DESIGN AND VALIDATION OF AN OPEN-SOURCE 3D PRINTABLE BIOREACTOR SYSTEM FOR EX VIVO BONE CULTURE

by

Brian A. Kunath

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Kingston, Ontario, Canada
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Abstract

In Canada, osteoporosis is a prevailing skeletal disease that is underdiagnosed and undertreated, with an annual economic cost of $4.6 billion. Trabecular bone is widely understood to undergo modelling and remodelling in response to biochemical and mechanical loads during physical activity. Further insights into the adaptation process of bone could help clinicians improve prevention and treatment methods, including physical activity recommendations, and lead to increased bone health. Previous studies have successfully implemented a polycarbonate (PC) bioreactor system to study trabecular bone adaptation in response to biochemical and mechanical stimulation in long-term *ex vivo* bone organ culture. However, the PC bioreactors are expensive and difficult to fabricate and have been limited to testing bone cores with maximum dimensions of 5 mm x 10 mm (height x diameter), which is below recommended standards for bone compression testing. Recent advancements in additive manufacturing can reduce fabrication cost and difficulty and allow for high dimensional precision in 3D printed designs with biocompatible material options. Thus, the first objective of the presented research was to develop an open-source 3D printable bioreactor with the photopolymer MED610™ that addresses the PC bioreactors fabrication and bone core height limitations. The second objective was to test the role of the MED610™ material on cell viability and determine a cleaning and sterilization protocol for MED610™ in cell and tissue culture applications. Finally, the third objective was to validate the 3D printed bioreactor in an *ex vivo* bovine trabecular bone study with mechanical stimulation by measuring the change in apparent elastic modulus over 21-days. Collectively, this thesis demonstrated that 3D printed MED610™ bioreactors cleaned and sterilized with a sonication and autoclave protocol are suitable for *ex vivo* bone organ culture and can replicate trends in trabecular bone apparent elastic modulus found in previous studies. Recommendations for next steps are provided including adjustments to the bioreactor design and continued biocompatibility and validation testing.
Statement of Co-Authorship

Chapter 1: Brian A. Kunath – manuscript composition
           Heidi-Lynn Ploeg – manuscript review
           Roshni Rainbow – manuscript review

Chapter 2: Brian A. Kunath – literature review, manuscript composition
           Heidi-Lynn Ploeg – manuscript review
           Roshni Rainbow – manuscript review

Chapter 3: Brian A. Kunath – study design and analysis, manuscript composition
           Heidi-Lynn Ploeg – study design, manuscript review
           Roshni Rainbow – manuscript review

Chapter 4: Brian A. Kunath – study design, data collection and analysis, manuscript composition
           Heidi-Lynn Ploeg – manuscript review
           Roshni Rainbow – study design, manuscript review

Chapter 5: Brian A. Kunath – study design, data collection and analysis, manuscript composition
           Kail Beloglowka – study design, data collection and analysis
           Heidi-Lynn Ploeg – study design, manuscript review
           Roshni Rainbow – study design, manuscript review

Chapter 6: Brian A. Kunath – manuscript composition
           Heidi-Lynn Ploeg – manuscript review
           Roshni Rainbow – manuscript review
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List of Abbreviations

2D.................................................................................................................. Two-dimensional
3D.................................................................................................................. Three-dimensional
%ΔE_{app}.............................................................................................. Percent change in apparent elastic modulus
AA.............................................................................................................. Antibiotic-antimycotic
A_x (mm^2)............................................................................................. Cross-sectional area
AM............................................................................................................. Additive manufacturing
ANOVA............................................................................................. Analysis of variance
AP................................................................................................................. Autoclave protocol
BM-MSCs.......................................................................................... Bone marrow mesenchymal stromal cells
CAD............................................................................................................. Computer-aided design
CNC............................................................................................................. Computer numerical control
CO_2........................................................................................................... Carbon dioxide
D (mm)...................................................................................................... Diameter
DMEM/F-12.......................................................................................... Dulbecco’s modified eagle/F-12 ham medium
E_{app} (MPa)............................................................................................. Apparent elastic modulus
ECM............................................................................................................. Extracellular matrix
EtO.............................................................................................................. Ethylene oxide
F (N).......................................................................................................... Force
FBS............................................................................................................ Fetal bovine serum
FDM........................................................................................................... Fused deposition modelling
g (m/s^2).................................................................................................. Earth’s gravitational field
H (mm)....................................................................................................... Height
HOQ.......................................................................................................... House of quality
ID (mm).................................................................................................... Inner diameter
IPA............................................................................................................ Isopropanol
MES.......................................................................................................... Minimal effect strain
MESm....................................................................................................... Minimal effect strain for bone modelling
MESp....................................................................................................... Minimal effective strain for bone microdamage and fracture
MESr....................................................................................................... Minimal effective strain for bone resorption
microCT................................................................................................. Micro-computed tomography
MP............................................................................................................. Manufacturer’s protocol
Chapter 1

Introduction

1.1 Motivation

Osteoporosis is a predominant skeletal disease that is more common in people over 40 years-old and is characterized by the deterioration of bone tissue and reduction in bone mineral density [1, 2]. As a result, osteoporosis can increase an individual’s bone fragility and chance of bone fracture [1]. With the increasing proportion of Canadians aged over 65 years old, musculoskeletal disease cases, such as osteoporosis, are predicted to increase [2]. Specifically, in Canada, 10% of adults over 40 years old and 21% of post-menopausal women are predicted to have osteoporosis [2]. In 2015-2016, over 2.2 million Canadians were diagnosed with osteoporosis with 130,000 fracture patients, which contributed to an annual economic cost of $4.6 billion [3]. Despite some provinces having improved their diagnosis and treatment methods, osteoporosis remains to be undertreated and underdiagnosed in Canada [2, 4]. Of the different kinds of osteoporotic fractures, hip fractures are considered to be the most significant with mortality rates of 22% and 33% for women and men, respectively, within the first 12 months post-fracture [2]. Therefore, there is a need for better understanding the properties of bone to help clinicians improve prevention and treatment methods for musculoskeletal disorders.

Further insights into bone properties and adaptation mechanisms can lead to improvements in bone health, fracture prevention, and treatment methods, including pharmaceutical, diet, and physical activity recommendations [5-8]. This thesis focuses on improving our understanding of physical activity as a therapy for healthy bone. A better understanding of the role of physical activity could improve computational models used to predict the mechanical behaviour of bone and help clinicians provide accurate patient-specific physical activity treatment. Researchers continue to study the different factors affecting bone adaptation, however, due to the complex structure and surrounding environment of bone in vivo, the mechanical and biochemical stimuli remain underdefined [9]. To better understand these
mechanisms and their interactions, in addition to in vivo methods, researchers may apply in vitro, ex vivo (tissue and organ culture), or in silico (computer simulation) methods [9]. While investigating the biochemical factors may improve our understanding of the impact that cells, such as osteocytes, and crosstalk with other tissues have, better understanding their interaction with mechanical factors can help us gain additional insights to bone diseases, such as osteoporosis [9]. Therefore, there is a need to develop new approaches that combine in vitro, ex vivo, and in silico methods.

Trabecular bone – the porous inner structure of vertebrae and the ends of long bones [10] – plays an important role in osteoporosis. Early bone loss generally occurs in trabecular bone [11], causing a reduction in strength and increasing the occurrence of fragility fractures in trabecular regions [12]. Trabecular bone is a complex heterogeneous and anisotropic tissue that behaves differently depending on anatomical location, age, and health condition [10]. In addition, trabecular bone is understood to adapt its structure to mechanical load [10] and has time and temperature-dependent behaviour, referred to as viscoelasticity [13]. As a result of its viscoelasticity, trabecular bone will relax when subjected to constant deformation. Although studying the mechanisms of trabecular bone in vivo is ideal, performing these studies can be difficult, time and resource intensive, expensive, and limited due to the complexity of the living system. Instead, in vitro and ex vivo methods are commonly used to study trabecular bone at the cellular and tissue structure levels [9]. While in vitro cell cultures are limited by their simple representation of the bone formation process and how well they can replicate the physiological diversity of bone, ex vivo bone culture methods preserve the three-dimensional (3D) bone structure and extracellular matrix and better represent the physical diversity across samples [9]. In silico models complement physical experiments with predictions of tissue local stress and strain fields.

Ex vivo bone culture methods, such as the Zetos 3D bone diffusion bioreactor and loading system, have been developed and are becoming more commonly used to model the process of bone adaptation [9, 14, 15]. The advantage of these ex vivo bioreactors is that they can keep bone alive for up to seven weeks while allowing for long-term bone response to biochemical and mechanical stimuli [16]. Additionally, these
bioreactors have been proven to mimic in vivo metabolism conditions by perfusing bone cores with culture medium to maintain pH, temperature, and oxygen and nutrient intake [14, 15]. Despite these advantages, these bioreactor systems are difficult and expensive to fabricate. As well, they are limited to testing bone cores with a maximum height and diameter of 5 mm and 10 mm, respectively, which is below the recommended height-diameter ratio of 1 to 2 for characterizing bone properties during compression [17] and is too small for bone-implant system testing [18]. With recent advancements in additive manufacturing (AM), 3D printing can ease the cost and difficulty of fabrication [19-21] while allowing for flexible design optimization [22], high dimensional precision [23], and biocompatible material options, such as MED610TM [24]. MED610™ is a photopolymer material composed of caprolactone acrylate (1.00-3.00%), 1,7,7-trimethyltricyclo[2.2.1.02,6]heptane (0.10-0.30%), 2-propenoic acid and 1,2-ethanediyl ester (0.10-0.30%), acrylic acid 2-hydroxyethyl ester (0.10-0.30%), acrylic acid (0.10-0.30%), camphene (0.10-0.30%), and glycerol propoxylated esters with acrylic acid (0.10-0.30%) [24]. The remaining MED610™ composition contains proprietary components. Although MED610™ is marketed as a biocompatible material with mucosal membrane for up to 24-hours, recent studies suggest that it is suitable for long-term cell and tissue culture if adequate cleaning is used [25, 26]. Therefore, as an alternative to polycarbonate machined bioreactors, we are establishing an open-source 3D printable bioreactor system fabricated from the photopolymer, MED610™. As an open-source design, this bioreactor system will offer fellow researchers a cost-effective 3D printable bioreactor chamber that is easy to implement for bone organ culture and can be fabricated with compatible 3D printers or 3D printing services. Additionally, the design is easily adjustable in computer-aided design (CAD) software and can prevent fellow researchers from dedicating the time and effort required to produce their own custom-made bioreactor systems.

1.2 Research Objective

The primary objective of this research was to design an open-source 3D printable bioreactor and loading system that can be used for applying mechanical and biochemical stimulation to individual trabecular bone
specimens for long-term bone organ culture studies. Using a combination of in vitro and ex vivo methods, the specific steps taken to complete this objective were:

1. Design a bioreactor for AM and larger bone core samples.
2. Test the role of the 3D printing material, MED610™, on bone cell viability and establish a cleaning and sterilization protocol for MED610™ use in cell culture.
3. Test the efficacy of the 3D printed bioreactor system in a long-term controlled ex vivo bovine trabecular bone test with mechanical stimulation.

1.3 Thesis Organization

This thesis document is composed of six chapters. Chapter One outlines the motivation and primary objective to design a new 3D printable bone organ culture bioreactor and loading system. Chapter Two provides a background of bone anatomy, properties, and mechanics as well as commonly used methods for testing and modelling trabecular bone. Chapter Three proposes the design of an open-source 3D printable bioreactor design for ex-vivo bone organ culture. Part of this initial work was presented as a virtual podium presentation at Canadian Bone and Joint Conference 2020 and poster presentation at Orthopaedic Research Society Annual Meeting 2020. Chapter Four establishes a cleaning and sterilization protocol for the 3D printable photopolymer MED610™ and reports on the long-term cytotoxicity of this material in cell culture. This work was accepted for a podium presentation at the World Congress of Biomechanics 2022 conference. Chapter Five reports on an ex vivo study performed with bovine trabecular bone cores using 3D printed bioreactors to test the efficacy of the MED610™ material and bioreactor design for bone organ culture. Lastly, the final conclusions, an insight into the future steps and improvements, and the clinical significance of this research are highlighted in Chapter Six. Additional figures and tables are provided in the appendices. The appendices also include studies regarding system compliance characterization for the Mach-1 mechanical testing system (Biomomentum, Laval, QB, Canada) and analytical and finite element analysis modelling of trabecular bone relaxation.
Chapter 2

Literature Review

2.1 Bone Structure, Properties, & Mechanics

2.1.1 Bone Anatomy & Structure

Bone is a complex biological material composed of organic matter (collagen fibres, non-collagenous proteins, and cells), inorganic matter (hydroxyapatite crystals), and water [10, 27-29]. Its biological and mechanical properties allow multiple critical functions within the body. Bone provides structural support, organ protection, facilitation of locomotion, maintenance of pH, mineral homeostasis, storage for growth factors and minerals, and formation of blood cells [30]. The structure of bone has a hierarchy (Figure 2.1) spanning from the nanoscale (mineralized collagen fibril) to the macroscale (whole bone) [28, 31]. The constituents at each level in the bone structure hierarchy change mechanical behaviour with time and are driven by biological, biochemical, and physical factors, therefore causing the structural hierarchy to influence the mechanical properties of bone at all scales [28].

Figure 2.1: Bone structure organization example showing the (a) macrostructure – cortical and trabecular bone; (b) microstructure – osteons and haversian systems; (c) sub-microstructure – lamellae; (d) nanostructure – collagen fibrils composed of collagen fibres; I and the sub-nanostructure – non-collagenous proteins, collagen molecules, and bone mineral crystals (reprinted from [32], with permission from Elsevier).
At the macroscopic level, bone is an anisotropic, heterogeneous, and viscoelastic structure and is divided according to function into two types: cortical bone and trabecular bone [10, 27, 33]. Cortical bone, also known as solid or compact bone, makes up approximately 80% by weight of the adult human skeleton [30] and provides the outer shell of the skeleton and long bone shafts [27]. Trabecular bone, also called spongy, porous, or cancellous bone, makes up the other 20% of bone by weight, but 80% by surface, in the human skeleton and is present at the end of long bones, throughout short bones, and in vertebrae [27, 31]. Trabecular bone has a porous ‘honeycomb network’ of trabecular plates and rods filled with bone marrow (cells, fat, and blood) [27, 30]. While cortical bone porosity ranges between 5%-10%, trabecular bone porosity is generally higher than 50% [34] and tends to vary from 75% to 95% [35]. Bone porosities between 15% and 50% are considered uncommon [34].

At the microscopic level, bone is classified into two categorizations, woven bone and lamellar [31, 35]. Woven (immature) bone structure consists of randomly aligned fibres and is formed quickly in response to factors such as fracture repair [34]. Lamellar (mature) bone contains thin fibres called lamella that are highly organized in stacked parallel layers that alternate by 90°, therefore, enabling lamellar bone to be stiffer and stronger than woven bone but causing it to form at a slower rate [35]. Although trabecular bone has a similar composition to cortical bone, it is organized in “packets” of lamellar bone, giving it an anisotropic and porous structure [10].

Bone contains specialized cells that aid in skeletal growth (modelling) during childhood and bone maintenance and adaptation (remodelling) in adulthood. These cells include osteoblasts, osteoclasts, bone-lining cells, and osteocytes [34, 35]. Osteoblasts are derived from mesenchymal stem cells and are primarily responsible for bone formation [27, 35]. Osteoclasts are multinucleated cells formed from the fusion of hematopoietic monocytes located in bone marrow [35]. The primary function of osteoclasts is to remove bone through the process of resorption. The differentiation of osteoblasts and osteoclasts from mesenchymal and hematopoietic stem cells, respectively, is activated by environmental factors including mechanical stress on the bone [35]. Osteoblasts acting on the bone surface produce bone by secreting an
unmineralized collagenous matrix, known as osteoid, and regulate its mineralization to begin hydroxyapatite formation [27]. Additionally, osteoclasts acting on the bone surface remove bone by creating an acidic environment that dissolves the mineralized bone matrix beneath them [35]. After mineralization of new bone, osteoblasts will either undergo cellular apoptosis (cell death) or differentiate into bone-lining cells or osteocytes [27]. Bone-lining cells are quiescent (inactive) osteoblasts derived from osteoprogenitor cells that provide a covering for the surfaces of all bones and help with the movement of ions between the bone and the body [34]. During remodelling, the lining cells form a canopy creating a bone remodelling compartment. Osteoblasts that become embedded in the bone matrix become osteocytes, the primary cell responsible for the regulation of the bone modelling and remodelling process [12, 27]. Osteocytes reside in cavities within the bone, called lacunae, and use their dendritic processes through small channels, known as canaliculi, to communicate with other osteocytes and bone-lining cells [12, 27, 36]. Using this network of connections, osteocytes monitor their environment. For example, osteocytes may undergo apoptosis in response to micro-damage in the bone matrix; and therefore, initiate remodelling processes, activating pre-osteoblasts and the recruitment of osteoclasts for bone resorption [27]. The coordination of bone cells gives bone the ability to adapt to its environment, yet how this process responds to biochemical and mechanical stimuli is poorly understood, making it a topic of research interest.

2.1.2 Mechanical & Time Dependent Properties of Trabecular Bone

Throughout life, the human body undergoes many physical activities. These activities subject bone within the body to a wide range of physiological loads and loading rates. As a result, bone is structured to withstand the various forms of loading and the associated strains. Mechanical strain ($\varepsilon$) is the relative measure of deformation or change in shape with respect to the material’s original shape and the strain rate is the rate at which the material deforms over time [34, 37, 38]. During skeletal locomotion, the primary direction of strain is axial, which includes compressive and tensile strains. If the direction of loading is eccentric (off-axis), bones are exposed to bending moments which lead to compression at one side of the bone and tension at the opposite side [37]. Physical activities, such as walking, running, and jumping, have
been recorded to cause repetitive loading at a frequency of about 2 Hz [31] with a range between 0.1-10 Hz [39] and compressive strains between approximately 300-950 με, 950-2100 με, and 1600-3450 με, respectively [9]. During these activities, bone strain rates typically vary between 1500-20,000 με/s [37]. More vigorous activities (e.g. sprinting) can cause strains and strain rates of up to 9000 με and 58,000 με/s, respectively.

Research suggests that the strain magnitude and strain rate during physical activity vary depending on the type of bone and anatomical location when undergoing the same exercise [9, 37]. In addition to the anisotropic ratio and trabecular orientation, the macroscale mechanical properties have been shown to correlate with apparent density [10, 12, 32] and bone volume fraction [10, 12, 31, 40, 41]. At the macroscopic level, bone is viewed as a continuum and measurements describe apparent mechanical properties. The apparent elastic modulus of trabecular bone at the macroscale has been shown to range between 10 and 4800 MPa when mechanically tested in compression (uniaxial and multiaxial), tension, and torsion tests [42-45]. The apparent elastic modulus represents the stiffness of the apparent bone structure and is characterized by the ratio of the stress and strain applied to the sample [46]. From a microscale perspective, tensile and bending tests have shown the elastic modulus of trabecular bone to vary between 0.8-16.9 GPa [47, 48]. At the nanoscale level, the elastic modulus correlates to local mineralization and is typically larger than values measured at the macro- and microscopic scales, ranging between 1.3-25 GPa [32, 41, 48] with most recordings closer to the higher limit. The variability in measurements can be attributed to trabecular bone’s anisotropic, heterogeneous, and viscoelastic properties, which cause the mechanical properties to be dependent on the direction of loading [10, 12, 41], rate of loading [13], anatomical site [10, 12], specimen size [12, 41], and the hierarchical level of bone considered [32]. An additional factor could include the testing environment of the bone; for example, compared to wet bone, dry bone tends to have a higher elastic modulus [31, 41].

