no significant difference in the max load ($p = 0.8684$), stiffness ($p = 0.4637$), or displacement at failure ($p = 0.9094$). e) Pure uniaxial load-displacement curve generated from suture testing to failure.
4.4 Discussion

The goal of this study was to design, fabricate, and characterize a pure uniaxial loading device for small-scale specimens, such as fibers, tissues, and cell-seeded elastomers. This device was designed to be simple, reliable, and cost-effective with the flexibility to grow with a user’s testing needs. Loading platforms such as these are an important part of biomedical research laboratories. Here we demonstrate the development of a platform motivated by a biologic need. From the tests outlined in this study, we were able to design, construct, and validate an accurate, reproducible, and therefore reliable small-scale pure uniaxial loading device. The device enables equal and opposite loading of synthetic and biologic specimens on a horizontal axis in wet and dry environments. The microactuator characterization verified the necessary precision required for our intended work and that system performance adequately applies pure uniaxial load to small-scale specimens.

From comparison of the suture results from the fixed axis tests it was determined that the devices were comparable in performance given that there was not a statistically significant difference in structural properties. From the results of the pure uniaxial loading it was determined that load cell performance was identical. This further suggests that pure uniaxial testing was accomplished, including adequate alignment, and synchronization of the microactuator travel.

The testing of small-scale specimens in the biomedical engineering field is becoming routine. However commercial systems can be cost-prohibitive when
investment is weighed against intended use. For example, the FX-4000 Flexcell and STB-150 Strex systems cost more than $10,000 [Iwadate and Yumura, 2009] while the Bose ElectroForce BioDynamic Test Instrument can exceed $50,000 [Huang et al, 2015]. As such, more and more researchers are developing their own systems to meet their mechanical testing needs within budgetary constraints. Inasmuch as the machining was done in-house using aluminum stock, the only expenses incurred were the cost of the commercial components which were under $4000.00. Given that these commercial components meet the most critical needs of the fixture, reliable movement, load, and guaranteed alignment, it is not overly challenging to design and fabricate these platforms. Platform design essentially reduces to physically connecting the commercial components. Furthermore, in stimulating scaffolds and sheets, such as required in soft tissue research, planar biaxial loading is utilized. A pure uniaxial system could be augmented with a second system to create a cost-effective biaxial machine. Utilizing similar approaches, a wide variety of specialized platforms and machines can be fabricated to meet user-specific testing needs.
CHAPTER V
SOLUBLE PROTEIN ACTIVITY OF OSTEOCYTE CELLS STIMULATED BY PURE UNIAXIAL LOADING

5.1 Introduction

In the body, bone is known to be an organ with the unique ability to constantly repair and remodel itself to meet physiologic and mechanical demands [Burr and Allen, 2013]. This function is, in part, controlled by the bone’s ability to transduce mechanical signals to cellular responses. This translational ability in cells is known as mechanotransduction [Ingber, 1997]. Mechanotransduction is theorized to allow bone cells to perform standard maintenance through bone remodeling [Turner and Pavalko, 1998].

Bone remodeling is the process by which the resorption of bone by osteoclasts and the formation of bone by osteoblasts occur sequentially in a coupled manner on a given bone surface [Burr and Allen, 2013]. It is well understood that by utilizing this ability, bone adjusts the strain experienced back to physiologically acceptable levels [Cervinka et al., 2014; Falcia et al., 2015; Frost, 1986]. This mechanism allows for bone to remain in a constant state of repair and mechanical adaptation [Burr and Allen, 2013].
Evidence increasingly supports the idea that the osteocyte cells in bone respond to mechanical and hormonal cues to coordinate the response of osteoblast and osteoclast cells [Bellido, 2014; Burr and Allen, 2013; Robling et al., 2008]. Due to the osteocytes’ location in the well-distributed lacunocanalicular system of the bone, they are able to ideally sense variations in mechanical signals. Osteocytes are exposed to many types of mechanical forces during standard loading including fluid shear, strain, and compression. How these loads elicit a specific cellular response in the bone remodeling process is unknown. Fluid shear has historically been accepted as a major loading mode for inducing mechanotransduction in these cells. While this type of loading may be accepted as having a predominant role in generating a cellular response, the contribution and combination of other loading modes is still unknown [Burr and Allen, 2013]. To confound the situation, primary mechanical loading can induce secondary fluid shear. Studies have addressed the strain experienced by the osteocyte within the lacunocanalicular system and have estimated that the direct strains applied to the osteocyte during loading may be orders of magnitude higher than previously assumed [Han et al., 2004; Nicolella et al., 2006]. For this reason it is important to also consider the effects of direct loading on cells in this type of research.

