Improving Large Scale Culture of Anchorage Dependent Cells

A Major Qualifying Project

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Abstract

Currently, chronic diseases are one of the most common and expensive health conditions in the United States, with 133 million individuals suffering from at least one chronic disease. Tissue engineering, particularly mesenchymal stem or stromal cell (MSC) approaches, show great potential in meeting the need for treating chronic conditions. Current MSC therapies involve the use of microcarriers for the proliferation of cells. Microcarriers are difficult to accurately seed cells onto and keep cells in suspension without shearing off, but also difficult to fully harvest cells off of. Currently, there is a need for an improved scaffold to promote proliferation of adherent cells by maximizing surface area to volume ratio within bioreactors. This project aims to solve this gap in technology by constructing a 3-D scaffold of a novel material that would solve the issues in the production of MSC's. A literature review was conducted to discover materials that were nontoxic, allowed cellular adhesion and could be formed into a 3-D scaffold for insertion into a bioreactor. Multiple experiments were performed to quantify cell seeding and proliferation on varying materials in order to select a material for final design. After four experiments a multilayer scaffold of stainless steel mesh was selected for final design. A proof of concept to compare overall efficiency to cell production gold standard could not be performed due to COVID-19 issues.

Acknowledgements

The team would like to give thanks to our MQP advisors Professor Page, PhD and Professor Rao, PhD for their constant guidance and assistance during this project. We would also like to thank Professor Ambady, PhD for teaching us the BrdU assay and cell culturing advice as well as use of his lab for our experiments and Maryam Masroor Shalmani for assistance creating polystyrene samples. In addition, we would also want to thank Lisa Wall (Lab Manager) for her support and assistance with our lab space in Goddard Hall.

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Chapter 1: Introduction

Currently, chronic diseases are one of the most common and expensive health conditions in the United States, with 45% of the population or 133 million individuals suffering from at least one chronic disease [1]. Globally, the prevalence of chronic diseases is growing at an unprecedented rate with an expected increase of 57% by 2020. Other estimations suggest that by 2020 chronic diseases will be responsible for approximately 75% of deaths world-wide [2]. Chronic diseases include such conditions as cancer, diabetes, hypertension, stroke, heart disease, respiratory diseases and arthritis [1]. Due to the rising levels of these diseases and the world's population growing older and living long, with an estimate that by 2050, 17% of the world's population will consist of individuals that are 65 years and older [3], there is also a growing clinical need to provide new and more effective treatments for these conditions.

Tissue engineering, particularly mesenchymal stem or stromal cell (MSC) approaches, show great potential in meeting the need for treating the millions suffering from chronic conditions. Due to their superior clinical potential and properties, such as low immunogenicity, no teratoma risks, as well as immunomodulatory and migratory features, MSCs are already the leading cell type in clinical trials [4,5,6]. However, clinical trials are limited by the quantity and quality of MSC production. Cultivating MSC for clinical applications possess quite a challenge, because high-grade MSCs require suitable growth surfaces that cells can attach to and proliferate on, as well as exposure to an effective, ideally, chemically defined medium (CDM) for expansion [7]. Two-dimensional static culture systems that are commonly utilized for the expansion and cultivation of MSC possess important limitations, such as reduced cell numbers and compromise of cell functions [8].

The overarching goal of this project is to develop a novel system or design to enable increased efficiency of large-scale growth of human mesenchymal stem cells (MSCs) by maximizing surface to volume ratio within a bioreactor. There is a growing need for the production of these cells and a market share estimated to reach 2,518.5 Million USD by 2026 [9]. Need for effective MSC production processes is expanding each year. Processes for various clinical conditions such as treatment of severe graft-versus-host disease to cartilage repair are all assisted through the development of MSC production processes [10].

The overall aims of this project include four major objectives. These objectives are: maximizing surface area to volume ratio of the system to allow for maximum cell adhesion and growth, increasing surface area utilization within the scaffold system, ease the harvesting process and increase harvesting efficiency, while creating a process that would be applicable to the industry. While keeping these objectives in mind, potential scaffold designs were explored and are outlined in the later chapters. These scaffold designs were tested to ensure that they meet at least one of the main four objectives and the corresponding results were quantified. These results provided an understanding of which scaffold designs possess the most industry potential.

1.1 Project Scope

The goal of this project is to step away from the current, yet very limited "gold standard" of both 2-dimensional MSC cultivation and cultivation of MSCs on spherical microcarriers by creating a new scaffold design to allow for cell adherence in media suspension within bioreactors.