During quasi-static loading below levels that cause damage, the elastic properties of trabecular bone are linear and can therefore be characterized with the generalized Hooke’s Law to determine stress and
strain of the bone structure [12, 41]. However, mechanical properties assessed with quasi-static tests do not always align with those measured with repetitive or high strain rates [31]. For example, at high strain rates, toughness and stiffness slightly decrease and increase, respectively [31]. Trabecular bone displays both elastic and viscous behaviours, which is referred to as viscoelasticity and implies that the stress-strain response of trabecular bone is time and temperature-dependent [13, 49]. Common bone viscoelastic behaviours include loading rate dependence, creep, hysteresis, and stress relaxation [13]. Creep behaviour is quantified as the increase in strain over time when constant stress is applied [13, 34, 49]. Fatigue occurs over time due to cumulative creep loads from cyclic loading and can cause a decrease in stiffness as well as potentially cause microcracking or fracture [10, 12]. During cyclic loading, a phase lag referred to as hysteresis typically occurs and causes the loss of mechanical energy [13]. Stress relaxation is quantified as the decrease in stress over time when the specimen is exposed to a constant strain. Trabecular bone viscoelasticity has been well-researched [40, 50-57], yet its specific role in trabecular bone adaptation is poorly understood. Although the role of viscoelasticity specifically in trabecular bone adaptation has not been well-documented, daily low magnitude (0.2 – 0.6 g, where g = earth’s gravitational field (9.8 m/s²)) and high frequency (30-45 Hz) vibrations have been shown to inhibit bone resorption [58, 59]. Additionally, high-intensity resistance and impact training with high magnitudes (> 80% repetition maximum or ground reaction forces > 4 x body weight) and low frequency (< 8 repetitions) have been shown to help improve bone mineral density [60, 61].

2.1.3 Bone Adaptation

Bone is understood to self-regulate and has a complex behaviour in response to mechanical loading. In the 19th century, it was proposed that bone adaptation is related to its mechanical environment such that the bone structure will adapt to orient itself corresponding to principal stress directions from mechanical loads on the bone [62, 63]. This theory is now commonly referred to as “Wolff’s Law”. Currey, Frost, and Burr further hypothesized that while optimizing stiffness, bone architecture is regulated to minimize fracture risk through the processes of modelling and remodelling [34, 64, 65]. Frost proposed the
“Mechanostat,” theory, which suggested a range of mechanical strain in which bone adaptation occurs (Figure 2.2) and a minimal effective strain (MES) required for initiating bone adaptation [9, 64, 66]. The Mechanostat theory proposed that below 50-100 με, there was a disuse-mode remodelling region (MESr) and caused the resorption (removal) and weakening of bone. Mechanoically controlled bone adaptation and apposition begins in the modelling region (MESm) between 1000-1500 με and can increase up until approximately 3000 με. Between the MESr and MESm thresholds, mechanically induced bone adaptation is minimized. Above the microdamage threshold (MESp) of 3000 με, bone is susceptible to microdamage with fracture likely to occur around 25,000 με. However, these threshold ranges are approximations, and precise mechanically controlled bone remodelling thresholds remain unknown [9]. In addition to mechanical strain, bone adaptation also depends on loading rate; however, how it affects bone adaptation remains unclear [67].

Figure 2.2: The mechanical usage window as defined by Frost’s Mechanostat theory for bone response to mechanical loading [68].
Bone adaptation is defined by two processes: modelling and remodelling. Bone modelling primarily occurs during childhood growth and consists of the change and formation of bone geometry in response to physiological loading and its required function [27, 30]. Modelling works in combination with bone remodelling throughout childhood but does not continue into adulthood. Bone remodelling is the primary adaptation process in adults and involves the replacement of old bone, through resorption, with newly formed bone to replace fatigued and damaged areas. Osteocytes, osteoblasts, and osteoclasts each play an important role in bone remodelling. Osteocytes, with a life span up to 50 years, play a key role in the conversion process of extracellular mechanical loading to intracellular biochemical modulation, known as mechanotransduction [9, 12]. Although the process by which osteocytes initiate and facilitate bone remodelling is not fully understood [9], research suggests that mechanotransduction impacts osteoblast and osteoclast behaviour, molecule activation, and gene expression [12]. The bone remodelling process has four phases: activation, resorption, reversal, and formation and takes 3-4 months to occur in adult human bone (Figure 2.3) [30]. The activation phase is initiated when osteocytes detect microdamage and bone-lining cells leave areas of the bone surface in response to various factors, such as inflammatory signals [27]. For example, during damage, pre-osteoblasts and bone-lining cells release the cytokine receptor activator of nuclear factor kappa-B ligand (RANKL), which promotes the formation and activation of osteoclasts from pre-osteoclast precursors [27]. The resorption phase occurs within the first few weeks, in which multinucleated osteoclasts (life span up to one month) attach to the exposed bone surface that the bone-lining cells left and create an acidic environment that breaks down the bone mineral. In the reversal phase, RANKL is blocked by osteoprotegerin (OPG), a glycoprotein that inhibits osteoclast formation and activity and which is secreted by mature osteoblasts, causing osteoclast apoptosis and recruitment of mononuclear osteoblast cells [27]. As well, pre-osteoblasts are activated by the release of calcium, phosphate, and growth factors during resorption and recruited to initiate bone formation [30]. Following the resorption stage, osteoblasts (life span up to six months) adhere to the surface and produce osteoid to form the new bone matrix. After 10-15 days, the osteoid begins to mineralize with up to 70% of the bone mineral content being
completed and the remaining 30% finishing after a few months [27]. At the end of remodelling, remaining osteoblasts either die or differentiate into bone-lining cells or osteocytes.

Figure 2.3: Bone remodelling process in response to mechanical loading, involving line cells (LC), osteocytes (OCY), osteoclasts (OCL), and osteoblasts (OBL) (reprinted from [69], with permission from Elsevier).

2.2 Experimentation Methods for Characterizing Bone Mechanics

2.2.1 Evaluation Methods for Bone Mechanical Behaviour

Characterizing the mechanical properties of bone is an important part of understanding bone behaviour and how it can react at bone-implant interfaces and in response to load-bearing situations [12]. As described in section 2.1.2, the mechanics of bone vary across the different hierarchical levels of the bone structure [32]. Bone is a living tissue and as a result, the mechanical properties are affected by the individual’s age and underlying health conditions [41]. The mechanical properties are also affected by environmental conditions, such as temperature [13], wet versus dry bone [31, 41, 70], and viable (live) versus non-viable (cadaveric) bone. Various mechanical loading methods (e.g. nanoindentation and bulk compression tests) have been combined with non-viable, in vivo, in vitro, and ex vivo bone testing methods.
to study bone mechanical properties in different conditions and at the different structural levels [71]. The emphasis of this thesis focuses on *ex vivo* culture. Brief discussions on non-viable bone, *in vivo*, and *in vitro* methods are provided as contrast to *ex vivo* methods.

### 2.2.2 Non-Viable Bone Test Methods for Bone Mechanical Behaviour Characterization

Non-viable bone testing is the mechanical testing of non-living bone tissue obtained from fresh-frozen bone (animal or human donor tissue) cadaveric samples [72]. Fresh-frozen samples are obtained from cadaveric tissue, preserved at -20°C (wrapped in saline-soaked gauze and sealed), and then thawed before performing mechanical testing. Cadaveric specimens may also be preserved using a formaldehyde embalming solution [72, 73] or soft embalming methods [74, 75]. Although non-viable bone mechanical tests are simplified without the need for maintenance of viability in contrast to *in vitro*, *in vivo*, and *ex vivo* methods, these tests are limited to non-viable bone mechanical property assessments (e.g. apparent elastic modulus and strength) [71] and by the accuracy of these measurements [76, 77]. Non-viable bone samples are understood to be limited in their accuracy for several reasons. First, bone has been shown to undergo post-mortem changes with decreases in shear compliance [76]. After death, bone is prone to desiccation, causing the bone to dry out over time [77] and exhibit increased brittleness and stiffness [70]. Though non-viable samples can be rehydrated using saline solution, the bone will deteriorate over time. Preservation methods can also affect the mechanical properties. Different freezing techniques can cause changes in viscoelastic properties over time [76]. The effect of long-term embalming methods is still up for debate depending on applications. While some studies have shown that long-term formaldehyde embalming affects bone stiffness [73] and yield and failure stresses [78], others have suggested that formaldehyde embalmed bone cadavers have similar mechanical characteristics to fresh-frozen cadavers [72] and are viable options for bone implant testing [79].
2.2.3 In Vivo Testing Methods for Bone Mechanical Behaviour

Some limitations of testing with non-viable bone can be addressed through in vivo studies carried out under institutional review for ethics approval. In vivo bone testing refers to the study of bone behaviour within a living organism [80]. In vivo studies are used in examinations of various research topics, for example: metabolic bone disease biochemical factors; bone development and growth; bone adaptation; bone repair due to trauma; and, impaired bone healing due to bone diseases such as osteoporosis [81]. Before preclinical studies are performed on human subjects, in vivo studies are often performed with animal models, such as ovine, porcine, and murine models [9]. For example, to quantify the strain of bones in vivo strain gauges are applied on the surface of the bone of interest [37]. These gauges measure strains and strain rates along the bone surface during physical activities and quantification of bone deformation and fatigue damage [82]. However, the accuracy of strain gauges is limited due to difficulties with bonding gauges on the bone surface, temperature-dependent electrical resistance, and calibration. Alternatives to strain gauges are extensometers and bone staples with strain gauges [37, 82]. Although these methods avoid bonding issues, they are susceptible to gauge failure and damage during surgical implantation [37]. All three methods are invasive and limited in that they can only measure the strain at one location along the bone; and therefore, without several strain gauges applied along the bone surface, the strain distribution cannot be measured [82]. Despite providing an accurate physiological environment, in vivo conditions are complex and challenging to control due to bone interacting with the surrounding tissues (e.g. muscles, tendons, and ligaments) and systemic factors. Additionally, in vivo animal models are expensive to maintain and require a large number of samples to identify statistically significant experimental outcomes [81]. Alternatives to in vivo studies are offered by in vitro or ex vivo studies which can provide pre-assessments before proceeding with in vivo models.

2.2.4 In Vitro and Ex Vivo Test Methods for Bone Mechanical Behaviour

In vitro bone testing refers to the investigation of live bone in a controlled environment isolated from the original organism and can be divided into three categories: cell culture, tissue culture, and organ
culture [9]. In contrast to in vivo, in vitro testing is generally less resource and time intensive to conduct [80]. In addition, in vitro testing allows for a higher degree of parameter control and optimization by using a controlled environment [83]. In vitro studies can be controlled to include or remove biochemical and mechanical factors such as growth hormones and fluid flow shear stress [9]. This control can ease study replication, allowing for a simplified experimental design and data analysis process.

In vitro testing is often performed at the cellular level using either two-dimensional (2D) or three-dimensional (3D) cell culture [84]. In 2D culture, cells are grown in 2D monolayers. As a result of this 2D monolayer, 2D cultures are typically simple and efficient to perform and often allow for homogeneous growth and proliferation [84]. Additionally, 2D cell culture test setups (e.g. flow chambers) allow for the study of cell responsiveness to mechanical stimuli such as fluid-induced shear stress [9, 85, 86]. However, despite 2D culture allowing for easier cell proliferation and mechanical load assessment, it is a simplification of the in vivo environment and often ignores important cell-cell interactions required for mechanotransduction [9, 84].

Compared to 2D cell culture, 3D cell culture methods simulate a more accurate physiological microenvironment by mimicking the 3D microstructure of specific tissues and organs [9, 84, 87-89]. A key advantage to using 3D culture is that it can assess cell proliferation, differentiation, and response to biochemical and mechanical stimuli while allowing for cell-cell and cell-extracellular matrix (ECM) interactions within a 3D microenvironment [84]. There are many 3D bone culture methods, including the seeding of bone cells in scaffolds, which can be fabricated from a variety of natural and/or synthetic polymers [83, 89]. Solid porous scaffolds have been shown to be successful for bone regrowth and allow for cell-cell interaction and nutrient diffusion through interconnected porous networks [89]. Hydrogel scaffolds provide an alternative to solid porous scaffolds and have a hydrophilic structure that supports bone cell growth, proliferation, and nutrient permeability; however, they are often not favoured for bone cultures with mechanical load due to their low mechanical strength and stiffness [90, 91]. In long-term static culture, increases in cell mass and matrix deposition reduce the effective porosity in scaffolds and
limit cell proliferation and nutrient diffusion [9, 92]. However, scaffolds can be implemented in bioreactor systems with dynamic culture to improve cell distribution and mechanical stimulation and allow for fluid transport and the control of culture conditions, such as nutrient supply, pH, temperature, and waste removal [9, 93]. Although in vitro 3D cell culture methods allow for a great deal of control, they are often limited to how well they can replicate the physiological diversity of bone and the stages of the remodelling cycle [9, 88].

Bone organ culture, also known as ex vivo or explant culture, refers to the growth or maintenance of explanted bone tissue in vitro [80, 81]. Similar to in vitro 3D cell culture, ex vivo culture allows for the assessment of cell-cell interactions and provides a less complex environment than in vivo animal studies that can be controlled to allow for the removal of systemic factors and evaluation of independent biochemical and mechanical responses [81]. However, unlike in vitro 3D cell culture, ex vivo culture preserves the natural 3D bone structure, cell diversity, and ECM, allowing for natural cell-cell and cell-ECM interactions [9, 81, 94]. By preserving the bone structure, the mechanical response to load includes change in tissue strain, fluid-induced shear stress, and streaming potentials [9]. Additionally, ex vivo bone culture can be used to study the remodelling process of bone ECM associated with biochemical and/or mechanical stimulation [94].

Bone organ culture is commonly performed with dynamic culture in bioreactor systems [9, 94]. For example, ex vivo procedures have been employed to study bone growth, bone-cartilage metabolism, cancer cell effects in bone, stem cell roles in bone repair, and the effect of mechanical loading on bone behaviour [94, 95]. Ex vivo bone culture analyses also include micro-computed tomography (microCT) scanning, and static and dynamic histomorphometry which measure bone microstructure (e.g. bone density, volume, porosity) [94] and bone formation (e.g. mineral apposition rate) [81], respectively. Bone response to mechanical loading is a critical research topic in understanding the role of mechanical stimulation in bone maintenance [81]. Bioreactor systems have been favoured for assessing the effect of mechanical loading thanks to their ability to control mechanical stimuli and media circulation for long-term studies [9]. Despite
the advantages of *ex vivo* methods, bone specimen dimensions can be limited due to the absence of the tissue’s vasculature system and dependence on diffusion [9]. As well, studies are often limited to testing animal tissue due to limited availability of human tissue [81]. When available, human bone tissue is limited to surgical samples with pathologies, such as osteoporosis.

2.3 Trabecular Bone Mechanical Loading in Bioreactors

2.3.1 Design of Bone Bioreactor Systems with Mechanical Loading

Bioreactor systems open the door of opportunities for mechanically stimulating bone cells and tissues by implementing 3D microenvironments with dynamic culture conditions similar to *in vivo* [9]. The design of bioreactor systems requires several key components to guarantee the maintenance of cells and tissues, including material biocompatibility, stability, contamination prevention, and nutrient and temperature regulation. The bioreactor material is considered biocompatible for cell and tissue culture applications if it does not adversely affect the biological tissue and allows for pharmacological acceptability, meaning it does not cause an inflammatory response or is not toxic to the tissue [25, 33]. The material must be chemically inert and not degrade over time, even after multiple experiments. To prevent contamination of the sample, the bioreactor system must be sterilizable and be closed to contaminants (yet gas permeable) to ensure all parts in contact with media and tissue remain uncontaminated [96]. Bioreactors are implemented for *in vivo, in vitro, and ex vivo* studies. *In vivo* and *in situ* bioreactors model the body as a bioreactor to use the body’s natural regenerative ability to promote tissue growth [97]. *In vivo* and *in situ* bioreactors are invasive and complex to perform and, therefore, will not be further discussed in this literature review. Sections 2.3.2 and 2.3.3 highlight commonly used *in vitro* 3D bone cell culture and *ex vivo* bone explant culture methods, respectively, for studying bone response to mechanical stimulation with bioreactors.
2.3.2 *In Vitro* 3D Bone Culture Studies with Bioreactors and Mechanical Stimulation

The bone *in vivo* environment is subjected to complex mechanical load combinations including bending, compression, shear, tension, and torsion [93, 98]. Of these load types, shear stress is hypothesized to be the primary type of stress transmitted to bone cells *in vivo*; and therefore, has been consistently applied in studies with *in vitro* bioreactors for 3D bone cell culture [93, 99]. To assess mechanical stimulation *in vitro*, various bioreactor applications are available for 3D bone cell culture. The simplest 3D bioreactor culture method is the spinner flask model [96, 98, 99]. Spinner flask bioreactors employ stirring methods (e.g. magnetic stir bar) to expose suspended cells, organoids, or cell-seeded scaffolds in a culture media vessel to fluid-induced shear stress. In contrast to static cell culture, the application of shear stress with spinner flasks promotes osteogenic differentiation of stem cells [99]. In spinner flask applications with scaffolds, if too large of a scaffold size is used, nutrient diffusion and waste removal throughout the scaffold can be limited causing cell necrosis at the scaffold centre [98, 99]. Increasing the media flow can improve nutrient delivery in spinner flasks but can increase the shear stress at the outer surface of scaffolds and lead to cell death [98, 99]. An alternative bioreactor is the rotating-wall vessel (RWV) bioreactor. This bioreactor places cell-seeded scaffolds between an inner stationary cylinder and an outer rotational cylinder with culture medium to stimulate bone cells using rotational fluid-induced shear stress [99]. Both spinner flask and RWV bioreactors struggle to allow efficient media perfusion into scaffolds [99, 100]; however, in comparison to spinner flasks, RWV bioreactors can improve oxygen supply and are less likely to cause turbulence during shear stress application [96].

Perfusion-based bioreactor systems offer another alternative and can improve the media perfusion versus shear stress struggle of spinner flasks and RWV bioreactors. Though there are commercial perfusion bioreactor systems, most perfusion systems have been custom-made to meet design and specimen requirements needed for specific projects [99]. Perfusion bioreactors typically consist of four key components: a culture medium reservoir, peristaltic pump, bioreactor (or growth) chamber, and a tubing circuit (Figure 2.4) [99]. The peristaltic pump is used to apply a continuous laminar culture medium flow
through samples (e.g. cell-seeded scaffold) enclosed within the bioreactor chamber. As a result of the culture medium perfusion, cells are continuously subjected to shear stress, which can increase osteogenic differentiation, mineralization, and cell viability at the interior scaffold regions compared to static, spinner flasks, and RWV bioreactors [96, 98-100]. The shear stress of culture medium on samples, such as cell-seeded scaffolds, can be manipulated by controlling the medium flow rate, with lower rates (< 1.0 mL/min) improving cell proliferation and viability and higher rates (< 2.0 mL/min) promoting osteogenic gene expression [98, 100].

Figure 2.4: Layout of perfusion bioreactor with a culture medium reservoir, growth chamber, and pump all connected using a tubing circuit (reprinted from [99], with permission from Elsevier).

Perfusion bioreactors combined with mechanical loading aim to replicate physiological loading conditions and further improve osteogenic marker expressions, differentiation, proliferation, and ECM synthesis [93, 98, 100]. Compression loads are commonly applied using commercial materials testing systems with a bioreactor containing mobile pistons to apply uniaxial compression directly to scaffolds and perpendicular to culture medium flow within the bioreactor (Figure 2.5) [101]. By combining this bioreactor type with a Zetos compression system, Bouet et al. [101] demonstrated that on-off cyclic compression could be used to apply physiologically appropriate mechanical stimulation and increase matrix production using in vitro 3D cell culture of 1.5x10⁶ primary calvarial osteoprogenitor cells seeded in calcium phosphate
bioceramic scaffolds (Figure 2.5). Uniaxial cyclic compression has also been applied parallel to culture medium flow using similar loading setups as Bouet and colleagues [101], with results that show increased bone marrow stromal cell proliferation and early osteogenic differentiation [102, 103]. However, compression bioreactor systems for in vitro 3D cell culture can struggle with retaining long-term sterility due to the insertion of a force-producing mechanism and are limited to applying loads to the scaffold and not directly to cells [98]. While mechanical loading may benefit culture medium perfusion, load applications with force-producing mechanisms, such as pistons, that enter the bioreactor chamber may increase the chance of sample infection [98]. Furthermore, individual bone cells in vitro only provide a simplified representation of the mechanical stimulation of bone behaviour in vivo. To address this, researchers have explored applying mechanical stimulation with ex vivo bioreactors and bone tissue explants to preserve the natural bone 3D environment and the bone marrow.