In understanding the cellular interactions during bone remodeling, the influential signaling pathway needs to be addressed. A more recently identified signaling pathway known to regulate, at least in part, bone mechanotransduction is the canonical Wnt signaling pathway. Two major inhibitors of this pathway, and bone formation, are sclerostin and Dickkopf-1 (Dkk-1) [Bonewald and Johnson, 2008]. Osteocytes specifically
are known to express high levels of these bone forming-inhibitory proteins. Protein levels are thought to be altered with changes in strain, yet the magnitude of effect from specific types of strains that trigger these responses remain unclear [Burr and Allen, 2013]. To begin to tease out the effects of loading mode on cellular activity, in this work osteocyte-like MLO-Y4 cells were mechanically loaded in order to correlate tensile stimuli to soluble activity. Given that soluble activity studies represent average responses, the goal was to utilize small volumes to obtain a more representative mean.

5.2 Methods

To quantify cellular response of osteocytes to mechanical load, first cellular metabolic activity in response to a range of strains was quantified. A lactate dehydrogenase (LDH) stain was executed on adherent cells post-loading. This created a color intensity that correlated to metabolic activity level in each individual cell. The metabolic activity was used as a means of quantifying changes in cellular activity. These changes were used to identify strain regions where protein expression was expected to fluctuate due to increases or decreases in cellular activity. From this data, levels of strain correlating to increased and decreased cellular activity were identified. After this activity was measured for a range of strains, cells were loaded at specific uniform strains. These strains were chosen to encompass the high and low levels of activity found from the LDH stain. The soluble activity of the cells in terms of sclerostin and Dkk-1 was measured using standard ELISA kits and correlated to substrate strain.
5.2.1 Loading Platform Development

For application of a range of strains on cells under identical culture conditions, a stepped profile was designed and fabricated using a range of cross-sectional thicknesses (0.635-2.667 mm) [King et al., 2015]. PDMS was chosen as the substrate material given its elastic nature, optical transparency and previous research indicating that PDMS did not impact osteocyte proliferation, viability, sclerostin expression, or the presence of gap junctions in MLO-Y4 cells when compared to glass [York et al., 2012]. A profile was designed that on loading would generate a range of strains for a given load. Multiple profile designs were investigated. Modeling the profile material as PDMS, finite element analysis was utilized to determine the uniformity of the loading. Both fixed end loading and pure uniaxial loading studies were completed. From the preliminary work, a pure uniaxial load was required to generate a uniform strain.

A mechanical loading device for generating pure uniaxial loading was designed and fabricated for this study [King et al., 2016]. This loading device incorporated a system of dual load cells and microactuators, and was designed to accommodate a sample reservoir and a temperature-controlled environmental chamber. In addition, a sample clamping system was developed to prevent sample preloads. Utilizing this preload prevention system, the CTI-coated stepped profile was placed between the friction clamps, pulled taut and locked in place to eliminate unintended external loading during cell culture and experimental setup.
5.2.2 Strain Correlation to Cellular Activity

For cellular experimentation osteocyte-like MLO-Y4 cells, a gift from Dr. Lynda Bonewald, were utilized [Rosser and Bonewald, 2012]. Minimum essential alpha medium (α-MEM, Gibco) was supplemented with 5% fetal bovine serum (FBS) (Hyclone), 5% fetal calf serum (FCS) (Hyclone), and 1% penicillin/streptomycin (Pen/Strep, Invitrogen) for use in cell culture. Cells were maintained in an environment of 5% CO₂ and 37°C inside of 25 cm² flasks coated with rat tail collagen type I (CTI) (BD Bioscience) at a concentration of 5 µg/cm² in .02 M acetic acid. Cells were passaged between 70-80% confluence using .25% trypsin-EDTA.

Cells were cultured on the stepped profiles following sterilization and coating with 5 µg/cm² CTI. Cells were seeded on the profiles at a density of 2 x 10⁴ cells/cm². During the cell seeding process, profiles were locked in the preload prevention system. The cell suspension was then balanced on top of the stepped profile by utilizing the surface tension between the cell suspension and the CTI-coated stepped profile. This setup was left in an incubator for 30 min to promote cellular adhesion to the PDMS. Following the 30 min incubation, additional medium was added to the culture to prevent exuberant evaporation of media and cellular dehydration. Cells were maintained in culture for 3 days prior to experimentation.
5.2.3 Loading of Stepped Profiles

Profiles were loaded at a rate of 104 mm/s (52 mm/s per actuator), utilizing both actuators for pure tension. Overall strains of 5.0 and 7.5% were applied for 1 hour. Gauge lengths were measured for each sample prior to loading in order to calculate input displacements needed to achieve these overall strains. Cells were cultured on 5 substrates per experiment. During testing, 1 substrate was used as a control and the 4 substrates were subsequently loaded to the desired strain. Samples were kept hydrated and at 37°C during testing by utilizing a heated media bath [King et al., 2016]. Testing medium (2% FBS and 2% FCS) was utilized to minimize the known effects of serum on stimulated cells. HEPES was added to the testing medium at a concentration of 10 mM to maintain pH during experimentation. Images were taken of the substrates before and after loading for non-contact step strain measurements. Experiments were performed in triplicate and repeated on 3 separate days.

Immediately after loading, substrates were carefully removed from the system. Cells were washed with warm Hank’s Balanced Salt Solution (HBSS) and LDH stain was applied for 2 hours. After staining, cells were fixed with 4% paraformaldehyde and imaged. Two images were taken of the stained cells near the middle and edge of each step. This resulted in 2 images per step for all 9 steps for each stepped profile. A MATLAB algorithm was written to process the images and quantify the average activity of the cells. Specifically, the code converted the images to grayscale. Cells were then located, outlined, and cellular intensity was measured. The average intensity of 100
random cells per image was found and normalized to the average background intensity. Normalizing the values to the background was important when using this specific profile due to the varying step thicknesses. The values from the two images were averaged together to generate the average cellular response of the cells per step. From this data, a metric for activity of loaded cells was established.

Utilizing the images taken of the profiles before and after application of load, step strain measurements were calculated. ImageJ was used to find the edge of each step and distances were measured along the lengths of the steps before and after application of the load. From these values, step strains were calculated for each sample in order to correlate applied strain to cellular activity.

5.2.4 Correlating Strain and Soluble Factor Expression

Once the cellular activity was correlated to strain, soluble activity at specific strains was quantified. Two strains were selected from the metabolic activity data to represent strains inducing high and low levels of activity anticipating that the cells’ soluble factors would change between these two activity levels.

Cells were seeded on PDMS substrates of uniform thickness as previously described. Pure tensile loads were applied to the cells at the chosen substrate strains. After loading, the cells were washed in warmed HBSS and incubated in 1.5 ml of fresh medium for 2 hrs. The medium was then collected and centrifuged at 2000 RCF for 15 min to remove large particles. The remaining medium was collected and stored at -80°C
for ELISA until all mechanical loading had been completed. Experiments were performed in triplicate on 3 separate days. ELISA assays were performed to quantify soluble activity of the target proteins, sclerostin and Dkk-1. These assays (Boster Immunoleader Mouse Sclerostin/SOST ELISA Kit EK1179 and Boster Immunoleader Mouse DKK1 ELISA Kit EK0925) were utilized with standards and samples set with the assumption of the minimum concentration of the target protein in the samples for the purpose of dilution. A standard plate reader (Biotek Synergy H1 hybrid reader) was utilized to obtain the optical density (OD) of the wells, and protein concentration was obtained by comparing the sample OD to the generated standard curve. The final concentration for all samples was calculated by normalizing the average control value for each loading regime to 0.

5.3 Results

Results were presented in several ways in order to bring a fuller understanding of the response of the cells to the applied load.