Figure 2.5: Section view of a bioreactor system with a mobile piston for mechanical loading and perpendicular media perfusion through a seeded scaffold within the bioreactor (adapted from [101]).
2.3.3 *Ex Vivo* Bone Studies with Bioreactors and Mechanical Stimulation

Many bioreactor methods used for *in vitro* 3D bone cell culture can also be implemented with *ex vivo* bone culture. This section provides a brief outline of common bioreactor and mechanical stimulation methods that have been applied for the study of bone mechanics with explanted bone tissue. Most often, *ex vivo* bone bioreactor systems are custom-built for specific research objectives [81] and allow for the maintenance of culture conditions for explanted bone tissue with mechanical stimulation [94]. In *ex vivo* studies, various forms of mechanical loading have been performed to assess explanted bone tissue response to mechanical stimuli, including hydrostatic pressure, shear stress, vibration, and compressive strains. Dynamic hydrostatic pressure has been applied to bone explant cores maintained with static culture medium in a bioreactor by delivering compressed air to surround the bone tissue [81]. By regulating pressure with a controlled air cylinder and pressure transducer, this bioreactor system demonstrated that hydrostatic pressure enhanced osteocyte viability in trabecular bone cores [81, 104].

Similar to *in vitro* 3D bone cell culture methods, explanted bone tissue is most commonly studied with perfusion bioreactors due to their simple and effective nutrient delivery and waste removal methods [81]. With perfusion bioreactors, explanted bone specimens can be mechanically stimulated by fluid-induced shear strains from continuous culture media circulation [81]. Adjusting the rate of culture medium perfusion through the bioreactor directly changes the amount of shear stress applied to the bone sample. In bioreactors, uniaxial load applications, such as compression, tension, and bending, offer mechanical stimulation options for bone tissue with marrow. Combining perfusion bioreactors with compression loading for *ex vivo* bone explant culture can allow for extended culture periods with physiologically relevant mechanical stimulation [81]. However, mechanical property characterization with compression testing is affected by the orientation and geometry of trabecular bone specimens [10, 17, 105]. Due to trabecular bone’s anisotropic nature, compression loads are recommended to be applied in line with the principal direction of the trabecular structure to avoid under-estimation of the mechanical properties in the principal direction [10, 17]. To prevent material buckling, the inaccurate characterization of bone mechanical
properties due to boundary effects, and continuum assumptions, cylindrical specimens with an aspect ratio – the ratio of height to diameter – between 1 and 2 and a minimum diameter of 5 mm should be used [17].

Previous studies have used a perfusion compression system, known as the Zetos system, with a custom-built bioreactor and loading system (Figure 2.6) [14, 15, 81]. The Zetos system allowed for individual trabecular bone core (with marrow) maintenance and compression loading while continuously perfusing culture medium through the bioreactor chamber. Real-time measurement of the mechanical properties (stiffness) could be made while applying physiological loads by using the Zetos loading system and computer-controlled static and dynamic loads with various strain rates. Other groups have performed studies with similar bioreactor and loading approaches, such as Bose Electroforce loading systems (TA Instruments) and bioreactors adapted for trabecular bone compression [94, 106]. As an alternative, researchers have also studied mechanical stimulation in perfusion bioreactors with vibration-induced shear stresses to prevent bone matrix deformations [81].

![Figure 2.6: Schematic representation of the Zetos (a) loading system, (b) bioreactor chamber, and (c) perfusion system layout (adapted from [14]).](image)

2.4 Summary

In summary, trabecular bone is a complex biological material with anisotropic, heterogeneous, and viscoelastic mechanical behaviour that remodels itself and adapts to biochemical and mechanical stimuli. Although mechanical strains between 1000-3000 με are approximated to promote bone adaptation, precise mechanically controlled bone remodelling thresholds remain unknown. Trabecular bone mechanical
behaviour has been studied with non-viable, in vivo, in vitro, and ex vivo bone testing methods. The emphasis of this thesis focuses on the application of ex vivo culture in bioreactor systems. Ex vivo bioreactors with mechanical loading maintain explanted bone tissue with marrow, allowing for an accurate representation of in vivo bone response to mechanical stimulation. Therefore, bioreactors for ex vivo culture with mechanical loading offer unique and promising applications for the study of trabecular bone adaptation to mechanical stimulation.
Chapter 3

Design of an Open-Source 3D Printable Bioreactor for Ex Vivo Trabecular Bone Culture with Compressive Load Application

This chapter has been written as an independent work in preparation for a short communications submission to the Journal of Biomechanics For the benefit of this thesis, this chapter contains additional material that will not be included in the submission due to the publisher’s strict word and figure limit.

3.1 Abstract

The role of mechanical stimulation on bone adaptation is not well understood and is the subject of many studies working towards improved prevention and treatment options to maintain bone health. One approach applied in previous studies implemented a polycarbonate (PC) bioreactor system for assessing the effects of mechanical and biochemical stimulation on trabecular bone adaptation in ex vivo bone organ culture. Despite the success of the PC bioreactor system in long-term organ culture, the PC bioreactor chamber limited the size of bone cores to a maximum height of 5 mm and is difficult and expensive to fabricate. Therefore, this paper has proposed a design for an open-source 3D printable bioreactor with the photopolymer, MED610™, that addresses the limitations of the PC bioreactors and offers fellow researchers and engineers an alternative cost-effective and accessible bioreactor system for bone organ culture. Design validation testing will follow in future publications in the form of MED610™ cell viability studies and ex vivo bone organ culture studies.

3.2 Introduction

The modelling and remodelling processes of bone are widely understood to be promoted by mechanical stimulation [31, 35, 66]. Bone adaptation in response to mechanical loading is dependent on the magnitude and rate of the applied strain [64, 67]. Frost’s Mechanostat theory suggests that bone resorption occurs below strains of 100 με (microstrain) while bone adaptation occurs between strains of...
1000 με and 3000 με [64, 66]. Above 3000 με, bone is susceptible to microdamage with fracture likely to occur around 25,000 με. However, these ranges are rough approximations, and more accurate thresholds have yet to be determined [9]. A better understanding of the role of mechanical load on bone adaptation could assist clinicians with improving prevention and treatment measures, such as physical activity recommendations, for degenerative bone diseases like osteoporosis [6, 8, 107].

The role of mechanical stimulation on bone adaptation is an important and well-studied area of research, though due to the complex bone structure and environment in vivo, mechanical stimuli are difficult to quantify [9]. In comparison to in vivo methods, in vitro or ex vivo techniques offer more control of experimental factors to study bone adaptation at the cellular and tissue structure levels. Ex vivo bone culture preserves the three-dimensional (3D) bone structure including marrow and allows for controlled mechanical stimulation while maintaining an environment for long-term studies [9, 81, 94, 95]. A polycarbonate (PC) bioreactor system combined with a mechanical loading system has been successfully used in previous research to study the effect of mechanical load and biochemical stimuli on trabecular bone adaptation [14, 15, 108]. Although the PC bioreactors have proven biocompatibility and control of culture conditions in bone organ culture, they are difficult and expensive to fabricate and require access to a professional machine shop. The PC bioreactors were limited to testing specimens with a maximum height and diameter of 5 mm and 10 mm, respectively, to ensure sufficient culture medium nutrient diffusion into the centre of bone specimens. However, bone testing standards require a height to diameter ratio between 1 and 2 for accurate measurement of bone mechanical properties and to avoid specimen buckling [17]. A larger bioreactor chamber might also allow for testing of a variety of sample types, for example, a bone-implant system.

Recent additive manufacturing (AM) advancements offer a more cost-effective, accessible, and faster prototyping method compared to traditional computer numerical control (CNC) and injection moulding fabrication [19, 21]. Polyjet 3D printing has high dimensional precision [22] for flexible design optimization [23] with biocompatible materials [25]. Therefore, the objective of this work was to design a 3D printable bioreactor system for bone organ culture with compressive load application that addresses the
fabrication and specimen size limitations of the machined PC bioreactors used in previous studies. The bioreactor design proposed is offered as an open-source design to provide researchers with an accessible, cost-effective bioreactor design for bone organ culture that can be fabricated with compatible 3D printers or 3D printing services.

### 3.3 Design Requirements and Functional Specifications

Following a formal mechanical design process [109], the 3D printable bioreactor system design requirements and functional specifications were defined and assessed in a “house of quality” HOQ evaluation matrix (provided in Appendix). The design requirements were assigned an importance rank between 1 (least important) and 5 (essential). Firstly, the bioreactor design must (rank 4) be able to maintain an individual bone specimen with an aspect ratio (height to diameter) between 1 and 2 to follow bone compression testing standards for accurate mechanical property measurement [17]. Secondly, the bioreactor material and fabrication process must (rank 4) be easy to implement, cost-effective, and accessible. For ease of repeating the fabrication process, it would be beneficial for the design to be reusable, though this was not considered essential (rank 2) for the success of the bioreactor design. As an additional design requirement, the bioreactor design should (rank 2) fit bone core heights up to 14 mm to allow for bone-implant system testing with implant insertion depths up to 13 mm [18]. For the bioreactor design described in this paper, this additional improvement was not considered essential.

To replicate the success of the PC bioreactors in past studies, the new bioreactor design must be sterilizable, non-toxic, maintain aseptic conditions during organ culture, and provide bone tissue with physiological conditions (37°C temperature and 5% CO₂) and a nutrient supply. These requirements are essential (rank 5) to ensure the success of the bioreactor system in long-term bone organ culture for up to at least 3-weeks to replicate previous studies [14, 15]. If these requirements are not met, contamination or bone cell necrosis may occur during organ culture and alter results. Lastly, the bioreactor system must be capable of applying mechanical compression loads (rank 5) and quantifying the apparent elastic modulus.
of individually maintained bone specimens to study the bone adaptation response to mechanical loading in long-term (rank 4) culture.

Functional specifications for the 3D printable bioreactor system with compressive load application are outlined in Table 3.1. Target criteria (Table 3.1) are based on proven methods used by Vivanco et al. [14] and Meyer et al. [15] and standard conditions required for successful bone organ culture.

Table 3.1: Functional specifications and their target criteria for a 3D printable bioreactor system with compressive load application.

<table>
<thead>
<tr>
<th>System Component</th>
<th>Functional Specification</th>
<th>Target Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical loading application</td>
<td>Sample aspect ratio between 1-2</td>
<td>Height ≤ 14 mm, diameter ≤ 10 mm</td>
</tr>
<tr>
<td></td>
<td>Mechanical compression loading</td>
<td>0 – 4000 με</td>
</tr>
<tr>
<td></td>
<td>No. of mobile pistons for loading</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3D printable</td>
<td>MED610™ material</td>
</tr>
<tr>
<td>Fabrication process</td>
<td>Minimize fabrication cost</td>
<td>&lt; $1000 [CAD $]</td>
</tr>
<tr>
<td></td>
<td>Minimize post-fabrication processing (support material removal)</td>
<td>&lt; 48 hours</td>
</tr>
<tr>
<td></td>
<td>Minimize No. of bioreactor chamber parts</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Material</td>
<td>Biocompatible material</td>
<td>&gt; 21-day tissue contact</td>
</tr>
<tr>
<td></td>
<td>Gas or steam sterilizable material</td>
<td>EtO gas, autoclave at 132°C for 4 minutes</td>
</tr>
<tr>
<td>Organ culture application</td>
<td>Chamber specific reservoir</td>
<td>Yes or no</td>
</tr>
<tr>
<td></td>
<td>CO₂ and temperature regulation</td>
<td>5 ± 0.5% CO₂, 37 ± 0.5°C</td>
</tr>
<tr>
<td></td>
<td>Culture medium circulation</td>
<td>≥ 6.6 mL/hour</td>
</tr>
<tr>
<td></td>
<td>Maintain long-term aseptic culture</td>
<td>Yes or no, &gt; 21-days</td>
</tr>
<tr>
<td></td>
<td>No. of seals in bioreactor</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

The functional specifications are also listed in the HOQ (provided in Appendix), with their relation to each design requirement highlighted. Additionally, a comparison of the PC bioreactor and the proposed 3D printable bioreactor design with respect to the design requirements, displayed in the HOQ, highlights that the new bioreactor system addresses the PC bioreactor’s sample size and fabrication limitations. However, validation is required to confirm the biocompatibility, sterility, and efficacy of the 3D printable MED610™ bioreactor in long-term organ culture.
3.4 Bioreactor System Design

3.4.1 Bioreactor Chamber Design

The proposed bioreactor design was adapted from the PC bioreactor chamber implemented by Vivanco et al. [14] and Meyer et al. [15]. The bioreactor consists of cylindrical body (Figure 3.1 (A) and (B)) and cap parts (Figure 3.1 (C) and (D)), which attach by an M24 x 2.0 mm thread connection. Both parts contain a slot to fit an X-ring and have flats on their top surfaces to help grip them for assembly. The primary function of the bioreactor body is to house a single bone core (with an aspect ratio greater than or equal to one) in a cylindrical alcove (11.5 mm diameter) with dimensions up to 11 mm in height (H) and 10 mm in diameter (D). The alcove was designed to have the same diameter as the bioreactor top insert to ease the removal of support material after fabrication. Additionally, the alcove diameter is slightly larger in diameter (1.5 mm) than the maximum bone core diameter to ensure that the bone is fully submerged in culture medium during organ culture and not restrict lateral expansion of the bone core. Inlet and outlet channels are connected to the alcove to allow for the circulation of culture medium throughout the bioreactor. The openings of the inlet and outlet channels are tapered for the secure insertion of luer tubing connectors. The outlet luer and horizontal fluid channels were designed to go through to the alcove to ease support material removal. Furthermore, the horizontal fluid channel goes through to the outer diameter of the bioreactor body. Detailed mechanical drawings of the bioreactor body and cap parts are provided in the Appendix.
Figure 3.1: (A) Isometric and (B) section views of the bioreactor chamber body. (C) Isometric and (D) section views of the bioreactor chamber cap.

3.4.2 Fabrication Process and Material

The bioreactor model was designed in SolidWorks 2019 (SolidWorks Corp., Dassault Systemes, Waltham, MA, USA) and 3D printed with an Objet30 Prime printer, MED610™ material, and SUP706B™ water-soluble support material (Stratasys, Edina, MN, USA). MED610™ is classified by the manufacturer as a biocompatible material for permanent skin contact and up to 24-hours of mucosal membrane contact [110]. However, recent studies have demonstrated that the biocompatibility of MED610™ is dependent on the cleaning and sterilization protocol used [25, 26]. If properly cleaned and sterilized, MED610™ is suitable for cell and tissue culture applications [25, 26, 111, 112]. Ngan et al. has recommended a sonication protocol to clean MED610™ and mitigate the effect of leachates from the material in tissue culture [25]. Additionally, MED610™ is autoclavable for up to four minutes at 132°C [110]. Overall, the bioreactor is readily printable with a low fabrication cost (∼$16.20/bioreactor) and quick print time (< 3 hours).

3.4.3 Bioreactor System Assembly

The assembly of the bioreactor system contains four main components: the bioreactor chamber, culture medium reservoir, peristaltic pump and tubing, and a mechanical loading system. The system
assembly also contains sapphire pistons and X-ring and O-ring seals. All components in the system assembly are sterilizable by steam or gas sterilization.

3.4.3.1 Bioreactor Chamber Assembly

In the bioreactor assembly, a single bone core specimen is contained within the alcove of the bioreactor chamber and placed between two 10 mm x 10 mm (H x D) sapphire pistons (Figure 3.2). Each piston is positioned within a silicone rubber X-ring to provide seals at the top and bottom of the bioreactor. The piston-X-ring interfaces are lubricated with a food-grade silicone lubricant (No. 1204K32, McMaster-Carr, Elmhurst, IL, USA) to minimize translational friction during piston displacement due to compression loads. Additionally, silicone rubber O-rings with inner diameters (ID) of 19 mm (S1001-1x19, Marco Rubber & Plastics, Seabrook, NH, USA) and 11 mm (S1001-.7x11, Marco Rubber & Plastics) are positioned in O-ring slots between the body and cap parts to prevent medium leakage at the bioreactor thread interface. Lastly, a custom-made MED610™ tapered plug is inserted in the horizontal fluid channel to close it and prevent leakage out of the bioreactor. Detailed drawings of the O-rings, sapphire piston, MED610™ plug, X-ring, and bioreactor chamber assembly are provided in the Appendix.

Figure 3.2: (A) Isometric and (B) section views of the bioreactor chamber assembled with a 10 mm x 10 mm (H x D) bone core, tapered plug seal, sapphire pistons, and O-ring and X-ring seals.
3.4.3.2 Chamber Specific Culture Medium Reservoir

Each chamber specific culture medium reservoir consists of a rimless 9 mL borosilicate glass culture tube and a custom-made MED610™ stopper. The stopper has a three-tiered tapered O-ring section (8 mm ID silicone O-ring, S1001-1.5x8, Marco Rubber & Plastics) to accommodate the inner diameter tolerance of the culture tubes (Figure 3.3). Two needles (18-gauge) are inserted through the needle channels of the stopper to provide an inlet and outlet for the culture medium reservoir. A 60 mm long piece of 0.053” (outer diameter) polytetrafluoroethylene (PTFE) tubing (No. 5335K16, McMaster-Carr) is attached to the outlet needle to draw media from the bottom of the media reservoir. When assembled, the medium reservoir can store up to 8 mL of culture medium. To change culture medium, the stopper, needles, and PTFE tubing can be easily removed from the culture tube and inserted into a new tube with fresh medium. Stopper dimensions are provided in the Appendix.

![Figure 3.3](image_url)

**Figure 3.3:** (A) Isometric and (B) section views of the custom-made 3D printable MED610™ culture tube stopper with a 3-tiered tapered O-ring section.

3.4.3.3 Peristaltic Pump and Tubing

The purpose of the peristaltic pump and tubing circuit is to supply nutrients to the bone core and remove waste by circulating culture medium from the medium reservoir to the bioreactor chamber and back (Figure 3.4). Medium is drawn from the outlet of the medium reservoir with gas permeable Tygon® E-Lab tubing (1.52 mm ID, Ismatec No. 06460-36, Cole-Parmer Canada, Montreal, QB, Canada) and directed through 2-stop PharMed® BPT pump tubing (1.52 mm ID, Ismatec No. 95723-36, Cole-Parmer Canada) in
a 24-channel peristaltic pump (Ismatec™ ISM939D, No. 78000-41, Cole-Parmer Canada). From the pump tubing, medium is then directed through Tygon® tubing to the bioreactor and then back to the medium reservoir inlet. Fluid flow is controlled with the peristaltic pump and is capable of consistent flow rates between 0.03 to 3.0 mL/min for the pump tubing ID used in this system. Additionally, since the pump in this system has 24 channels, one pump can control culture medium flow for up to 24 bioreactor chambers at a time, each with their own culture medium reservoir. Polypropylene male luer to 1/16” hose barb adapters (Masterflex No. 45518-22, Cole-Parmer Canada) and polyvinylidene fluoride 1/16” barbed male-male adapters (No. 53055K111, McMaster-Carr) were used for tubing connections to the bioreactor, peristaltic pump, and medium reservoir.

3.4.3.4 Mechanical Loading System

The bioreactor system uses a Mach-1 mechanical loading system (Biomomentum, Laval, QB, Canada) to apply displacement-controlled load applications. The loading fixture consists of a flat indenter (MA263, Biomomentum) in contact with a custom-made ball-and-socket adapter which contacts the top
sapphire piston of the bioreactor assembly to apply uniaxial compression to bone specimens (Figure 3.5). The ball-and-socket fixture corrects for non-parallel surfaces at contact interfaces. Silicone lubricant is applied between contact interfaces to reduce contact friction. Time, force, and vertical displacement are measured with a multiple-axis 250 N (F_x and F_y: ± 0.0125 N; F_z: ± 0.0065 N) load cell (MA242, Biomomentum). System compliance was assessed with ten aluminum 7075-T6 reference bodies [113] and determined to be linear (provided in appendices).

![Figure 3.5: Layout of the bioreactor system and fixture assembly with the Mach-1 mechanical loading system.](image)

### 3.5 Discussion

Although the proposed bioreactor design addresses the fabrication and specimen size limitations of the PC bioreactor used by Vivanco et al. [14] and Meyer et al. [15], it has its own limitations. Although Ngan et al.’s [25] work indicated the successful use of MED610™ in long-term cell culture, due to little to no research in recorded literature, it is unknown if the MED610™ material or leachates affect bone cell
behaviour during the bone adaptation response to mechanical stimulation. Secondly, though the alcove diameter was designed to fully encompass the bone core without restricting it for the ease of support material removal, it may reduce the pressure of culture medium flow into the bone and thus, limit the diffusion of culture medium through the bone core. As a result, the supply of nutrients to the bone core centre could be limited and lead to cell necrosis. Additionally, the increased bone core height could also limit the diffusion of culture medium into the bone core centre. Therefore, testing is required to validate the biocompatibility of the MED610™ material in long-term bone culture and assess the diffusion of culture medium to the bone core centre. Thirdly, it is unknown if repeated autoclave cycles over time will weaken the MED610™ material, similar to polycarbonate. This could limit the repeatable use of the 3D printed bioreactor and incur additional 3D print and material costs. Lastly, the bioreactor design can only fit bone core heights up to 11 mm, which may still be too small for testing larger samples, for example, a composite bone-implant system [18].