5.3.1 Loading Platform Development

The stepped profile used to generate the strain field under uniform loading is shown in figure 5.1. The range of thicknesses, 0.635-2.667mm, resulted in strain patterns as a function of step. The bowtie shape was arrived upon after completion of FEA on a series of step designs. Exaggerated here under tensile loading from a fixed
end, the bowtie profile resulted in a more uniform strain pattern in contrast to other designs such as the ramp profile shown, figure 5.1b. Osteocytes are shown seeded on the CTI-coated stepped profile in figure 5.1c. The pure uniaxial loading machine is illustrated in figure 5.2a. The platform, consisting of 2 microactuators riding on a guide rail system ensures pure uniaxial alignment. The performance of the platform for non-biologic testing has been previously characterized [King et al., 2016]. For the biologic work, the heated bath (figure 5.2b) and fixtures (figure 5.2c) were fabricated. The fixtures consist of two clamps secured with a brace. The stepped profiles are clamped in the fixtures and then cultured. The brace is used to handle the samples and is removed once the fixtures are in the loading machine. In this way, the flexible substrates (and cells) do not experience any loads prior to testing. Following testing, the brace is placed back on the samples for removal from the loading machine.
Figure 5.1 – Multi-strain profile for cellular loading. a) Elastic profile consisting of varying thicknesses was utilized in order to apply a range of strains to cells under identical culture conditions. The (+) and (-) were used to give an orientation to the profile. This was important to the design due to thicknesses increasing by 0.127 mm in an alternative manner starting from the middle step (0.635 mm) to the step on the (-) side then to the step on the (+) side and so forth maximizing at 2.667 mm. b) FEA simulations were performed to find the optimal shape of the profile for uniform loading from several candidates. c) Cells were grown on these profiles coated in CTI as seen in the SEM image.
Figure 5.2 – Pure uniaxial loading device. a) A pure uniaxial loading device was generated for this research. The device was designed to load cells adhered to a substrate while maintaining a b) hydrated and temperature regulated environment by suspending the sample in a heated hydration bath. c) Precautions were taken to assure that no load was applied to the samples prior to experimentation by utilizing a bar to lock the friction clamps in a set position in culture.

5.3.2 Strain Correlation to Cellular Activity

Typical LDH images post-loading on the stepped profiles are shown in figure 5.3a. Figure 5.3b shows the randomly selected cells analyzed for stain intensity. 100 cells per image were selected to give an average reading. The LDH activity was quantified initially for 2 strains selected based upon preliminary trial-and-error work,
data not shown. The average intensity of the cells, calculated by the MATLAB algorithm was compared to the strain on each step. Scatter plots showing LDH intensity for 5 and 7.5% overall strain applied to the cell-seeded substrates with each dot representing the average of 200 cells were generated, figure 5.4.

![Figure 5.3](image)

Figure 5.3 – Before and after pictures of the images processed with the MATLAB algorithm. a) Cells were stained using an LDH stain to measure metabolic activity. b) One-hundred cells were randomly selected from each image with 2 images taken per step for color intensity measurement which was used as a measurement of cellular metabolic activity.
Figure 5.4 – Scatter plots of the cellular activity to strain were generated. These plots at the a) 5 and b) 7.5% overall strain display average cellular metabolic on each step (averaged from 200 cells per step with the control normalized to 1) compared to the measured step strain. Step strain was calculated from images taken pre- and post-application of the load. From this data 6 and 12% strains were found to correlate to high and low levels of cellular activity, respectively.

In order to better visualize where these points were on the profile this plot was altered to show the average step strain vs LDH activity by step, figure 5.5, and by binned steps, figure 5.6. The results were also placed into column graphs for each step, figure 5.7a and 5.7b, as well as for each bin, figure 5.7c and 5.7d. Two-tailed t-tests were performed on the bins for each load and significance was found between each consecutive bin, thick to middle and middle to thin bin for both overall loads. From these plots, regions of high and low activity were located and the corresponding strains were applied to cells for the soluble activity testing. These regions were found at 6 and 12% strain for the high and low activity levels, respectively. Loading was then applied to
cells seeded on substrates of constant thickness to generate 6 or 12% strain under loading. The constant thickness allowed for a larger volume of cells to be loaded resulting in soluble activity quantifiable with standard ELISAs.