Despite these limitations, this paper proposes a 3D printable bioreactor system for ex vivo bone culture with mechanical loading that addresses the limitations of a PC bioreactor chamber used in previous research. The advantage of 3D printing the bioreactor offers users the ability to quickly apply design changes at a low cost compared to traditional CNC and injection moulding fabrication processes [19, 21]. Additionally, in contrast to common commercial bioreactor systems, this design is unique in that each bioreactor chamber has its own specific culture medium reservoir. However, the proposed 3D printable bioreactor is still in the design process. Future validation testing is required to assess the functional specifications of the 3D printable bioreactor outlined in the HOQ, with priority given to the highest weighted functional specifications with respect to the design requirements. Specifically, the biocompatibility and sterilizability of the MED610™ material should be assessed in long-term in vitro cell culture and a cleaning and sterilization protocol should be established to minimize the effect of MED610™ leachates before the bioreactor is used for organ culture. Repeated in vitro cell culture studies with the same 3D printed parts will also address the reusability of the MED610™ material and the ability to regulate CO₂
and temperature. Furthermore, validation testing is required to confirm the ability of the bioreactor to apply a continuous culture medium flow to enclosed samples without causing leakage at the bioreactor seals. The bioreactor design should be employed in a bone organ culture study to investigate the bioreactor’s capability to supply an efficient culture medium flow for maintaining a 10 mm x 10 mm (H x D) bone core while maintaining aseptic conditions and supplying daily mechanical compression loads. Following validation tests, the open-source, 3D printable bioreactor design will continue to be updated with required design adjustments as needed.
Chapter 4

Establishing a Cleaning and Sterilization Technique for 3D Printed MED610™-Based Tissue Culture Devices for Long-Term Cell Culture

This chapter has been written as an independent work in preparation for a journal article submission to Biomedical Materials.

4.1 Abstract

In recent years, polyjet 3D printing has become a promising prototyping technique for biomedical applications due to its high dimensional precision and ability to print with biocompatible materials, such as MED610™. Although marketed to have limited mucosal membrane biocompatibility, the biocompatibility of MED610™ in cell and tissue culture has been shown to be dependent on the cleaning and sterilization protocol applied. Therefore, the objective of this study was to establish a cleaning and sterilization protocol for 3D printed MED610™ culture devices, including a custom-made 3D printed bioreactor. Four cleaning and sterilization protocols – the manufacturer’s protocol (MP), a sonication protocol (SP), an autoclaving protocol (AP), and SP with autoclaving (SP+A) – were tested across three separate cell culture studies with MED610™ constructs and static and perfusion bioreactors. Conditioned medium from each sterilization group was applied to Saos-2 cell line cultures in 96-well plates (15,000 cells/well, 37°C, 5% CO₂) and changed every other day. Cell viability was assessed with a CellTiter-Blue assay and compared to positive (conditioned medium from an autoclaved polycarbonate bioreactor) and negative (unconditioned medium) controls. In the static construct study, no significant differences (p > 0.05) were observed for MP and SP+A compared to the controls after seven days. For the static bioreactor study, the MP and SP+A cell viabilities were significantly different from the controls, though the SP+A viability was 9.67% and 15.9% higher compared to the negative and positive control, respectively. After 21 days, the SP+A cell viability in the perfusion bioreactor study was not significantly different from the positive control but was 13.0% higher.
than the negative control. This study demonstrated that cell viability was not adversely affected by MED610™ cleaned and sterilized with a combination of sonication and autoclaving. However, further testing is warranted to confirm the role of MED610™ on cell behaviour during direct contact.

4.2 Introduction

With recent advancements in additive manufacturing (AM), the application and development of three-dimensional (3D) printed devices in biomedical and tissue engineering are becoming more common [19, 114, 115]. Compared to traditional manufacturing methods, such as computer numerical control (CNC) or injection moulding, 3D printing offers research and development engineers a cost-effective, easier, more accessible, and faster prototyping method from computer-aided design (CAD) models and facilitates design optimization [19-21]. Additionally, 3D printing can be used to fabricate multiple custom-made parts at the same time [26] with high precision (as low as 20-30 μm) [23]. Furthermore, 3D printing offers flexible mechanical property optimization of designs by adjusting printing options such as print layer orientation and thickness [22, 116]. 3D printing has been recently employed for cell and tissue engineering to generate bone scaffolds [20, 22, 111, 117], microfluidic devices [19, 23, 118], bioreactor devices [21, 119], and culture equipment such as well plates [19, 120]. The most common 3D printing methods are: fused deposition modelling (FDM), stereolithography (SLA), selective laser sintering (SLS), and inkjet-based techniques [23]. However, the biocompatibility of 3D printing materials for biomedical applications remains a major concern [25, 26, 121]. Materials are considered biocompatible for cell and tissue culture if they do not negatively affect biological tissue during contact or cause an inflammatory response and enable a tissue response as if it were in its natural host environment [25, 33, 121]. In recent years, polyjet printing techniques have become increasingly promising for tissue engineering applications due to their high precision printing with biocompatible materials, such as MED610™ (Stratasys, Edina, MN, USA), and water-soluble support material [25]. Thus, 3D printing with MED610™ could open doors for simplifying the fabrication process of tissue culture devices, such as bioreactors, such as well plates, scaffolds, and bioreactors.
MED610\textsuperscript{TM} biocompatibility has been evaluated by the manufacturer, Stratasys [110], following the standard ISO 10993-1 [122]. The manufacturer lists MED610\textsuperscript{TM} as biocompatible for permanent skin contact but limited to 24-hour mucosal membrane contact [110]. While some researchers have agreed with the manufacturer that MED610\textsuperscript{TM} is not suitable for cell and tissue culture environments [123, 124], other researchers have suggested that the biocompatibility of MED610\textsuperscript{TM} is dependent on the cleaning method used [25, 26]. If adequate cleaning and sterilization protocols are used, the effect of MED610\textsuperscript{TM} leachates can be minimized and allow for MED610\textsuperscript{TM} use in cell and tissue culture [25, 26, 111, 112].

The purpose of this study was to establish a cleaning and sterilization protocol for various MED610\textsuperscript{TM}-based 3D printed culture devices, including a custom-made perfusion bioreactor chamber. The biocompatibility resulting from various cleaning and sterilization protocols for printed devices were assessed via a long-term cell viability assay using the Saos-2 human osteosarcoma cell line. As a comparison, machined polycarbonate (PC) bioreactors and polystyrene tissue culture plates were also implemented as experimental controls. PC bioreactors, similar to the MED610\textsuperscript{TM} bioreactors utilized in this study, have been previously demonstrated to allow for the culture of bone tissue for long-term studies for up to 7 weeks [14-16, 108].

4.3 Methods and Materials

4.3.1 Reagents and Chemicals

Unless stated otherwise, all cells, culture reagents, and chemicals were obtained from Millipore Sigma (Burlington, MA, USA).

4.3.2 MED610\textsuperscript{TM} and SUP706B\textsuperscript{TM} Materials

For this study, parts were 3D printed using MED610\textsuperscript{TM} and the SUP706B\textsuperscript{TM} support material (Stratasys). MED610\textsuperscript{TM} is a photopolymer with the composition listed in Table 4.1 [24]. A significant percentage of the composition, provided by Stratasys [24], contains proprietary content, which could
include cytotoxic components. The support material, SUP706B™, is a water-soluble polymer material and is comprised of the materials listed in Table 4.1 [125].

Table 4.1: MED610™ and SUP706B™ compositions and percentage breakdowns.

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight-%</th>
<th>Component</th>
<th>Weight-%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Proprietary</td>
<td>95.2-98.4</td>
<td>Total Proprietary</td>
<td>36.3-78.7</td>
</tr>
<tr>
<td>Caprolactone Acrylate</td>
<td>1.00-3.00</td>
<td>Propane-1,2-diol</td>
<td>10.0-30.0</td>
</tr>
<tr>
<td>1,7,7-Trimethyltricyclo[2.2.1.02,6]heptane</td>
<td>0.10-0.30</td>
<td>Polyethylene Glycol 400</td>
<td>10.0-30.0</td>
</tr>
<tr>
<td>2-Propenoic Acid, 1,2-Ethanediy1 Ester</td>
<td>0.10-0.30</td>
<td>Acrylic Acid, 2-Hydroxyethyl Ester</td>
<td>1.00-3.00</td>
</tr>
<tr>
<td>Acrylic Acid, 2-Hydroxyethyl Ester</td>
<td>0.10-0.30</td>
<td>4-Methoxyphenol/Mequinol</td>
<td>0.10-0.30</td>
</tr>
<tr>
<td>Acrylic Acid</td>
<td>0.10-0.30</td>
<td>2,6-Bis(1,1-Dimethylethyl)-4-Methylphenol</td>
<td>0.10-0.30</td>
</tr>
<tr>
<td>Camphene</td>
<td>0.10-0.30</td>
<td>Heptane</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>Glycerol, Propoxylated, Esters with Acrylic Acid</td>
<td>0.10-0.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.3 3D Printing and Support Material Removal

Three MED610™ constructs were designed in SolidWorks 2019 (SolidWorks Corp., Dassault Systemes, Waltham, MA, USA) and divided into three separate 3D print jobs. Four MED610™ rectangular constructs (Figure 4.1 (B)) with dimensions of 15 mm x 15 mm x 4 mm (length x width x height) were printed in the first print job. The second print job contained two scaled-down versions of the body piece of a custom-made bioreactor design (Figure 4.1 (C)) adapted from the PC bioreactor used by Vivanco et al. [14] and Meyer et al. [15]. Two cap and body pieces of the full-scale bioreactor design (Figure 4.1 (D)) were printed in the third job. All parts were printed using a Stratasys Objet30 Prime 3D printer with MED610™ and SUP706B™ support material. Parts were printed with a high-quality glossy finish setting (Objet Studio Ver. 9.2.11.6825). Detailed dimensioned part drawings are provided in the Appendices.

After printing, all visible exterior and interior support material was manually removed from parts with a pick tool. Parts were then individually washed in a high-pressure waterjet and air-dried. Following the manufacturer’s support removal protocol, parts were then fully submerged in a 1% w/v sodium metasilicate (Na₂SiO₃) and 2% w/v sodium hydroxide (NaOH) solution [110]. After soaking for 24 hours,
the parts were soaked in a 5% v/v acetic acid for 1 minute while stirring to neutralize pH. Once removed from the acetic acid solution, parts were individually rinsed under running tap water for 1 minute and then soaked in distilled water for an additional 5 minutes.

Figure 4.1: (A) Polycarbonate bioreactor body (left) and cap (right) parts (positive control); (B) MED610™ rectangular constructs; (C) bottom (right) and top (right) views of the scaled-down MED610™ bioreactor body part; and (D) MED610™ bioreactor body and cap parts.

4.3.4 Cell Viability Assay

All cell viability studies were completed using the Saos-2 human osteosarcoma cell line. Unless specified otherwise, cells were cultured in McCoy’s 5A culture medium with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Cells were plated onto 96-well plates at a seeding density of 15,000 cells/well and cultured in 100 μL of fresh culture medium at 37°C and 5% CO₂. After 24 hours, culture medium was removed and replaced with conditioned medium (Day 0 and every two days thereafter). At specified time points, cell viability was assessed using the Cell-Titer Blue assay kit (No. G8080, Promega, Maddison, WI, USA) and a Synergy H1 plate reader (BioTek, Winooski, VT, USA). After adding the assay dye, well-plates were incubated at 37°C and 5%CO₂ for 4-hours before measuring fluorescence with an excitation and emission filter pair of 550 nm and 600 nm, respectively. Background assay fluorescence was accounted for measuring the fluorescence of the assay dye in 100 μL of fresh culture medium.
4.3.5 Sterilization Protocol Screening

An initial treatment screening study was performed using four MED610™ rectangular constructs to compare three sterilization methods to the manufacturer’s recommended cleaning method. Each construct was randomly allocated to a specific sterilizing protocol test group. The first test group (MP) followed the manufacturer’s protocol which required soaking the constructs in analytical grade isopropanol (IPA) for 30 minutes [110]. The second test group (SP) followed a sonication protocol created by Ngan et al. [25], which found that sonication was able to remove MED610™ leachates and minimize adverse affects from MED610™ on cell viability and differentiation in cell culture. This protocol required the sonication (40 kHz) of a construct in IPA for 2 hours, followed by another 2-hour sonication period in Milli-Q water (Millipore Sigma). During sonication, the construct was fully submerged with a minimum volume ratio of 10:1 for the solvent to construct in a covered glass beaker. The sonication solvent was replaced with fresh solvent every hour. After sonication, the construct was rinsed in 80% ethanol and dried under UV light for 2 hours. The third test group (AP) was autoclaved at 132°C for 4 minutes. The fourth protocol (SP+A) involved the sonication protocol followed by autoclaving at 132°C for 4 minutes. Using aseptic culture techniques, sterilized constructs were individually placed in sterile 60 mm x 15 mm culture dishes with culture medium at 37°C and 5% CO₂ for 7 days. Negative (unconditioned medium) and positive (medium with an autoclaved PC bioreactor (121°C for 15 minutes)) controls were placed in sterile culture dishes under the same conditions. Culture medium was changed every two days with half-conditioned medium. Cell viabilities of test groups conditioned media (n = 6 wells/group) were assessed on days 2 and 7 and compared to the negative and positive.

4.3.6 Conditioned Medium Study Under Static Conditions Using MED610™ Sterilized Bioreactors

The SP+A cleaning protocol was further evaluated for complex 3D printed MED610™ shapes using two 3D printed scaled-down bioreactor body parts. Here, the printed parts were cleaned using the MP and SP+A protocols and placed in two sterile tissue culture dishes with culture medium. Culture medium was changed every second day with full conditioned medium. Cell viabilities of the test groups (n = 6
were assessed on day 7 and compared to negative (unconditioned medium from a sterile culture dish) and positive control (conditioned medium from autoclaved PC bioreactor in a sterile culture dish) groups.

**4.3.7 Conditioned Medium Study Under Perfusion Using MED610™ Sterilized Bioreactors**

The SP+A sterilization protocol was then tested over 21-days in a perfusion bioreactor study. Here, the MED610™ bioreactor was assembled into a closed-system consisting of the bioreactor, a glass medium reservoir, tubing circuit, and a 24-channel peristaltic pump (Ismatec™ ISM939D, No. 7800041, Cole-Parmer Canada, Montreal, QB, Canada) (Figure 4.2). The bioreactor chamber assembly consisted of a MED610™ plug, MED610™ bioreactor body, MED610™ bioreactor cap, sapphire pistons, silicone X-rings, and silicone O-rings. The tubing circuit consisted of 2-way stop platinum-cured silicon tubing, polytetrafluoroethylene (PTFE) tubing, Tygon® E-Lab tubing, polypropylene luers, and polyvinylidene fluoride 1/16” connectors.

![Figure 4.2: Polycarbonate and MED610™ bioreactors (A) assembled with media reservoirs (B), and a peristaltic pump tubing circuit (C) to perfuse culture media through the bioreactors.](image)

Prior to assembly, all non-MED610™ components were autoclaved, while MED610™ parts were cleaned using the SP+A protocol. After assembling the bioreactor system using aseptic techniques, culture medium was perfused through the system with a flow rate of 6.6 mL/hour [14, 15] at 37°C and 5% CO₂.
Every other day, culture medium in the system was replaced with fresh medium and used as conditioned medium for well-plate culture medium changes. An autoclaved PC bioreactor was assembled following the same protocol and used as a positive control. A negative control consisted of a sterile dish containing culture medium. Conditioned medium was removed from all groups (n = 12/group) on days 7 and 21 to assess cell viability. Table 4.2 outlines a summary of the methods for the static construct, static bioreactor, and perfusion bioreactor studies.

Table 4.2: Sterilization and cleaning study methods summary table. MP: manufacturer’s protocol; SP: sonication protocol; AP: autoclave protocol; SP+A: sonication and autoclave protocol.

<table>
<thead>
<tr>
<th>Study</th>
<th>Parts Used (Amount)</th>
<th>Sterilization &amp; Cleaning Methods Tested</th>
<th>Media Change Method</th>
<th>Cell Viability Timepoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static Construct</td>
<td>Rectangular construct (x 4)</td>
<td>MP, SP, AP, SP+A</td>
<td>Half-conditioned</td>
<td>Days 2 and 7</td>
</tr>
<tr>
<td>Static Bioreactor</td>
<td>Scaled down bioreactor body (x 2)</td>
<td>MP, SP+A</td>
<td>Full conditioned</td>
<td>Day 7</td>
</tr>
<tr>
<td>Perfusion Bioreactor</td>
<td>Full-scale bioreactor body and cap (x 1 of each)</td>
<td>SP+A</td>
<td>Full conditioned</td>
<td>Days 7 and 21</td>
</tr>
</tbody>
</table>

4.3.8 Statistical Analysis

Statistical analysis of data was performed with MATLAB 2019b (MathWorks, Portola Valley, CA, USA) and Origin 2021b (OriginLab, Northampton, MA, USA). Group normality was assessed with Shapiro-Wilk tests and confirmed normality in the sterilization screening and static bioreactor studies but found a lack of normality across all groups in the perfusion bioreactor study. In the sterilization screening and static bioreactor studies, test group cell viabilities were compared to the positive and negative controls with a one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test. Test group cell viabilities in the perfusion bioreactor study were compared to the positive and negative controls with Kruskal-Wallis ANOVA and Dunn’s multiple comparison post hoc test. A statistical significance of $\alpha = 0.05$ was assumed in all statistical analyses.
4.4 Results

4.4.1 Comparison of Sterilization Protocols

Cell viabilities corresponding to the MP and AP cell viabilities were not significantly different compared to the control groups on day 2 (Figure 4.3 (A)). Cell viabilities corresponding to the SP and SP+A methods were both found to be significantly different compared to the negative (SP: p = 0.013; SP+A: p < 0.001) and positive controls (SP: p = 0.015; SP+A: p < 0.001). No significant differences were observed for MP and SP+A compared to the controls (Figure 4.3 (B)) on day 7. Although SP and AP were significantly different from the negative control (SP: p = 0.001; AP: p = 0.009) and were lower by 10.92% and 9.19%, respectively, they were not significantly different compared to the positive control. In addition, AP was not significantly different compared to MP and SP+A and was 4.77% and 6.83% lower than each, respectively. SP was significantly different from the SP+A group (p = 0.021) with an 8.60% lower cell viability. The positive control was significantly different compared to the negative control on day 7 (p = 0.025), with a percent change of -8.18%. Overall, the results showed that the MP and SP+A are more favourable than the SP and AP methods for use in static cell culture with simple MED610™ constructs.
Figure 4.3: Relative fluorescence units (RFUs) data indicating cell viability for the negative control (unconditioned medium), positive control (conditioned medium from autoclaved PC bioreactor), and the different cleaning protocol groups (n = 6) on days (A) 2 and (B) 7. MP: manufacturer’s protocol; SP: sonication protocol; AP: autoclave protocol; SP+A: sonication and autoclave protocol. (*p-values < 0.05)
4.4.2 MED610™ In Vitro Static Bioreactor Cell Culture Viability

Following the results obtained from the sterilization protocol screening study, the MP and SP+A methods were tested with a MED610™ bioreactor design in static cell culture. Statistical significance was observed in the difference in cell viability compared to negative and positive controls for both the MP and SP+A methods (Figure 4.4). SP+A cell viability was 9.67% and 15.9% higher than the negative and positive controls, respectively, whereas the MP cell viability was negligible.

![Relative fluorescence units (RFUs) indicating cell viability](image)

**Figure 4.4:** Relative fluorescence units (RFUs) indicating cell viability for the negative control (unconditioned medium), positive control (conditioned medium from autoclaved PC bioreactor), manufacturer’s protocol (MP), and the sonication and autoclave protocol (SP+A) groups (n = 6) on day 7 for the static bioreactor study (*p*-values < 0.05).

The results of the static bioreactor study indicated that the SP+A cleaning protocol was able to clean and sterilize the MED610™ bioreactors for use in cell culture. Steps for the sonication protocol by Ngan et al. [25] and the additional autoclave step are outlined in Table 4.3. Table 4.3 also provides the steps used for removing support material.
Table 4.3: Summary outline of the sonication and autoclave (SP+A) protocol for cleaning MED610™ parts.