Figure 5.5 – Strain vs LDH activity results displayed by step. Step 1 correlated to the thickest step while step 9 correlated to the thinnest step.
Figure 5.6 – Strain vs LDH activity results displayed by bin. Bins were set up the same as in Table 3.3 with the thick bin (steps 1-3), middle bin (steps 4-6), and thin bin (steps 7-9).
Figure 5.7 – LDH activity plotted in column graphs. This was performed for a-b) each step on the profile as well as for c-d) the three bins of step sizes. Significance was found between the bins in both the c) 5% overall strain (thick to middle ($p < 0.0001$), middle to thin ($p < 0.0001$)) and the d) 7.5% overall strain (thick to middle ($p < 0.0001$), middle to thin ($p < 0.0001$)).
5.3.3 Correlating Strain and Soluble Factor Expression

ELISA concentrations were determined for each sample by comparing the OD to the standard curve. The 9 samples for each strain were then normalized to the average control concentration for the same experiments. Standard two-tailed t-tests at a 95% confidence interval were performed on the data between the protein levels at the lower strain and the protein levels at the higher strain, as well as the protein concentration at each strain compared to the control which was normalized to 0. The results are displayed as column charts, figure 5.8. Statistical analysis revealed that while sclerostin was significantly different at 6 and 12% strain (p < 0.04), Dkk-1, was not significantly different. Specifically, we found that sclerostin activity at 6% strain was decreased 101% over controls. Furthermore at 12% strain, sclerostin activity was increased 121% over activity at 6%. Similar trends were noted with Dkk-1 activity. Specifically, we found that Dkk-1 activity at 6% strain was decreased 21% over controls. Furthermore at 12% strain, Dkk-1 activity was increased 21% over activity at 6%.
Figure 5.8 – Two-tailed t-test column charts of the soluble activity data were generated with the control normalized to 0. There was a significant difference between the 6 and 12% strain for the a) Sclerostin (p < 0.04), however there was no significant difference between the 6 and 12% strain for the b) Dickkopf-1. There were no significant differences in the different strain levels compared to unloaded cells for either of the target proteins.

5.4 Discussion

Osteocyte-like MLO-Y4 cells were mechanically loaded to correlate tensile strain to soluble activity. This was done by first finding regions in a range of strains where cellular activity was elevated over a control and then by finding where the cellular activity was decreased from further application of strain. Two levels of 6 and 12% strain were found to correlate to high and low cellular metabolic activity, respectively, due to overloading. These two strains were applied to the cells and the soluble activity of two target proteins, sclerostin and Dkk-1, were measured and statistically compared.
The scatter plot data from the LDH staining, figure 5.4a & 5.4b, showed activity which peaked between strains of 4 and 6%. This activity then decreased with increasing strain. Since these values normalized to a control of 1, the data showed that the average metabolic activity for cells experiencing lower substrate strains initially increased for both overall strains (5 and 7.5%). However, cells experiencing the higher substrate strains experienced a decrease in activity similar to the activity level of the control. This increase in cellular activity at the lower strains is consistent with mechanical activation of the cells, with a decrease in cellular activity at the higher strains consistent with cellular overloading and damage.

Utilizing 6% strain as the elevated activity strain and 12% strain as the decreased activity strain consistent with cellular overloading, the cells were strained at these levels and medium containing soluble factors was collected. ELISA tests were performed on these samples for concentrations of the proteins sclerostin and Dkk-1. Two-tailed t-tests were performed on the results of the ELISAs to compare protein concentrations at the two tested strain levels as well as protein concentrations at each strain compared to the control. Our results suggest that low tensile strains decrease sclerostin activity, consistent with bone formation, while higher tensile strains increase sclerostin activity, consistent with inhibition of bone formation and possibly damage.

These initial decreases in protein expression agree with in vivo experiments by Robling et al. where loaded mice ulnae exhibited a decrease in both sclerostin and Dkk-1 under loading [Robling et al., 2008]. With application of higher strain, soluble concentrations of the target proteins rose back to a concentration similar to that
displayed by the unloaded control. This parabolic activity suggests there may be an ideal range of strain where these cells respond to load. Once these cells become overloaded, their protein levels are consistent with unloaded levels. This may be due to cellular damage or death.