<table>
<thead>
<tr>
<th>Sonication and Autoclave Protocol for Sterilizing MED610™ Bioreactors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Remove all visible support material from parts.</td>
</tr>
<tr>
<td>a. Remove visible exterior and interior support material using a pick tool.</td>
</tr>
<tr>
<td>b. Thoroughly wash parts in a high-pressure water jet to remove remaining visible support material.</td>
</tr>
<tr>
<td>2. Follow manufacturer’s support removal to remove any remaining support material.</td>
</tr>
<tr>
<td>a. Soak parts in 1% Na₂SiO₃ and 2% NaOH caustic soda solution for 24 hours.</td>
</tr>
<tr>
<td>b. Soak parts in 5% acetic acid solution for 1 minute to neutralize caustic soda pH.</td>
</tr>
<tr>
<td>c. Rinse parts with water for 1 minute and then soak in distilled water for 5 minutes.</td>
</tr>
<tr>
<td>3. Sonicate parts in a covered glass beaker with IPA (10x volume of parts) for 2 hours. The solvent should be replaced every hour.</td>
</tr>
<tr>
<td>4. Sonicate parts in a covered beaker with Milli-Q water (10x volume of parts) for 2 hours. The solvent should be replaced every hour.</td>
</tr>
<tr>
<td>5. Rinse parts in 80% ethanol.</td>
</tr>
<tr>
<td>6. Dry parts in a biosafety cabinet under UV light for a minimum of 2 hours.</td>
</tr>
<tr>
<td>7. Autoclave parts at 132°C for 4 minutes.</td>
</tr>
</tbody>
</table>

4.4.3 MED610™ Cell Viability with Perfusion Bioreactor System for In Vitro Culture

In the perfusion bioreactor study, the cell viability of the SP+A group was significantly different from the negative control on days 7 and 21 (day 7: p = 0.001; day 21: p < 0.001) (Figure 4.5). The SP+A cell viability was 11.0% and 13.0% higher than the negative control on days 7 and 21, respectively. The SP+A cell viability was not statistically different from the positive control on days 7 and 21.
Figure 4.5: Relative fluorescence units (RFUs) indicating cell viability for the negative control (unconditioned medium), positive control (conditioned medium from autoclaved PC bioreactor), and the sonication and autoclave protocol (SP+A) groups (n = 12) on days (A) 7 and (B) 21 (*p-values < 0.05).
4.5 Discussion

3D printing is a promising fabrication technique for biomedical devices and tools. Polyjet 3D printing has been of specific interest recently due to its ability to 3D print biocompatible materials, such as MED610™. Although MED610™ is listed as biocompatible for up to 24-hours of mucosal membrane contact [110], recent studies have found the biocompatibility of MED610™ to depend on the cleaning and sterilization methods used [25, 26]. Without proper cleaning and sterilization, polyjet-printed materials can leach chemicals from incomplete resin polymerization and that may adversely affect cell development [26]. Therefore, the purpose of this study was to establish a cleaning and sterilization protocol to minimize the effect of MED610™ leachates in a custom-made 3D printed MED610™ bioreactor chamber. To achieve this, several cleaning and sterilization protocols, including the manufacturer’s recommended protocol [110] and a sonication protocol made by Ngan et al. [25], were implemented with MED610™ constructs and static and perfusion bioreactors to assess the effect of these protocols on potential leachates and their effect on cell viability.

In the treatment screening study, the MP and SP+A protocols had comparable cell viabilities to the control groups, suggesting that either method can be used for long-term culture with MED610™. Although the SP and AP cell viabilities were significantly different from the negative control after 7 days, they both had significantly higher fluorescence measurements compared to the background fluorescence. Further testing would be required to confirm if the significant differences between the SP and AP groups with the negative control was attributed to cell death over time or due to the groups having lower initial cell seedings than the negative control. Notably, the MP protocol was not significantly different from the negative control after 7 days. Ngan et al. [25] found that after 3-days the MP had comparable cell viability to the SP and negative control groups but had significantly more cell death after 10-days. If the static construct study in the current study had been extended to 10-days, a similar observation could have been observed, though further testing would be required to confirm this. In the static bioreactor study, the MP group had insignificant cell viability compared to the control and SP+A groups and therefore agreed with Ngan et al.
Additionally, the static bioreactor results indicated that the combination of sonication and autoclaving did not adversely affect cell viability in long-term 2D culture with indirect MED610\textsuperscript{TM} contact. The perfusion bioreactor study further confirmed that the SP+A method may be a suitable method to reduce any negative biological effect from MED610\textsuperscript{TM} for long-term culture.

In both the static and perfusion bioreactor studies, the cell viability of the SP+A group was significantly higher than the negative control. It is uncertain whether this was a coincidence or if MED610\textsuperscript{TM} cleaned with the SP+A method can improve cell viability. In literature, few studies have addressed the influence of MED610\textsuperscript{TM} on cell behaviour, with most studies only highlighting the biocompatibility of MED610\textsuperscript{TM} [25]. Schmelzer et al. [123] were the first to address MED610\textsuperscript{TM} toxicity in cell culture by comparing the roles of ABS, PLA, polycarbonate, and MED610\textsuperscript{TM} on human skin epidermal keratinocytes and bone marrow mesenchymal stromal cells (BM-MSCs). In the same study, the materials were rinsed with 100% ethanol, washed with de-mineralized water, and then sterilized using ethylene oxide gas. Schmelzer et al. [123] found that MED610\textsuperscript{TM} leachates caused a decrease in cell proliferation and an increase in differentiation when in indirect contact with cells. When in direct contact with cells, MED610\textsuperscript{TM} was also observed to increase cell death [123]. Thus, Schmelzer et al. [123] have cautioned against prolonged direct contact with MED610\textsuperscript{TM} for cell culture. However, Ngan et al. [25] found that a sonication cleaning protocol minimized cell death in cell cultures with MED610\textsuperscript{TM} contact. Ngan et al. [25] observed that sonicated MED610\textsuperscript{TM} samples had similar cell viability and differentiation to control groups in 2D and 3D \textit{in vitro} cultures after 10-days and had a negligible foreign-body response when implanted \textit{in vivo} in rats for 2-weeks; therefore, suggesting that MED610\textsuperscript{TM} can be used in cell and tissue culture. Currens et al. [26] further confirmed that MED610\textsuperscript{TM} can be used in cell culture depending on post-print treatment methods. They suggested that a NaOH and Na\textsubscript{2}SiO\textsubscript{3} solution reduced the effect of MED610\textsuperscript{TM} leachates in direct contact studies with bovine artery endothelial cells and Madin-Darby canine kidney cells for over 72 hours. Studies by Chen et al. [117] and Mustahsan et al. [20] found that different MED610\textsuperscript{TM} scaffold
coating and calcitonin receptor treatments improve cell proliferation, osteogenic differentiation, and bone matrix mineralization.

Although the results of this study found MED610™ is a promising material for cell and tissue culture, there were several limitations. In each cell culture study, the initial number of cells seeded in each well was approximate and may not have been consistent across all wells. This could have caused significant differences in viability between the controls and the AP and SP groups in the protocol screening study and the SP+A and negative control groups in the static and perfusion bioreactor studies. Additionally, this study only tested MED610™ biocompatibility in cell culture using indirect conditioned media studies. As a result, the results may not imitate the direct contact cell response that would occur in a 3D printed MED610™ bioreactor during long-term bone organ culture. Furthermore, this study only observed the effect of MED610™ on cell viability and did not address how MED610™ affects cell behaviour. Lastly, a repeat of the perfusion bioreactor study (provided in the Appendix) struggled to replicate results seen in this chapter; however, the cause for replication failure remains unknown and requires further investigation. Despite these limitations, this study demonstrated that cell viability was not affected by contact with constructs printed from MED610™ that were cleaned and sterilized with a combination of sonication and autoclaving.

4.6 Conclusion

This study demonstrated that the combination of sonicating and autoclaving 3D printed MED610™ parts can negate the toxicity of MED610™ leachates on Saos-2 cell lines in 2D culture. While further studies are warranted to repeat the perfusion bioreactor test and assess the role of MED610™ on cell behaviour and cell viability, the findings of this study support continuing developments of 3D printed bioreactors for organ culture.
Chapter 5

Mechanical Loading of *Ex Vivo* Bovine Trabecular Bone in 3D Printed Bioreactor Chambers

This chapter has been written as an independent work in preparation for a journal article submission to the Journal of Biomechanics.

5.1 Abstract

Previous *ex vivo* bone culture methods have successfully implemented polycarbonate (PC) bioreactors to investigate bone adaptation to mechanical load; however, they are difficult to fabricate and have been limited to a 5 mm maximum specimen height. The objective of this study was to validate a custom-made 3D printed MED610™ bioreactor system that addresses the limitations of the PC bioreactor and assess its efficacy in *ex vivo* bone culture. Twenty-three viable trabecular bone cores (10 mm height by 10 mm diameter) from an 18-month-old bovine sternum were cultured in MED610™ bioreactors with culture medium at 37°C and 5% CO₂ for 21-days. Bone cores were ranked based on their day 0 apparent elastic modulus (E_{app}) and evenly separated into a “Load” group (n = 12) and a control group (n = 11). The Load group was loaded five times per week with a sinusoidal strain waveform between -1000 and -5000 με for 120 cycles at 2 Hz. E_{app} was assessed on day 0, 8, and 21 using quasi-static tests with a -4000 με applied strain. Over 21-days, the Load group E_{app} increased 26.4% more than the control group (p = 0.074), and no visual culture contamination was observed. This study demonstrated that bone organ culture in 3D printed MED610™ bioreactors replicated E_{app} trends found in previous studies with PC bioreactors. However, further studies are warranted with a larger sample size to increase statistical power and histology to assess cell viability and bone mineral apposition.
5.2 Introduction

In Canada, over 10% of adults over the age of 40 and 21% of post-menopausal women are estimated to suffer from osteoporosis [2]. Osteoporosis is a skeletal disease that involves the deterioration of bone tissue, leading to increased bone fragility and chance of fracture [1, 2]. Between 2015 and 2016, over 2.2 million osteoporosis diagnoses were made in Canada, with over 130,000 fracture patients, contributing to an annual economic cost of $4.6 billion [3]. Fragility fractures in men and women over the age of 50 are projected to increase by an average of 24% across Canada by the year 2030 [2]. Hip fractures are recorded to be the most fatal osteoporotic fracture type, with 12-month post-fracture mortality rates as high as 22% and 33% for women and men, respectively [2].

Further insights into the biomechanical behaviour of bone could lead to the advancement of bone fracture prevention and treatments, such as physical activity recommendations [6, 8, 107]. Mechanical stimulation is one of several factors understood to promote bone modelling and remodelling [31, 35, 66]. Bone tissue response is dependent on the applied magnitude and rate of strain [64, 67]. Below strains of 100 με bone resorption occurs, while strains between 1000-3000 με are theorized to promote bone adaptation [64, 66]. Above these strains, bone is susceptible to microdamage with fracture likely to occur around 25,000 με. However, these ranges are approximations and exact thresholds are yet to be identified [9].

Bone response to mechanical stimulation can be evaluated using various techniques with in vivo or ex vivo applications [9]. Ex vivo bone organ culture methods allow for the mechanical stimulation of bone in long-term culture studies while providing a controlled environment that replicates accurate physiological conditions [9, 81, 94, 95]. Previous ex vivo bone studies have successfully implemented a polycarbonate (PC) bioreactor system combined with a mechanical loading system to investigate bone adaptation of trabecular bone specimens in response to mechanical load [14, 15, 108]. Compressive bulk strains between 2000 and 4000 με have been shown to cause a higher increase in ex vivo trabecular bone apparent elastic modulus (E_app) compared to non-loaded samples [14, 15, 126-128].
Despite the advantages of the PC bioreactor system, the PC bioreactors have been limited to testing cylindrical bone specimens with maximum dimensions of 5 mm x 10 mm (height (H) x diameter (D)). Additionally, PC bioreactors are difficult and expensive to manufacture. According to mechanical testing standards, to prevent sample buckling and ensure accurate bone mechanical property measurement, an aspect ratio – the ratio of height to diameter – between 1 and 2 should be applied [17]. Previous \textit{ex vivo} trabecular bone core studies limited core height to increase diffusion of culture medium nutrients into the core centre [14, 15, 108]. It is not known whether larger cores can be maintained in long-term culture. With the advancing technology of additive manufacturing, 3D printing can reduce fabrication costs and difficulty [19-21] and allow for flexible design optimization [22], high dimensional precision [23], and biocompatible material options [25]. To address the limitations of the previous PC bioreactors, a custom-made 3D printable bioreactor using the photopolymer MED610\textsuperscript{TM} has been developed. However, the biocompatibility of MED610\textsuperscript{TM} in bone organ culture remains unknown. Although MED610\textsuperscript{TM} is classified as a biocompatible material with mucosal membrane contact for up to 24-hours, recent publications suggest that if adequately cleaned, MED610\textsuperscript{TM} is suitable for long-term culture experiments [25, 26]. Therefore, the objective of this study was to test the 3D printed bioreactor system in a long-term \textit{ex vivo} bovine trabecular bone experiment with 10 mm high bone cores. Efficacy was assessed by quantifying the bone adaptation response to mechanical loading using the change in $E_{\text{app}}$ over 21 days.

5.3 Methods

5.3.1 MED610\textsuperscript{TM} Part 3D Printing and Preparation

Bioreactor chambers were designed in SolidWorks 2019 (SolidWorks Corp., Dassault Systemes, Waltham, MA, USA) and adapted from the Zetos design employed by Vivanco et al. [14] and Meyer et al. [15]. Bioreactor parts were 3D printed with an Objet30 Prime 3D printer and MED610\textsuperscript{TM} and SUP706B\textsuperscript{TM} support material (Stratasys, Edina, MN, USA). After printing, all visible exterior and interior support material was removed with a pick tool and a high-pressure waterjet bath. Remaining support material was
removed in a 1% w/v sodium metasilicate (Na$_2$SiO$_3$) and 2% w/v sodium hydroxide (NaOH) solution, following the manufacturer’s recommendations [110]. Parts were then cleaned with a sonication protocol [25] and autoclaved for 4 minutes at 132°C. Custom-made MED610™ test tube stoppers for culture medium reservoirs were sterilized with the same cleaning process. Detailed mechanical drawings of 3D printed parts are provided in the appendix.

5.3.2 Preparation of Bovine Trabecular Bone Cores

A bovine sternum (Figure 5.1 (A)) was excised from an 18-month-old animal with no known diseases or infections and obtained from a local abattoir. Bone core sample preparation began within two hours after the time of slaughter. Under sterile conditions, the sternum trabecular bone was sectioned at cartilage intersections, called the sternebrae, using an Exakt 312 diamond-coated pathology saw (Exakt Advanced Technologies, Norderstedt, Germany) with continuous water irrigation. Trabecular bone sections were cut into 12 mm thick slices (Figure 5.1 (B)) and then cored into 12 mm x 10 mm (H x D) samples (Figure 5.1 (C)) with a 10 mm diameter custom-made coring bit and a vertical mill (CX605, Busy Bee Tools, Concord, ON, Canada). After coring, up to eight bone cores at a time were clamped and milled with a 4-fluted endmill (No. 8918A53, McMaster-Carr, Elmhurst, IL, USA) to obtain a height of 10 mm and ensure samples had flat and parallel surfaces (Figure 5.1 (D)). While waiting to be cored and milled, samples were placed in sterile dishes with sterile Dulbecco’s Modified Eagle/F-12 Ham medium (DMEM/F-12, MilliporeSigma, Burlington, MA, USA), supplemented with 10% fetal bovine serum (FBS, MilliporeSigma) and 5% antibiotic-antimycotic solution (AA, MilliporeSigma), to prevent samples from drying out and obtaining any infections. Bone specimens were submerged and constantly irrigated with sterile 0.9% sodium chloride (NaCl) solution at 4°C during the coring and milling procedures. NaCl solution was aspirated and replaced often to maintain sample visibility. Coring and milling were performed at slow speeds (~300-400 rpm) to prevent bone exposure to significant heat generation and damage. A total of 40 bone cores were obtained and washed in DMEM/F-12 medium. Cores were placed in 24-well plates with DMEM/F-12 medium and incubated at 37°C and 5% CO$_2$ for 4-days to allow time for recovery. Of the 40
original bone cores prepared, 23 viable bone cores that best met dimensional requirements without visible machining damage were selected for this study.

![Images](A) (A) 18-month-old bovine sternum. (B) Exakt 312 diamond-coated pathology and sternum segment for trabecular bone slicing. (C) Polypropylene coring clamp with cored sternum slice, fixed to vertical mill for bone core fabrication. (D) ABS holder and insert with the stainless-steel mill clamp and eight bone cores during the bone core milling process.

5.3.3 Bone Core Ex Vivo Culture

After the 4-day recovery period, each bone core was placed in a sterile MED610™ bioreactor system between two sapphire pistons and assembled with a sterile medium reservoir and tubing circuit (Figure 5.2). All bone cores were assembled in bioreactors within 48 hours following the 4-day recovery period. Bone cores were maintained in the bioreactors for 21-days at 37°C and 5% CO₂ with constant culture medium perfusion at 6.6 mL/hour [14, 15] using two 24-channel peristaltic pumps (Ismatec™ ISM939D, No. 7800041, Cole-Parmer Canada, Montreal, QB, Canada). The culture medium contained DMEM/F-12
supplemented with 10% FBS, 1% AA, and 10 μg/mL of L-ascorbic acid 2-phosphate (MilliporeSigma). Each bone core’s reservoir contained 6 mL of culture medium and was replaced with fresh medium three times a week. Throughout the study, no visible signs of contamination were observed in culture medium reservoirs or bone core samples.

Figure 5.2: MED610™ bioreactors containing trabecular bone cores with continuous culture medium perfusion.

5.3.4 Mechanical Load Treatment

Twenty-four hours post-bioreactor assembly, bone cores were ranked from lowest to highest $E_{\text{app}}$. Bone cores were divided based on their rank into two groups with equal mean $E_{\text{app}}$, a no-load control group (n = 11) and a load treatment group (n = 12). $E_{\text{app}}$ ranks were acquired using a quasi-static compression test on day 0 with a 10 N preload followed by an applied strain of -4000 με at a loading rate of 50 με/s. Additional quasi-static tests were performed on day 8 and 21 to assess the change in $E_{\text{app}}$ of bone cores in each group throughout the study. A summary table of culture and loading timepoints for this study are provided in the Appendix. Cyclic loading was applied to the load group 5-days a week for 21-days to mechanically stimulate bone cells. Load group bone cores were given two days without load to allow time for recovery. For the loaded group, bone cores were subjected to an initial 10 N preload followed by a
sinusoidal load with a maximum change in applied strain of 4000 με (-1000 to -5000 με) for 120 cycles at 2 Hz. In both the quasi-static and cyclic load tests, bone cores were tested in random order at room temperature with a Mach-1 mechanical testing system (Biomomentum, Laval, QB, Canada) and a lubricated ball-and-socket joint fixture (Figure 5.3).

Figure 5.3: MED610™ bioreactor and fixture assembly with the Mach-1 mechanical testing system. Bone cores were tested and stimulated at room temperature but remained assembled in their closed-loop bioreactor systems with culture medium.

Mechanical testing system compliance was characterized using aluminum reference bodies [113] (discussed in appendix) and accounted for in all mechanical tests. Time, force, and displacement data were recorded with a multiple-axis 250 N (± 0.0065 N) load cell (MA242, Biomomentum) and analyzed in MATLAB 2019b (MathWorks, Natick, MA, USA). $E_{app}$ was calculated by assuming Hooke’s law (Equation (5.1)) and using the last 50% of the linear force-displacement curve (Figure 5.4; $R_{avg}^2 > 0.99$); where $F$ represents force (N), $H$ is the sample bone core height (mm), $\delta$ is the axial displacement due to compression (mm), and $A_c$ is the bone core cross-sectional area (mm$^2$).

$$E_{app} = \frac{FH}{\delta A_c}$$

(5.1)

The percent change of $E_{app}$ (%$\Delta E_{app}$) was calculated between days 0 and 8, days 0 and 21, and days 8 and 21 using Equation (5.2), where the subscripts indicate the days compared.
\[
\% \Delta E_{\text{app}} = \left( \frac{E_{\text{app,day }} - E_{\text{app,day } Y}}{E_{\text{app,day } Y}} \right) \times 100\%
\] (5.2)

Figure 5.4: Sample representative force-displacement plots from quasi-static loading of bovine trabecular bone cores for the (A) control and (B) Load groups on day 0 of the study. The linear fit shown (red) was performed with the last 50% of the force-displacement curve and demonstrates the apparent bone core stiffness used to determine the apparent elastic modulus of bone cores.
5.3.5 Statistical Analysis

All statistical analyses were performed in MATLAB 2019b and Origin 2021b (OriginLab Corporation, Northampton, MA, USA). Results are presented as the mean ± the standard deviation. Mean $E_{app}$ differences between the load and control groups were compared with percentage differences. Group normality was evaluated with Shapiro-Wilk tests and confirmed a lack of normality across all groups. A Wilcoxon rank-sum test was applied to assess $E_{app}$ significance between the load and control group for all 23 bone cores and ensure that the groups were comparable on day 0. All other analyses excluded the six bone cores removed after day 8 (load group: $n = 9$; control group: $n = 8$). The statistical significance between the load and control group $E_{app}$ was assessed with a Wilcoxon test on day 0, 8, and 21. Additional Wilcoxon tests were performed to analyze the difference in $\%\Delta E_{app}$ between the load and control groups for day 0-8, 0-21, and 8-21 and evaluate the effect of mechanical loading on trabecular bone stiffness. The statistical significance of $E_{app}$ and $\%\Delta E_{app}$ within each group were evaluated with Friedman’s non-parametric repeated measures analysis of variance (ANOVA) and Dunn’s multiple comparison post hoc test. A significance level of 5% ($\alpha = 0.05$) was assumed for all statistical analyses.

5.4 Results

The mean and standard deviation $E_{app}$ for all 23 bone cores tested on day 0 was 56.4 ± 16.4 MPa. After day 8, three bone cores were discarded from each group due to continuous bioreactor leakage causing them to dry out and therefore, were not included in the $E_{app}$ results presented. A Wilcoxon rank-sum test confirmed that there was no significant difference in the initial mean $E_{app}$ between the load and control groups on day 0 when comparing all 23 bone cores. Table 5.1 displays the mean $E_{app}$ values with the standard deviations computed for the load and control groups on days 0, 8, and 21.
Table 5.1: Mean apparent elastic modulus ($E_{app}$) values (± standard deviation (SD)) observed for the load and control groups on days 0, 8, and 21.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean $E_{app}$ (SD) [MPa]</th>
<th>Day 0</th>
<th>Day 8</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load (n = 9)</td>
<td></td>
<td>59.4 (± 17.0)</td>
<td>88.5 (± 36.2)</td>
<td>92.8 (± 41.3)</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td></td>
<td>55.4 (± 13.0)</td>
<td>55.4 (± 12.7)</td>
<td>66.6 (± 17.1)</td>
</tr>
<tr>
<td>Percentage Difference</td>
<td></td>
<td>6.88%</td>
<td>46.1%</td>
<td>32.9%</td>
</tr>
</tbody>
</table>

Friedman’s non-parametric repeated measures ANOVA showed that the $E_{app}$ was significantly different across the observation time points for the load ($p = 0.003$) and control groups ($p = 0.008$). For the load group, the $E_{app}$ on day 0 was significantly different from the $E_{app}$ measured on day 21 ($p = 0.003$) but days 0 and 8 ($p = 0.055$) and days 8 and 21 ($p = 1.00$) were not significantly different. The control group $E_{app}$ was not significantly different for the comparisons between days 0 and 8 ($p = 1.00$) and days 0 and 21 ($0.073$), though days 8 and 21 were significantly different ($p = 0.008$). The load and control groups had no significant differences for $E_{app}$ values on day 0 ($p = 0.815$), day 8 ($p = 0.059$), and day 21 ($p = 0.277$). Although $E_{app}$ had no significant differences between the load and control groups on each day, the load group $E_{app}$ values were 6.88%, 46.1%, and 32.9% higher for days 0, 8, and 21, respectively (Figure 5.5). A higher percent change in the load group $E_{app}$ was observed between day 0-8 compared to day 8-21 (Table 5.2). However, the opposite was observed in the control group.
Figure 5.5: Measured apparent elastic modulus evaluated for load treated and control bone core groups on days 0, 8, and 21 (*p < 0.05).

Table 5.2: Mean percent change in apparent elastic modulus (%ΔE_{app}) for the load and control groups between days 0-8, 0-21, and 8-21. SD: standard deviation.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean %ΔE_{app} (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 – 8</td>
</tr>
<tr>
<td>Load (n = 9)</td>
<td>46.5 (± 30.3)</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>0.0329 (± 8.40)</td>
</tr>
</tbody>
</table>

Friedman ANOVA tests showed that within both the load and control groups, the %ΔE_{app} for all day comparisons were significantly different (load: p = 0.002; control: p = 0.010) (Figure 5.6). For the load group, the day 0-21 %ΔE_{app} was statistically different from day 8-21 (p = 0.001) but was similar to day 0-8 (p = 0.472). Though %ΔE_{app} for load group days 0-8 and 8-21 were not significantly different (p = 0.102), the mean %ΔE_{app} for day 0-8 was 46.5% and day 8-21 was 4.04%. For the control group, day 0-8 %ΔE_{app} was significantly different from control days 0-21 (p = 0.037) and 8-21 (p = 0.018).
Figure 5.6: Percent change in apparent elastic modulus (%ΔE_{app}) between days 0 and 8, 0 and 21, and 8 and 21 for the load and control groups (*p < 0.05).

Wilcoxon tests found that the load and control %ΔE_{app} values were significantly different for day 0-8 (p < 0.001) but not for day 8-21 (p = 0.059). Although the %ΔE_{app} for the load group was not significantly different from the control for day 0-21 (p = 0.059), it was more than twice as large (53.4% versus 20.9%).

5.5 Discussion

Mechanical loading is understood to promote trabecular bone adaptation, though due to the complex structure of bone, how it does remains to be well-defined [31, 35, 66]. Further insights into the role of mechanical loading in bone adaptation may improve bone disease prevention and treatment methods. Ex vivo bone culture techniques have been successfully employed using bioreactors with mechanical loading, such as the PC-based Zetos perfusion bioreactor, to provide a physiologically relevant culture environment in which to study the role of mechanical loading during trabecular bone remodelling [14, 15, 126, 127]. Custom-made 3D printed MED610™ bioreactors were designed to address the core height and
fabrication limitations of PC bioreactors. However, Med610™ toxicity in tissue culture is unclear, and the 3D printed MED610™ bioreactor design has yet to be validated for ex vivo bone organ culture. Therefore, the purpose of this study was to test the efficacy of the proposed MED610™ bioreactor design in an ex vivo trabecular bone study, with 10 mm high bone cores, for 21 days by monitoring the change in E$_{app}$. Previous studies found that the E$_{app}$ of trabecular bone cores increased with increased bone formation [15] and that mechanically stimulated bone cores with increased bone formation had improved osteocyte viability [128].

In this study, an initial E$_{app}$ range of 28.6 – 89.5 MPa was observed across all 23 bone cores with a mean of 56.4 ± 16.4 MPa, which is comparable to previous results found by Vivanco et al. [14] for 10 mm diameter by 5 mm high bovine sternum trabecular bone cores (treatment: 50.77 ± 11.85 MPa (± standard error of mean (SEM)), control: 30.09 ± 7.70 MPa (± SEM)). However, the day 0 average E$_{app}$ observed is comparably less (∼83% less) than the day 1 average (136 ± 12.8 MPa (± standard error)) found by Meyer et al. [15]. Though these studies also tested bovine sternum trabecular bone, variance in E$_{app}$ should be expected across different animals due to the anisotropic and heterogeneous nature of trabecular bone as well as the age and health of the animal [12, 41]. Variability across sample trabecular structure could have masked significant differences between the load and control groups E$_{app}$ despite the load group having average E$_{app}$ values 46.1% and 32.9% higher on days 8 and 21, respectively.

Although the load group E$_{app}$ was not significantly different from the control, in comparison to the control group, the load group had a much higher (46.5% versus 0.0329%) change in E$_{app}$ between days 0 and 8 and a lower (4.04% versus 20.7%) change between days 8 and 21. Though the E$_{app}$ changes were small, these results suggest that the loaded bone cores experienced more bone formation within the first eight days of the experiment, while the non-loaded bone cores experienced more bone formation over the last 13 days compared to the load group. Vivanco et al. [14] observed a similar trend in which they found that the E$_{app}$ of bovine trabecular bone loaded daily with a maximum strain of -4000 με increased by 38% more within the first seven days compared to the last 11 days of the study. Additionally, Vivanco et al. [14] found that a non-loaded control group had a 14% higher E$_{app}$ change within the first seven days compared
to the following 11 days, whereas this study found that the control group $\% \Delta E_{\text{app}}$ was 20.7% higher during the last 13 days compared to the first eight days. Trabecular bone has a rapid membranous bone formation healing process in response to injury [129]. Woven bone mineralization and osteoid formation have been shown to form within 1-2 weeks for small animals and humans, respectively [129]. Therefore, it is possible that the load group $E_{\text{app}}$ increase over the first eight days occurred due to osteoid formation in response to injuries sustained during the bone core preparation. Meyer et al. [15] found that prostaglandin E2 (PGE2) concentrations in a non-loaded control group were elevated and similar to bone cores treated with mechanical load (maximum change of -2000 µε) after eight days but decreased after day 12 and were significantly different from the treatment groups by day 15. PGE2 levels are vital to bone formation during injury response, with elevated levels increasing bone mineral content and strength [130]. Thus, one would have expected that the load group $E_{\text{app}}$ would have continued to increase after day 8 in response to the mechanical loading. The current study implemented daily cyclic loading with a max strain of -5000 µε, which may have caused bone fatigue or damage over time and attributed to the minimal $E_{\text{app}}$ increase between days 8 and 21. The elevated PGE2 levels observed by Meyer et al. [15] suggest that an increase in the control group $E_{\text{app}}$ would have been expected before day 8 in response to injuries sustained during core preparation.

Overall, the load group $\% \Delta E_{\text{app}}$ over 21 days was not significantly different from the control group but increased by more than twice (53.4% versus 20.9%) the control. Any significant difference between the load and control group $\% \Delta E_{\text{app}}$ could have been masked by the large variance in the load group $E_{\text{app}}$, by type 2 statistical error. Despite this, the results suggest that an adequate amount of viable bone cells were present to promote bone adaptation during the 21-day culture. However, it is unclear if the MED610™ material of the bioreactor affected bone cell behaviour and the bone adaptation process. Ngan et al. [25] demonstrated in 2D and 3D in vitro cultures that MED610™ did not inhibit cell viability and differentiation after 10-days. As well, Ngan et al. [25] found that MED610™ did not produce a significant foreign-body response when implanted in vivo for 2-weeks. In addition, we performed a 21-day in vitro study with an indirect contact
approach using the Saos-2 cell line and conditioned medium obtained from a MED610™ bioreactor. We observed that if adequately cleaned, MED610™ does not inhibit cell viability during long-term indirect contact, agreeing with results found by Ngan et al. [25]. To further confirm the role of MED610™ in the 3D printed bioreactor design during direct contact with trabecular bone, a repeat study should be performed with histological analyses to measure bone mineral apposition and cell apoptosis rates.

This study had several limitations. The sample size observed in each group was small and had considerable variance, specifically in the load group. Due to the small sample size, it was difficult to confirm the normality of individual data sets. Thus, choosing a non-parametric analysis for all comparisons may have increased the type II statistical error in some group comparisons. Additional possible sources of error include the bone structure variability [12, 41], end and side artefacts [42, 131, 132], and bone cell apoptosis. $E_{\text{app}}$ is dependent on the anisotropic and heterogeneous orientation of the trabecular structure which varies throughout the trabecular tissue [12, 41, 133]. Inconsistent sample trabecular orientation alignment with the loading direction may have increased variability during compression loading. Side and end artefacts that form during bone core extraction from trabecular tissue cause inaccurate estimations of $E_{\text{app}}$ during mechanical loading [132]. 10 mm diameter bone cores with an aspect ratio of 1 were prepared for this study to ensure sample dimensions were adequate for continuum assumptions [42, 131], prevent sample buckling [17], and minimize side artefact effects [131]. Inaccuracies due to end artefacts are assumed to have been minimized since small “toe” regions were consistently observed in sample force-displacement curves (Figure 5.4) [15]. Previous studies have confirmed medium diffusion and minimal cell apoptosis throughout 5 mm x 10 mm (H x D) trabecular bone cores during long-term culture [108, 128]. Although the bone core height was increased to 10 mm for this study to meet bone mechanical testing recommendations [17], it is unclear if the increased height limited medium diffusion to the centre of bone cores. A lack of medium diffusion could have reduced cell viability and bone hydration and caused apparent stiffness overestimates [134]. Lastly, bone core relaxation was observed during the controlled cyclic loads. Trabecular bone has time-dependent (viscoelastic) behaviour [13, 134]. As a result, trabecular bone relaxes and has strain rate-
dependent mechanical properties during mechanical loading, which could affect the accuracy of tissue loading applications.

Despite these limitations, this study demonstrated that an *ex vivo* bovine trabecular bone experiment with a 3D printed MED610™ bioreactor design could replicate \(E_{\text{app}}\) trends over 21-days in mechanically loaded bone cores found by Vivanco et al. [14]. However, future studies with a larger sample size and histological analyses are warranted to reduce the variability of measured mechanical behaviour within groups and assess cell viability and bone mineral apposition throughout organ culture.
Chapter 6
Conclusions and Future Work

6.1 Summary and Conclusions

The main purpose of this thesis was to design an open-source 3D printable bioreactor chamber with a mechanical loading system for the study of trabecular bone adaptation in response to biochemical and mechanical stimulation during *ex vivo* bone organ culture. To complete this objective, the following three subtasks were outlined and presented in Chapters 3-5: (1) Design a 3D printable bioreactor chamber for bone core heights larger than 5 mm to address the sample height limitation of the polycarbonate (PC) bioreactor; (2) Test the role of MED610™ on cell viability and determine a cleaning and sterilization method for 3D printed MED610™ parts; and (3) Assess the efficacy of the 3D printed bioreactor in a long-term *ex vivo* trabecular bone organ culture study with mechanical loading. To assist with these tasks, a compilation of bone biology, bone culture methods (*in vivo*, *in vitro*, and *ex vivo*), and bioreactor design literature were reviewed and presented in Chapter 2.

The first objective, presented in Chapter 3, highlighted the design process of a bioreactor chamber intended for additive manufacturing (AM) with the photopolymer MED610™. The design presented was adapted from a polycarbonate (PC) bioreactor design that has been successfully applied in previous *ex vivo* bone organ culture studies [14, 15]. However, the presented bioreactor was designed to address the difficult and expensive fabrication process and the maximum bone core height (5 mm) limitation of the PC bioreactor. With polyjet 3D printing, the bioreactor was designed to be readily printable with a low material cost (~$16.20/bioreactor) and fast print time (< 3 hours), which is significantly less than the cost (~$1000/bioreactor) and time (> 24 hours) required for fabricating the PC bioreactor. Though MED610™ compatible printers are expensive and may not be easily accessible for everyone, 3D printing services are readily available. The unique advantage of polyjet 3D printing compared to traditional manufacturing methods, such as computer numerical control (CNC), is that the printing time does not increase linearly
with the number of parts. While one bioreactor takes up to 3 hours to 3D print, 12 bioreactors can be 3D printed in under 9 hours. The bioreactor chamber was designed to fit bone core heights up to 11 mm and allow for bone core samples with an aspect ratio (height-diameter ratio) greater than one to follow bone compression testing standards. The bioreactor assembly consists of a MED610™ bioreactor chamber, medium reservoir, tubing circuit, peristaltic pump, and a Mach-1 mechanical loading system to allow for bone organ culture with culture medium circulation and mechanical loading. Validation testing of the MED610™ material and the efficacy of the bioreactor design are presented in Chapters 4 and 5, respectively.

The second objective, presented in Chapter 4, was completed by assessing the viability of Saos-2 cell lines in indirect contact with MED610™ cleaned and sterilized with four different protocols, which consisted of the manufacturer’s protocol (MP), a sonication protocol (SP), an autoclave protocol (AP), and a protocol with sonication and autoclaving (SP+A). The four protocols were tested across three separate Saos-2 cell line culture studies with conditioned medium from MED610™ constructs and static and perfusion bioreactors. Collectively, the three studies presented in this chapter demonstrated that conditioned medium from MED610™ cleaned and sterilized with a combination of sonication and autoclaving did not adversely affect the long-term viability of cells.

The efficacy of the bioreactor design was evaluated in Chapter 5 by testing trabecular bone in long-term ex vivo bone organ culture with the 3D printed bioreactor system. In this study, 23 viable bovine trabecular bone cores were individually cultured in MED610™ bioreactor chambers with culture medium circulation at 37°C and 5% CO₂. Over 21-days, bone cores loaded five times per week with a sinusoidal strain waveform (-1000 and -5000 με, 2 Hz, 120 cycles) had a higher (53.4% versus 20.9%) average increase in apparent elastic modulus (E_{app}) by more than twice the non-loaded bone cores. Additionally, this study demonstrated that 3D printed MED610™ bioreactor chambers could be used in long-term bovine trabecular bone organ culture to replicate E_{app} trends observed in previous studies with PC bioreactors [14, 15].
In summary, the research presented in this thesis demonstrated the design and validation of a 3D printable Med610\textsuperscript{TM} bioreactor design that addresses the limitations of the PC bioreactor and can be used for the study of trabecular bone adaptation to mechanical loading in long-term \textit{ex vivo} bone organ culture. The proposed bioreactor design has been offered as an open-source design to provide fellow engineers and researchers with a cost-effective and 3D printable bioreactor system for bone organ culture. In addition, the design has been made to be easily customizable with computer-aided design (CAD) software to save fellow researchers the effort required to develop a custom-made bioreactor system. The open-source bioreactor design will continue to be updated as required design adjustments are identified through additional validation testing.

6.2 Limitations and Future Work

Although the presented research demonstrated the role of MED610\textsuperscript{TM} on cell viability and the efficacy of the 3D printed bioreactor for bone organ culture, there were several limitations. The MED610\textsuperscript{TM} studies presented in Chapter 4 only tested the biocompatibility of MED610\textsuperscript{TM} with indirect cell culture contact through conditioned medium by measuring long-term cell viability. The results observed may not imitate the cell behaviour when subjected to direct contact with MED610\textsuperscript{TM} material. The initial cell seeding number implemented in each MED610\textsuperscript{TM} cell culture test were approximations and may not have been consistent across all seeded wells. This could have been a factor in the observed significant differences between the SP+A and negative control cell viabilities in the static and perfusion bioreactor studies.

The \textit{ex vivo} bone organ culture study had several limitations due to the methods and bioreactor design implemented. Firstly, the control and treatment bone core groups had small sample sizes and considerable variance, specifically in the treatment group, which could have reduced the power of the statistical comparisons. Secondly, it is unclear if the increased bone core height and bioreactor alcove diameter limited culture medium diffusion into the centre of bone cores. Reduced culture medium diffusion could have caused bone dehydration and, over time led to cell death and bone deterioration. While bone dehydration has been shown to cause the overestimation of $E_{\text{app}}$, deterioration of the bone structure could
have led to the underestimation of $E_{\text{app}}$. Though no visual deterioration of the outer bone structure was observed for any bone cores, additional analyses (histology and microCT) would be required to confirm if cell death and internal deterioration occurred. Thirdly, mechanical load was applied to bone cores at room temperature. Although bone cores were temporarily removed from 37°C for up to 15 minutes at a time, bone cores may have cooled down to room temperature during loading and caused the mechanical loading application to not be representative of \textit{in vivo} bone loading conditions. In regard to the bioreactor system, it is unknown if bone cores were exposed to leachates released from the MED610$^{\text{TM}}$ material over the duration of the experiment. Though the effect of leachates from MED610$^{\text{TM}}$ were assumed to have been mitigated by the sonication and autoclave cleaning protocol, if bone core exposure to MED610$^{\text{TM}}$ leachates were to occur it could inhibit bone cell viability and bone formation. Additionally, leakage occurred throughout the study at the piston-X-ring interfaces of six bioreactors and limited culture medium supply to the bone cores in these bioreactors. As a result, these six bone cores were observed to be dehydrated and, despite our best efforts to stop the leakage, had to be removed from the study and excluded from the control and treatment $E_{\text{app}}$ comparisons. Lastly, treatment group bone cores were observed to have a stress relaxation response when subjected to controlled cyclic strain. Bone core relaxation could have reduced measurement accuracy and the measured force during loading and, therefore caused the underestimation of $E_{\text{app}}$.