In previous work utilizing out-of-plane distention loading of cells on PDMS substrates, we found that intracellular sclerostin activity was significantly increased with substrate strains up to 10% strain in comparison to strains on the order of 20% [York et al., 2015]. This parabolic activity could potentially work in harmony with the soluble sclerostin levels found in our current study by the intracellular and soluble extracellular sclerostin levels working inversely to each other. The difference in the strain magnitude required to produce these parabolic responses of sclerostin could be explained by the complex loading differences produced in each study. Dkk-1 was analyzed in this study to further interrogate this issue understanding that sclerostin and Dkk-1 are noted to be proportional in their response as both inhibit bone formation.

This study investigated the effects of pure tensile mechanical strain on the activity and soluble protein concentration in osteocyte-like MLO-Y4 cells. The results of this study indicate that protein expression in osteocyte cells is significantly dependent on the magnitude of tensile strain. This was displayed with soluble sclerostin expressing significant differences in concentrations under the application of 6 and 12% strain. For this study, an elastic substrate for cellular loading [King et al., 2015] and a pure uniaxial loading device for loading cells in a hydrated temperature-controlled environment [King et al., 2016] were developed and characterized. In order to better understand the
cellular response of stimulated osteocytes, metabolic activity was first observed over a range of strains. This was done utilizing a substrate of varying thicknesses in order to apply a gradient of strains to cells under identical culture conditions. After this differential activity was established, the cells were loaded at uniform strains where soluble activity of the two inhibitory proteins, sclerostin and Dkk-1, was quantified and correlated to applied strain. A better understanding of these cells’ response to this loading pattern may allow for more insight into science behind bone and bone disease mechanics that cannot be explained by current experimentation alone.
CHAPTER VI

CONCLUSIONS

The economic and mental cost of bone related diseases in the United States continues to be a constant burden. In order to combat diseases such as these, models need to be developed that allow us to understand the basic science behind bone cell response to mechanical stimulation. Current models are ill equipped to study the complex, intricate nature of cellular interactions and mechanotransduction of bone cells. For this reason, in this work a model for investigating the effects of mechanically loaded osteocyte-like MLO-Y4 cells was designed, fabricated, and validated. This was done by addressing 3 specific aims for the project.

Specific Aim 1- Development of a Multi-Strain Profile for Cellular Mechanotransduction Testing.

Profiles were designed and fabricated, and out of 2 proposed designs for a PDMS stepped profile, profile B (the bowtie profile) was found to have the ideal mechanical properties for application of a range or strains to cells cultured under identical culture
conditions. This was verified by computer generated FEA models and mechanical testing.


A device was designed, fabricated, and characterized in order to apply a pure uniaxial load to bone cells adhered to a substrate. This device allowed for reproducible application of load to a biologic sample while remaining at physiologically relevant conditions. Verification performed on this device provided evidence of the device’s accuracy.

Specific Aim 3 - Soluble Protein Activity of Osteocyte Cells Stimulated by Pure Uniaxial Loading.

In order to provide validation of this model’s usefulness in cellular work, the profile and device designed for the first 2 specific aims were used together and cellular experimentation was performed on osteocyte-like MLO-Y4 cells. From this experimentation a pattern of cellular metabolic and soluble activity to direct strain was able to be established and was found to follow general trends displayed in other research. This success in cellular experimentation has shown the overall model to be a
success as a means of researching bone cell mechanotransduction and cellular interactions.

6.1.1 Future Work

This research has established the ground work for a novel microloading model to be used in bone research. This model was designed for specific current testing needs, yet the design was created in a simple manner to allow for expansion of the system to capabilities which address a wide range of clinical applications relevant to bone research. One major short coming of this model was that it focused on loading bone cells attached to an elastic substrate. Future iterations of this system hope to improve loading patterns on the osteocyte-like MLO-Y4 bone cells in a more native manner such as with a 3D gel. These additions to the system hope to improve the biomimicry capabilities in order to address a wide range of applications including fracture repair and distraction osteogenesis mechanisms and pathways. Where this loading device was designed and validated specifically for the current testing needs in bone research, it has been designed in such a way that it could be used for testing needs with other biologic samples such as fibers, tissue, and other cell types.
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