Before further bone organ culture studies are performed, the role of MED610$^{\text{TM}}$ on cell behaviour and the piston-X-ring interface leakage must be addressed. Firstly, the cell culture studies presented in Chapter 4 should be replicated to confirm the results of the MED610$^{\text{TM}}$ cell culture studies and increase statistical power. Furthermore, additional \textit{in vitro} cell culture studies are warranted to address the long-term viability and behaviour of cells in direct contact with MED610$^{\text{TM}}$. Secondly, the X-ring slot diameters of the bioreactor body and cap should be increased to provide a tighter seal between the pistons and X-rings. Following the adjustment, the piston-X-ring interface should be tested with fluid perfusion and mechanical loading to ensure leakage is prevented while minimizing translational friction between the piston and
X-ring. Too much friction between the piston and X-ring could increase the measured reaction force, and therefore over-estimate specimen stiffness. Several iterations of the X-ring slot adjustment may be required to optimize the piston-X-ring seal for minimal leakage and translational friction. After the bioreactor limitations have been addressed, future studies are warranted to repeat the ex vivo bone core study with a larger sample size and histological analyses to improve statistical power and assess bone cell viability and bone mineral apposition. Additionally, further analysis is required to assess the diffusion of culture medium into bone cores to ensure that sufficient nutrients are supplied to the bone tissue and that bone cell necrosis is prevented. Furthermore, future tests are required to characterize trabecular bone viscoelasticity in bone organ culture and minimize measurement and stimulation inaccuracies due to bone viscoelasticity.

### 6.3 Clinical Significance

In Canada, osteoporosis is an underdiagnosed and undertreated skeletal disease that increases an individual’s risk of bone fracture. Osteoporosis is more common in individuals over 40-years-old, with higher fracture risks correlating with increasing age. With Canada’s elderly population increasing, osteoporotic fracture cases are predicted to increase. Bone modelling and remodelling have been shown to be promoted by mechanical loads, such as physical activity. Thus, a better understanding of the bone adaptation response to mechanical load could help clinicians improve fracture prevention and treatments by prescribing patient-specific physical activity therapy.

The presented research highlighted the design of a 3D printable bioreactor system capable of maintaining and mechanically stimulating viable trabecular bone cores for long-term ex vivo bone organ culture. The proposed bioreactor has been provided as an open-source design with the potential to help fellow research scientists and engineers continue to study the adaptation mechanism of bone. The long-term goal of the presented bioreactor design and bone organ culture research is to improve our understanding of how bone adapts to mechanical stimuli during physical activity and help clinicians to prescribe patient-specific rehabilitation plans for bone diseases and fractures.
References


Appendix A: House of Quality
Appendix B: Mechanical Dimensioned Drawings

B.1 Bioreactor Chamber & Assembly

Figure B.1: Bioreactor chamber body V.011 mechanical drawing.
Figure B.2: Bioreactor chamber cap V.011 mechanical drawing.
Figure B.3: Bioreactor chamber body plug mechanical drawing.
Figure B.4: Mechanical drawing for 11 mm O-Ring for bioreactor chamber.
Figure B.5: Mechanical drawing for 19 mm O-Ring for bioreactor chamber.
Figure B.6: Mechanical drawing for sapphire piston cylinder.
Figure B.7: Mechanical drawing for X-Ring for bioreactor chamber.
Figure B.8: Bioreactor chamber assembly exploded view and parts list.
Figure B.9: Additional bioreactor chamber assembly views.
B.2 Stopper and O-Ring for Culture Medium Reservoir

Figure B.10: Mechanical drawing for culture medium reservoir stopper V.010.
Figure B.11: Culture medium reservoir 8-mm O-ring
B.3 Bone Coring Clamp

Figure B.12: Mechanical drawing for bone coring clamp base.
Figure B.13: Mechanical drawing for bone coring clamp slider head.
Figure B.14: Mechanical drawing for bone coring clamp lead screw.
Figure B.15: Mechanical drawing for bone coring clamp slider rod.
Figure B.16: Bone coring clamp assembly.
Figure B.17: Bone coring clamp assembly exploded view and parts list.
B.4 Bone Milling Clamp

Figure B.18: Mechanical drawing for bone core milling clamp base.
Figure B.19: Mechanical drawing for bone core milling clamp base insert.
Figure B.20: Mechanical drawing for bone core milling clamp for 10 mm tall bone cores with 10 mm diameters.
Figure B.21: Bone core milling clamp assembly and parts list.
Appendix C: Mach-1 Mechanical Test System Compliance Characterization

C.1 Abstract

The material provided in this appendix outlines the methodology and results used to characterize the Mach-1 mechanical test system (Biomomentum) compliance for the bovine trabecular bone core compression testing presented in Chapter 5. Mechanical compression loading of ten stiff aluminum 7075-T6 reference bodies and a comparison to stiffness results from FEA and previous mechanical testing showed that the system compliance of the Mach-1 system was linear.

C.2 Introduction

In mechanical uniaxial compression tests, the actual deformation of the sample is typically difficult to measure due to high material stiffness and small specimen length [1]. As a result, it is often difficult to apply extensometers to measure the direct sample deformation. This becomes even more difficult when applying mechanical compression loads to bone core samples that are enclosed in a bioreactor chamber. Due to this, users are often limited to measuring sample deformation with the mechanical system crosshead measurements. However, when testing small samples in compression tests, the system compliance can contribute a significant portion to the total measured (loading system and sample) displacement by the test system crosshead [1]. Additionally, compression loading causes the sample cross-sectional area to increase with increased strain, which increases the load applied and non-sample displacement.

The bioreactor system outlined in Chapter 3 includes the use of a Mach-1 mechanical testing system (Biomomentum) to apply mechanical compression load to 10 mm x 10 mm (height x diameter) bone cores maintained within the bioreactor chamber. Due to the bioreactor chamber material not being transparent or large enough to include digital image correlation or an extensometer, respectively, the sample deformation must be measured with the system crosshead. Therefore, the compliance of the Mach-1 test system must be characterized to compute the actual deformation of mechanically compressed bone cores. In a previous study [2], ten aluminum 7075-T6 reference bodies (RBs) with a structural stiffness range between 0.915-29.2 N/μm (58.3 MPa – 1.86 GPa) were used to characterize the system compliance of a mechanical loading system, known as the Zetos system. Thus, the objective of this study was to characterize the system compliance of the Mach-1 system by testing the RBs with uniaxial compression and validate it by comparing the adjusted stiffness range of the RBs with FEA and previous test results.
C.3 Methods

For this study, ten aluminum 7075-T6 RBs labelled 1 to 10 (RB1 – RB10) with a rigidity range between 0.915-29.2 N/μm (58.3 MPa – 1.86 GPa) were tested in random order six times with a Mach-1 mechanical testing system (Biomomentum) and custom-made ball-and-socket joint fixture with lubrication Figure C.1. RBs were tested in uniaxial compression tests with a preload of 10 N held for 30 seconds followed by a maximum load of 200 N with a loading rate of 0.001 mm/sec. Time, displacement, and force were measured with a multiple-axis 250 N (± 0.0065 N) load cell (MA242, Biomomentum) and analyzed in MATLAB 2019b. Sample compliance was calculated with a linear fit of the last 50% of data from the force-displacement plots ($R^2_{avg} = 1.00$). The measured RB stiffness results were compared to previously recorded stiffnesses found with a Zetos loading system and finite element analysis (FEA) [2]. Results were also compared to updated FEA results. To characterize the Mach-1 system compliance, the variation in the RB apparent compliance was plotted as a function of the updated RB FEA compliance results, where the $y$-intercept represented the system compliance [1]. The system displacement for each test was calculated as a function of force and the system compliance and then subtracted from the measured displacement to obtain the sample displacement. Comparisons between the adjusted RB stiffnesses with previous study findings and FEA were made via percent changes.

Figure C.1: Layout of the reference body (RB) and fixture assembly with the Mach-1 mechanical loading system.
C.4 Results and Discussion

Compression testing of the ten RBs yielded linear force-displacement plots (Figure C.2 (A) and (B)). The system compliance was determined to be linear across the range of RB stiffnesses tested with a value of 0.639 μm/N from the RB apparent compliance and RB FEA compliance relationship, shown in Figure C.3. Average RB stiffnesses found from the physical tests with adjustments for system compliance are provided in Table C.1. Despite ranging between 0.91% to 18.52% and 2.93% to 16.14% with the previous study Zetos and FEA stiffness results [2], respectively, the adjusted RB stiffnesses were comparatively closer to the updated FEA stiffness results with a percent change range between -2.43 to 9.06% (Table C.2). The higher end of the percent change range between the adjusted RB and the updated FEA stiffnesses were observed for the two stiffest RBs, which had considerably larger percent changes (9.06% and 5.99%) compared to the rest of the RBs (-2.43% to 1.77%). However, for the research presented in this thesis, the increase in percent change for the two largest RB stiffnesses was not necessarily of concern as 10 mm by 10 mm (height by diameter) trabecular bone cores were expected to have significantly lower stiffnesses based on results from previous literature with 5 mm by 10 mm trabecular bone cores [3,4].

Figure C.2: (A) Measured force-displacement plot of all six tests for a representative reference body (RB). (B) Average force-displacement plots of all ten RBs.
Figure C.3: Average reference body (RB) apparent compliance for all ten RBs plotted as a function of the RB FEA compliance. Linear fit provided with the coefficient of determination to show that the system compliance was linear across all ten RBs tested. The y-intercept of the linear fit, which represents the system compliance factor, was determined to be $0.639 \, \mu m/N$.

Table C.1: Mean reference body (RB) stiffness and standard deviation of the raw and adjusted RB stiffnesses from physical tests and the RB stiffnesses from a previous study [2] and FEA.

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<tbody>
<tr>
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<td>0.593 (0.00164)</td>
<td>0.953 (0.00400)</td>
<td>0.889</td>
<td>0.915</td>
<td>0.943</td>
</tr>
<tr>
<td>2</td>
<td>0.876 (0.00417)</td>
<td>2.00 (0.0220)</td>
<td>1.94</td>
<td>1.94</td>
<td>2.00</td>
</tr>
<tr>
<td>3</td>
<td>1.09 (0.00577)</td>
<td>3.56 (0.0683)</td>
<td>3.38</td>
<td>3.36</td>
<td>3.49</td>
</tr>
<tr>
<td>4</td>
<td>1.21 (0.00707)</td>
<td>5.43 (0.151)</td>
<td>5.13</td>
<td>5.13</td>
<td>5.37</td>
</tr>
<tr>
<td>5</td>
<td>1.33 (0.00734)</td>
<td>8.76 (0.314)</td>
<td>8.36</td>
<td>8.3</td>
<td>8.77</td>
</tr>
<tr>
<td>6</td>
<td>1.39 (0.00868)</td>
<td>12.3 (0.750)</td>
<td>12.2</td>
<td>11.9</td>
<td>12.6</td>
</tr>
<tr>
<td>7</td>
<td>1.43 (0.00497)</td>
<td>17.4 (0.849)</td>
<td>16.6</td>
<td>16.6</td>
<td>17.6</td>
</tr>
<tr>
<td>8</td>
<td>1.46 (0.00881)</td>
<td>22.0 (1.79)</td>
<td>20.4</td>
<td>20.7</td>
<td>22.0</td>
</tr>
<tr>
<td>9</td>
<td>1.48 (0.00772)</td>
<td>28.9 (2.72)</td>
<td>24.4</td>
<td>24.9</td>
<td>26.5</td>
</tr>
<tr>
<td>10</td>
<td>1.49 (0.0105)</td>
<td>32.8 (5.18)</td>
<td>28.9</td>
<td>29.2</td>
<td>30.9</td>
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</tbody>
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Table C.2: Percent change of the mean adjusted reference body (RB) stiffness compared with RB stiffnesses from a previous study (Zetos and old FEA) [2] and from updated FEA.

<table>
<thead>
<tr>
<th>Reference Body</th>
<th>Mach-1 Adj versus Zetos</th>
<th>Mach-1 Adj versus Old FEA</th>
<th>Mach-1 Adj versus Updated FEA</th>
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<td>7.21</td>
<td>4.16</td>
<td>1.09</td>
</tr>
<tr>
<td>2</td>
<td>2.93</td>
<td>2.93</td>
<td>-0.198</td>
</tr>
<tr>
<td>3</td>
<td>5.22</td>
<td>5.85</td>
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</tr>
<tr>
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<td>5.89</td>
<td>5.89</td>
<td>1.09</td>
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<tr>
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<td>4.81</td>
<td>5.57</td>
<td>-0.0903</td>
</tr>
<tr>
<td>6</td>
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<td>3.45</td>
<td>-2.43</td>
</tr>
<tr>
<td>7</td>
<td>4.66</td>
<td>4.66</td>
<td>-1.33</td>
</tr>
<tr>
<td>8</td>
<td>7.69</td>
<td>6.13</td>
<td>0.0639</td>
</tr>
<tr>
<td>9</td>
<td>18.5</td>
<td>16.1</td>
<td>9.06</td>
</tr>
<tr>
<td>10</td>
<td>13.4</td>
<td>12.2</td>
<td>5.99</td>
</tr>
</tbody>
</table>

C.5 Conclusion

In summary, the compliance of the Mach-1 mechanical testing system was determined to be linear for the RB stiffness range (0.953 N/μm to 32.8 N/μm) with a value of 0.639 μm/N. Additionally, the stiffnesses of the RBs adjusted using the system compliance align with the updated RB FEA stiffnesses with a percent change range of -2.43 to 9.06%.

C.6 References


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Figure D.1: Relative fluorescence units (RFUs) indicating cell viability for the negative control (unconditioned medium), positive control (conditioned medium from autoclaved PC bioreactor), and the sonication and autoclave (SP+A) groups (n = 12) on days (A) 7 and (B) 21 for the repeat of the perfusion bioreactor study (*p-values < 0.05).
Appendix E: Summary Table for Chapter 5 Timepoints

Table E.1: Timepoint summary of the Chapter 5 ex vivo bovine trabecular bone core study.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day -6</td>
<td>Received bovine sternum and prepared trabecular bone cores</td>
</tr>
<tr>
<td>Day -5 to Day -2</td>
<td>Recovery period in well-plate with culture medium (medium changed on day -4)</td>
</tr>
<tr>
<td>Day -2 to Day -1</td>
<td>Bioreactor and bone core assembly</td>
</tr>
<tr>
<td>Day 0</td>
<td>First set of quasi-static compression tests and $E_{\text{app}}$ ranking</td>
</tr>
<tr>
<td>Day 8</td>
<td>Second set of quasi-static compression tests</td>
</tr>
<tr>
<td>Day 21</td>
<td>Third set of quasi-static compression tests</td>
</tr>
</tbody>
</table>

*Media was changed every other day, starting from day 0

**Controlled cyclic load applied to treatment group 5-days a week (Monday-Friday) for 21-days
Appendix F: Chapter 5 *Ex Vivo* Trabecular Bone Force-Displacement Plots

![Appendix F: Chapter 5 *Ex Vivo* Trabecular Bone Force-Displacement Plots](image)

**Figure F.1**: *Ex vivo* bone core study force-displacement plots on day 0 for bone core specimens (A) core-113 (load); (B) core-123 (load); (C) core-124 (control); (D) core-125 (load); (E) core-126 (control); and (F) core-128 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination $(R^2)$. 
Figure F.2: *Ex vivo* bone core study force-displacement plots on day 0 for bone core specimens (A) core-129 (load); (B) core-211 (load); (C) core-213 (control); (D) core-222 (control); (E) core-224 (load); and (F) core-311 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Figure F.3: *Ex vivo* bone core study force-displacement plots on day 0 for bone core specimens (A) core-313 (control); (B) core-314 (load); (C) core-315 (load); (D) core-324 (control); and (E) core-411 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Figure F.4: *Ex vivo* bone core study force-displacement plots on day 8 for bone core specimens (A) core-113 (load); (B) core-123 (load); (C) core-124 (control); (D) core-125 (load); (E) core-126 (control); and (F) core-128 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Figure F.5: *Ex vivo* bone core study force-displacement plots on day 8 for bone core specimens (A) core-129 (load); (B) core-211 (load); (C) core-213 (control); (D) core-222 (control); (E) core-224 (load); and (F) core-311 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Figure F.6: Ex vivo bone core study force-displacement plots on day 8 for bone core specimens (A) core-313 (control); (B) core-314 (load); (C) core-315 (load); (D) core-324 (control); and (E) core-411 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Figure F.7: *Ex vivo* bone core study force-displacement plots on day 21 for bone core specimens (A) core-113 (load); (B) core-123 (load); (C) core-124 (control); (D) core-125 (load); (E) core-126 (control); and (F) core-128 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Figure F.8: Ex vivo bone core study force-displacement plots on day 21 for bone core specimens (A) core-129 (load); (B) core-211 (load); (C) core-213 (control); (D) core-222 (control); (E) core-224 (load); and (F) core-311 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination (R²).
Figure F.9: *Ex vivo* bone core study force-displacement plots on day 21 for bone core specimens (A) core-313 (control); (B) core-314 (load); (C) core-315 (load); (D) core-324 (control); and (E) core 411 (control). Linear fit for last 50% of data shown to determine apparent elastic modulus with coefficient of determination ($R^2$).
Appendix G: Human Trabecular Bone Relaxation Modelling

G.1 Introduction

The mechanical behaviour of trabecular bone has been shown to be time-dependent when subject to different strains and strain rates [1]. This time-dependent behaviour, commonly known as viscoelasticity, can play a significant role in bone remodelling and at the bone-implant interface when considering cases such as bone fracture and orthopaedic implant loosening [2, 3]. In 2015-2016, over 2.2 million Canadians were diagnosed with osteoporosis with 130,000 fracture patients which contributed to an annual economic cost of $4.6 billion [4]. Additionally, bone diseases, such as osteoporosis, are undertreated and underdiagnosed in Canada [5]. Previous studies have explored modelling the relaxation response of trabecular bone using empirical models to quantify the viscoelastic response; however, these studies were limited to testing cadaveric bovine bone at room temperature [2, 3]. Addressing these limitations can provide a better understanding of bone remodelling and help clinicians predict patient specific fracture risk. Therefore, the objective of this research is to quantify the relaxation response of human trabecular bone by applying analytical models to experimental relaxation data and then implementing them in finite element analysis (FEA).

G.2 Methods

5 mm x 10 mm cylindrical (height x diameter) trabecular bone samples (n = 86) were sliced, cored, and milled from human femoral heads obtained from seven patients (five female; ages varying between 66 to 87 years) who underwent hip replacement surgery. This study was approved by the Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (MECH-029-05) and patient written consent was provided. During sample preparation, the bulk and dry densities of bone cores were measured, and the CT mineral density was obtained by CT scanning bone cores. After testing, ash (mineral) density was obtained by heating samples at 700°C for 24 hours. Bone cores were individually tested in an ex vivo bioreactor chamber at room temperature.

Prior to relaxation testing, samples were pre-loaded to 10 N for 5 seconds. Samples were subjected to five constant strain loads in the following order, -2200 με, -3000 με, -3800 με, -4600 με, and -5400 με, for one-minute intervals with a 20-minute recovery period between each compression using a piezoelectric custom loading system. Time, force, and bone core displacement were measured using a load cell and strain gauges on the piezoelectric
actuator of the testing system. Bone specimen stress with respect to time was found from the measured force normalized to the cross-sectional area. Due to damage and having a lower stiffness than the testing system, 25 bone cores were removed from the analysis.

To quantify sample viscoelasticity, the following analytical models were fit to the experimental stress-time response (relaxation) of the bone core samples using the corresponding relaxation modulus: 3-Parameter Solid, 4-parameter Solid, Burgers 4-parameter model, and Generalized Kelvin-Voigt, also known as a Prony series model [2]. Model fitting was performed in MATLAB 2019b by minimizing the residual sum of squares between the experimental stress and data and each analytical relaxation model. A power law fit was modelled using the same approach as the viscoelastic models to investigate the relationship between bone density and the instantaneous and equilibrium relaxation moduli. A 3-term generalized Kelvin-Voigt analytical model was combined with ABAQUS 2017 FEA software to simulate the viscoelastic response of the trabecular bone cores using linear-elastic and hyperfoam material models, assuming isotropic and homogeneous properties. A bulk representation of the trabecular structure was implemented to reduce complexity and required computational power and focus on modelling the viscoelastic response. Comparisons were made between models using a coefficient of determination.

### G.3 Results and Discussion

Experimental data showed a nonlinear response, typical of viscoelastic materials (Figure G.1 (A)). The Generalized Kelvin-Voigt model provided the best fit to the human trabecular bone relaxation ($R^2_{avg} = 0.9882$, Figure G.1 (B)) despite underestimating the initial relaxation stress.
Increases in the instantaneous and equilibrium relaxation moduli correlated best with the bone ash density (Figure G.2 (A) and (B)) by a power law (instantaneous modulus: Table G.1; equilibrium modulus: Table G.2) and can also be predicted using the bone CT density (Figure G.2 (C) and (D)). The linear-elastic FE model with viscoelastic properties consistently predicted the relaxation response of trabecular bone ($R^2_{avg} = 0.8461$) with a higher coefficient of determination than the hyperfoam FE model ($R^2_{avg} = 0.6221$, Figure G.3), though more analysis is required to address the nonlinear viscoelasticity of the material.

Figure G.2: Representative power law fits at 5400 με for the ash density correlation with the (A) instantaneous and (B) relaxation equilibrium moduli and the CT density correlation with the (C) instantaneous and (B) equilibrium relaxation moduli.
Table G.1: Power law parameters for the instantaneous relaxation modulus correlation with bulk density, dry density, ash density, CT density, and mean Hounsfield units (HU).

| Strain [με] | Power Law Model Parameters: Instantaneous Relaxation Modulus | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Bulk Density | Dry Density | Ash Density | CT Density | HU |
| | A | B | A | B | A | B | A | B | A | B |
| 2200 | 4.51E-06 | 2.54 | 0.450 | 1.05 | 0.577 | 1.08 | 1.09 | 1.00 | 0.763 | 1.00 |
| 3000 | 2.81E-06 | 2.63 | 0.145 | 1.26 | 0.181 | 1.31 | 1.30 | 1.00 | 0.913 | 1.00 |
| 3800 | 5.20E-06 | 2.55 | 0.183 | 1.24 | 0.220 | 1.29 | 1.40 | 1.00 | 0.982 | 1.00 |
| 4600 | 7.33E-07 | 2.84 | 0.086 | 1.37 | 0.104 | 1.43 | 1.50 | 1.00 | 1.05 | 1.00 |
| 5400 | 1.21E-06 | 2.78 | 0.054 | 1.46 | 0.065 | 1.52 | 1.62 | 1.00 | 0.965 | 1.03 |

Table G.2: Power law parameters for the equilibrium relaxation modulus correlation with bulk density, dry density, ash density, CT density, and mean Hounsfield units (HU).

| Strain [με] | Power Law Model Parameters: Equilibrium Modulus | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Bulk Density | Dry Density | Ash Density | CT Density | HU |
| | A | B | A | B | A | B | A | B | A | B |
| 2200 | 4.02E-06 | 2.48 | 0.342 | 1.00 | 0.499 | 1.00 | 0.613 | 1.00 | 0.430 | 1.00 |
| 3000 | 4.15E-06 | 2.51 | 0.187 | 1.15 | 0.246 | 1.18 | 0.834 | 1.00 | 0.585 | 1.00 |
| 3800 | 2.85E-06 | 2.58 | 0.225 | 1.14 | 0.276 | 1.18 | 0.931 | 1.00 | 0.653 | 1.00 |
| 4600 | 7.93E-07 | 2.77 | 0.116 | 1.26 | 0.140 | 1.31 | 1.00 | 1.00 | 0.701 | 1.00 |
| 5400 | 2.18E-06 | 2.64 | 0.140 | 1.24 | 0.171 | 1.29 | 1.10 | 1.00 | 0.770 | 1.00 |

Figure G.3: Relaxation response of a representative trabecular bone core for all strains modelled by the Generalized Kelvin-Voigt model and the linear-elastic and hyperfoam FE models. Coefficient of determination shown for linear-elastic FEA (R²L) and hyperfoam FEA (R²H) models.
G.4 Conclusions and Future Work

Despite modelling the relaxation response of non-viable samples at room temperature and the bone behaving as a non-linear viscoelastic material, both the Generalized Kelvin-Voigt analytical and the linear-elastic FE models can predict the relaxation response of trabecular bone. Future work for this research should define a constitutive equation that can model the nonlinear viscoelastic behaviour of trabecular bone and then combine the model with FEA. Additionally, we aim to test viable trabecular bone cores using an improved experimental procedure and bioreactor system to validate the non-linear viscoelastic model.

G.5 References


Appendix H: Conference Abstracts

H.1 Orthopaedic Research Society 2020

Compressive Stiffness Measurement of Trabecular Bone Surrogates in a Bioreactor

Brian A. Kunath¹, Baixuan Yang¹, Roshni Rainbow¹, Heidi-Lynn Ploeg¹

¹Department of Mechanical and Materials Engineering, Queen’s University, Kingston, ON, Canada
Email: 14bak3@queensu.ca

Disclosures: Brian Kunath (None), Baixuan Yang (None), Roshni Rainbow (None), Heidi-Lynn Ploeg (None)

INTRODUCTION: The stiffness of a material is quantified as the material’s response to resist deformation due to an applied load and can be used to characterise a material’s behaviour, and the measurement method, when tested under different conditions. Previous studies have been performed to test the stiffness of trabecular bone cores in bioreactors such as the ZETOS bioreactor chamber [1]. With the redesign of the ZETOS bioreactor chamber used by Vivanco et al. [1], a calibration method is required to ensure that accurate mechanical properties are measured in and out of the bioreactor. Therefore, the objective of this study was to investigate different rigid polymers’ stiffness in and out of the bioreactor and determine if the bioreactor design has a significant effect on measured stiffness.

METHODS: For this study, two trabecular bone surrogates were investigated to compare stiffness in and out of the ZETOS bioreactor chamber, Ultem® (polyetherimide) and acetal copolymer (polyoxymethylene). Material samples with dimensions 10 mm×10 mm (height×diameter) were tested using a Bose ElectroForce 5500 (TA Instruments) at room temperature. These materials and dimensions were chosen to mimic trabecular bone cores without buckling [2], while also ensuring the samples fit within the bioreactor. Bioreactors were incubated at 37°C for 48 hours before testing to imitate in vivo temperature conditions. Parallel-surface and ball-and-socket adapters were used to apply compressive loads to samples while in and out of the ZETOS bioreactor. Prior to testing samples, LB 8801 silicone lubricant (Loctite) was applied to minimize friction between the x-rings and pistons. Three samples of each material were pre-conditioned using cyclic loading between 5 – 10 N force applied at 0.5 Hz for 5 cycles. After preconditioning, force and displacement measurements were recorded while samples were compressed to a maximum load of 150 N at a rate of 2 N/s and then unloaded. Tests were performed on each sample five times in and out of the bioreactor. The total stiffness of the system was calculated as the force over deflection. For each sample, a two-tailed
A t-test was performed assuming unequal variance and a significance level of 5% to compare the stiffness in and out of the bioreactor chamber using Microsoft Excel 2016.

**RESULTS:** Force-deflection data were observed to be similar for each material when tested in and out of the bioreactor (Figure H.1: (a)). The stiffness values, shown in Figure 1b, for the acetal copolymer showed no significant difference between being tested in and out of the bioreactor chamber (p > 0.05). The stiffness of the Ultem® polymer measured in the bioreactor was not significantly different than out of the bioreactor (p > 0.05) (Figure H.1: (b)).

**DISCUSSION:** These experiments demonstrated for two polymers, that their stiffness was not affected by the bioreactor. The bioreactor increased the testing system compliance resulting in an underestimation of sample stiffness; however, the friction between the piston and the bioreactor seals caused an overestimation of the sample stiffness. The bioreactor could cause an increase in trabecular bone surrogate stiffness if tests are performed without lubricant and at lab temperature. Limitations of the current study included the fast cooling rate of bioreactors during testing and the linear model used to calculate the stiffness of samples. For future tests, temperature control could be adopted, a model should be used to estimate sample stiffness, and nonlinear viscoelastic models can be used to test accuracy of mechanical behaviour even when samples are tested in a bioreactor.

**SIGNIFICANCE/CLINICAL RELEVANCE:** The results observed are significant as they provide initial steps toward calibrating mechanical loading systems and bioreactors such as the ZETOS bioreactor. Calibration ensures that the material stiffness, when tested in the bioreactor, is a true measure of the material’s properties.

**REFERENCES:**


**ACKNOWLEDGEMENTS:** We acknowledge the support of the Natural Sciences and Engineering Research Council of Canada (NSERC), Ploeg’s Research Initiation Grant, CONNECT! NSERC CREATE Grant, Queen’s University Charles Allan Thompson Undergraduate Student Research Award, and the Human Mobility Research Centre, Queen’s University, Kingston, ON, Canada.
IMAGES AND TABLES:

(a) Figure H.1: (a) Average force-deflection curves (± standard deviation) of the acetal and Ultem® polymers in and out of the bioreactor. (b) Total stiffness comparison between testing in and out of the bioreactor for the acetal and Ultem® polymers.

H.2 Canadian Bone and Joint Conference 2020

**Compressive Modulus of Trabecular Bone Surrogates in a Bioreactor**

Brian Kunath¹, Baixuan Yang¹, Roshni Rainbow¹, Heidi-Lynn Ploeg¹

¹Department of Mechanical and Materials Engineering, Queen’s University, Kingston, ON, Canada

**BACKGROUND:** Bone fractures caused by bone diseases, such as osteoporosis, are undertreated and underdiagnosed in Canada [1]. Bone adaptation has been shown to be affected by mechanical loading and loading rate, however, precise ranges remain unclear [2, 3].

**RATIONALE:** Previous studies have been performed to test the effect of load and loading rate using bioreactors, such as the ZETOS bioreactor [4, 5]. With modifications of the ZETOS bioreactor, a calibration method is required to ensure that consistent material stiffness is observed in and out of the bioreactor.

**PURPOSE:** The objective of this study was to investigate different rigid polymers’ stiffness in and out of the bioreactor and to determine if the bioreactor has a significant effect on sample stiffness.

**METHODOLOGY:** Two trabecular bone surrogates, acetal copolymer and Ultem®, were pre-conditioned and compressed to 150 N at room temperature in and out of the bioreactor to compare the compressive modulus in each condition. System compliance was approximated and corrected for using ASTM standards [6].
RESULTS: Compressive modulus results for both surrogates showed no significant difference between being tested in and out of the bioreactor (p > 0.05). However, the system compliance characterization caused over- and underestimates for the acetal and Ultem® polymers, respectively, due to non-linearity by less than 30%.

SIGNIFICANCE: This study provides calibration steps for a system that aims to provide clinicians with the ability to prescribe patient specific physical activity as treatment for bone diseases and injuries.

REFERENCES:


H.3 Orthopaedic Research Society 2021

Analytical Modelling of Human Trabecular Bone Viscoelastic Response to Compressive Loads

Brian Kunath¹, Sylvana Garcia-Rodriguez², Roshni Rainbow¹, Heidi-Lynn Ploeg¹

¹Dept. of Mechanical and Materials Engineering, Queen’s University, Kingston, ON, Canada, ²Dept. of Radiology,
University of Wisconsin, Madison, USA

Email: 14bak3@queensu.ca
INTRODUCTION: The mechanical behaviour of trabecular bone has been shown to be time-dependent when subject to different strains and strain rates, where strain is the stress-induced deformation relative to the material’s initial dimension. This time-dependent behaviour, commonly known as viscoelasticity, can play a significant role at the bone-implant interface when considering aspects such as orthopaedic implant loosening and implant fits [1, 2]. Several studies have previously explored modelling the relaxation response and creep response of trabecular bone using analytical models and finite element models (FEMs) to quantify the viscoelastic response in terms of stress and strain; however, these studies were limited to testing cadaveric bovine bone at room temperature [1, 2]. Addressing these limitations can provide a better understanding of how bone reacts to implant placements and designs. Additionally, it can improve quantifying strain during mechanical testing, allowing for a better understanding of the viscoelastic response of bone. Therefore, the objective of this study was to fit analytical models to experimental relaxation data of human trabecular bone and quantify the viscoelastic response.

METHODS: 5 mm x 10 mm cylindrical (height x diameter) trabecular bone samples (n = 76) were sliced, cored, and milled from human femoral heads obtained from seven patients (five female; ages varying between 66 to 87 years) who underwent hip replacement surgery. This study was approved by the Queen’s University Research Ethics Board and patient written consent was provided. Bone cores were individually tested in an ex vivo bioreactor chamber at room temperature. Samples were subjected to five compressive loads in the following order, 11 μm, 15 μm, 19 μm, 23 μm, and 27 μm, for one minute using a ZETOS piezoelectric custom loading system with a 20-minute recovery period between compressions. Prior to compressions, samples were pre-loaded to 10 N for 5 seconds. Time, force, and piezoelectric actuator (PZA) expansion were measured using a load cell and strain gauges on the PZA. The PZA expansion was converted to compressive deformation of the bone core. Bone specimen stress with respect to time was found from the measured force normalized to cross-sectional area. To quantify sample viscoelasticity, analytical models were fit to the experimental stress versus time data for 7 bone core samples using the corresponding relaxation modulus: Maxwell, 3-parameter solid, 4-parameter solid, and generalized Kelvin-Voigt, also known as a Prony series model [1]. Model fitting was performed in MATLAB 2019b by minimizing the residual sum of squares between the experimental stress data and each analytical relaxation model. Comparisons were made between the experimental data and models with a one-way ANOVA (α = 0.05).
RESULTS: Experimental data showed a nonlinear response typical of viscoelastic materials. For each sample, there was no significant differences in comparing the experimental results and those obtained from all of the analytical models, although the Maxwell model produced a linear fit to the data (Figure H.2: (a)). Among the models used, a 3-term Prony series of the generalized Kelvin-Voigt model was observed to consistently provide the least difference compared to the experimental data for the different compressive loads (Figure H.2: (b)). Nonetheless, an underestimation of stress was observed at the final stage for over 50% of samples when using this analytical model.

DISCUSSION: The results demonstrated that modelling relaxation data with analytical models can help characterize the viscoelastic behaviour of trabecular bone. There was no statistically significant difference between any of the models with respect to the experimental data, including the Maxwell model, which could be attributed to the noise within the experimental data. The 3-term Prony model resulted in the best fit for the experimental data. Further research would enrich this study to explore the underestimation that was observed at the final stages of relaxation for over 50% of the tests. Samples were tested at room temperature instead of 37°C, which is one limitation of this study since viscoelastic properties are temperature dependent. Other limitations include the testing of cadaveric instead of viable bone, and experimental data noise, which could have affected model accuracy. Future work will investigate viable bone and nonlinear viscoelastic models such as Schapery’s nonlinear constitutive model or the Boltzmann superposition integral. Comparison of analytical models with FEM could further develop these methods and provide more accurate means for quantifying the viscoelastic response of trabecular bone.

SIGNIFICANCE/CLINICAL RELEVANCE: The significance of this study is that it aims to provide a more accurate characterization method of trabecular bone viscoelasticity and to help orthopaedic researchers better understand the impact of implant design and placement on bone mechanical behaviour.

REFERENCES:

ACKNOWLEDGEMENTS: We acknowledge the support of the Natural Sciences and Engineering Research Council of Canada (NSERC), Ploeg’s Research Initiation Grant, and the Human Mobility Research Centre, Queen’s University, Kingston, ON, Canada.
INTRODUCTION: Trabecular bone’s viscoelastic behaviour plays a significant role at the bone-implant interface [1, 2]. Previous studies have modelled relaxation and creep responses of bovine trabecular bone using empirical models to quantify the viscoelastic response in terms of stress and strain [1, 2]; however, human trabecular bone may behave differently than bovine bone. Combining analytical models with finite element analysis (FEA) may provide a better understanding of the viscoelastic response of trabecular bone and the bone-implant interface.

OBJECTIVE: The objective of this study was to combine analytical models with FEA to simulate the relaxation response of human trabecular bone.

METHODS: 5 mm x 10 mm (height x diameter) cylindrical trabecular bone samples (n = 76) were prepared from femoral heads donated by seven hip replacement recipients (five females; ages 66 – 87 years). This study was approved by the Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (MECH-02905) and patient written consent was provided. Samples were individually tested in an *ex-vivo* bioreactor at room temperature and compressed for one minute at five strain loads (-2200 με, -3000 με, -3800 με, -4600 με, and -5400 με) with a 20-minute recovery period between each load. A 3-term generalized Kelvin-Voigt analytical model (R² =
0.99) was combined with ABAQUS 2017 FEA software to simulate the viscoelastic response of trabecular bone cores (n = 15) using linear-elastic and hyperfoam material models. The analytical model was used as a surrogate to analyze the significance of the FEA models.

RESULTS: The linear-elastic FEA (R² = 0.85) showed a stronger average coefficient of determination to the analytical model compared to the hyperfoam FEA (R² = 0.62), despite underestimating the early stress relaxation (Figure H.3:).

CONCLUSIONS: Despite using a linear-viscoelastic analytical model and bulk representations of the bone microstructure, the linear-elastic FEA was able to predict human trabecular bone relaxation.

REFERENCES:

Figure H.3: Relaxation response of a representative trabecular bone core for all loads. Coefficients of determination are shown for the linear-elastic FEA (R²L) and hyperfoam FEA (R²H) compared to the analytical model for each load.

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Open-Source 3D Printable Bioreactors for High Throughput Bone Organ Culture

Brian Kunath⁽¹⁾, Heidi-Lynn Ploeg⁽¹⁾, Roshni Rainbow⁽¹⁾

⁽¹⁾Dept. of Mechanical and Materials Engineering, Queen’s University, Kingston, ON, Canada

INTRODUCTION: A polycarbonate bioreactor (PCB) system has been previously employed for ex vivo bone studies [1]. While allowing for controllability over culture conditions, PCBs are difficult and expensive to fabricate. As an
alternative, we are establishing an open-source 3D printable bioreactor using the photopolymer, MED610. MED610 is listed as biocompatible for permanent skin contact but is limited to 24 hours for mucosal membrane contact [2]. While some studies agree that MED610 is not suitable for culture environments [3, 4], others suggest that its biocompatibility is dependent on the sterilization method used [5, 6]. Here, we report on long-term toxicity testing of MED610 using the human osteosarcoma Saos-2 cell line.

**METHODS:** Using the Stratasys Objet30 Prime 3D printer, MED610 rectangular constructs were printed and sterilized using four different methods: manufacturer’s protocol (MP) [2], sonication protocol (SP) [5], and MP or SP with autoclaving (MP+A, SP+A). Post-sterilization, constructs were placed in sterile dishes containing culture media with serum at 37°C and 5% CO₂. Conditioned medium was applied to cells cultured in 96-well plates (15,000 cells/well, n=6/group) every two days. Cell viability was assessed via CellTiter-Blue on days 2 and 7, and compared to positive (autoclaved PCB) and negative (sterile unconditioned media) controls using a one-way ANOVA and Tukey’s multiple comparison test (α = 0.05). Similarly, a static bioreactor test was performed with two MED610 bioreactors, one sterilized using MP and one using SP+A, with cell viability assessed on day 7.

To assess MED610 in a perfusion system, a printed bioreactor sterilized using the SP+A method and an autoclaved PCB (positive control) were assembled with sterile tubing and a peristaltic pump. Media was circulated (6.6 mL/hr [1]) through the chamber for 21 days, every two days, the system medium was changed, and conditioned medium was applied to cells in a 96-well plate. Cell viability was assessed on days 7 and 21.

**RESULTS:** No significant differences were observed for MP and SP+A compared to the controls for the static constructs. For the static bioreactor study, MP and SP+A viabilities were significantly different from the controls, though SP+A was 9.67% and 15.8% higher than the negative and PCB controls, respectively. In the perfusion study, cell viability using the SP+A conditioned medium was not significantly different compared to the PCB but was 13.0% higher than the negative control (Figure H.4:).

**DISCUSSION:** This study demonstrated that a combination of sonication and autoclaving did not adversely affect the biocompatibility of MED610 and that 3D printed MED610 bioreactors can be successfully sterilized using this method. While further studies are warranted to assess the role of MED610 in improving cell viability, these findings bring us closer to establishing an open-source printed bioreactor for bone culture.

**REFERENCES:**


![Fluorescence data from CellTiter-Blue assay indicating the cell viability of the negative control (sterile culture medium), positive control (autoclaved PCB), and the SP+A sterilization method on Day 21 for the perfusion bioreactor study.](image)

**Figure H.4:** Fluorescence data from CellTiter-Blue assay indicating the cell viability of the negative control (sterile culture medium), positive control (autoclaved PCB), and the SP+A sterilization method on Day 21 for the perfusion bioreactor study.