Chapter 2: Literature Review

2.1 Clinical Significance

Currently, chronic diseases such as cardiovascular disease, cancer, chronic lung diseases and diabetes, account for 60% of the deaths globally [11]. Moreover, the prevalence of chronic diseases is growing at an unprecedented rate with an expected increase of 57% by 2020. Other estimations suggest that by 2020 chronic diseases will be responsible for approximately 75% of deaths world-wide [2]. Chronic diseases include such conditions as cancer, diabetes, hypertension, stroke, heart disease, respiratory diseases and arthritis [1]. Due to the rising levels of these diseases and the world's population growing older and living longer, with an estimate that by 2050, 17% of the world's population will consist of individuals that are 65 years and older [3], there is also a growing clinical need to provide new and more effective treatments for these conditions.

Treatments for these chronic diseases, although various, are limited. Some of the treatments require less intervention, such as physical and psychological therapies, while others are more strenuous for the patient such as surgery and radiotherapy. Many of the chronic conditions are managed via medication, however such treatments have been shown to be very burdensome both for patients and the medical providers [12]. Chronic diseases are also widely accepted to not have a cure and cannot be prevented by vaccination, nor do they disappear [13]. For these reasons there is both a clinical need for novel treatments and demand for possible cures.

Tissue engineering, particularly mesenchymal stem or stromal cell (MSC) approaches, show great potential in meeting this need for treating the millions suffering from chronic conditions. Due to their superior clinical potential and properties, such as low immunogenicity, no teratoma risks, as well as immunomodulatory and migratory features, MSCs are already the leading cell type in clinical trials [4,5,6]. However, clinical trials are limited by the quantity and quality of MSC production. Cultivating MSC for clinical applications possess quite a challenge, because high-grade MSCs require suitable growth surfaces that cells can attach to and proliferate on, as well as exposure to an effective, ideally, chemically defined medium (CDM) for expansion [7]. Two-dimensional static culture systems that are commonly utilized for the expansion and cultivation of MSC possess important limitations, such as reduced cell numbers and compromise cell functions [8].

The growing global need for the production of human MSCs has been estimated and predicted to comprise a market share of $2,518.5 Million USD by 2026 [9].

Currently, chronic diseases are one of the most common and expensive health conditions in the United States, with 45% of the population or 133 million American individuals suffering from at least one chronic disease [1]. According to a report by the National Health Council, the United States' economic burden due to chronic disease is estimated to be $1.3 trillion annually [13].

To quantify the need for MSC therapies present in the US, a special analysis was done on one of the most prevalent chronic conditions which is osteoarthritis. Osteoarthritis was selected for treatment quantification because therapies and treatments of this condition based on application of MSCs have been proven to be both safe and successful in phase I clinical trials [6].

In one clinical trial it was found that the injection of MSCs with a count between 20 and 24 million cells during one round of treatment produced positive results. Not only was the treatment able to reduce pain in the joints affected with osteoarthritis, but the effects of the

treatment lasted for up to 6 months. The treatment was also able to increase the thickness of cartilage at the affected sites, reversing some of the effects of osteoarthritis [6].

In another clinical trial it was shown that a single injection of 2 million MSCs derived from adult adipose, or fat, tissue was able to reduce pain significantly by clinical standards. Furthermore, the one injection of MSCs increased the overall function of the arthritic joints that were treated using the MSCs [14].

Using the data from the clinical studies mentioned above, in combination with estimates of 14 million people in the US suffering from osteoarthritis of the knee joint, we are able to calculate an estimation for the clinical demand of MSCs for patients with osteoarthritic knees [15]. By multiplying the number of cells used successfully per treatment by the number of patients that require this treatment we can calculate that the demand of MSCs for the treatment of this condition ranges between 28 and 308 million MSCs.

Based on the particular analysis done above and the projected market growth described in the previous section, it is evident that there is a great need for the production of human MSCs in extremely high quantities annually. This need in turn creates demand in the biomedical industry for new designs and systems for MSC expansion that can make these production numbers possible.

2.2 Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs) are a specialized class of cells of self-renewing cells that are able to differentiate into a multitude of other cell lineages [4]. Unlike embryonic stem cells, MSCs are adult stem cells and can be found in various areas of the body in adults. MSCs have the potential to differentiate into cells of the mesodermal lineage including osteocytes, adipocytes and chondrocytes. [16]. Beyond their ability for differentiation MSCs also possess the advantage of being immunomodulatory, or in other words they are able to produce and release such anti-inflammatory compounds as cytokines and immune-receptors. These immunomodulatory features have made MSCs a great potential means for treating chronic diseases. MSCs have also been shown to induce and assist in tissue regeneration, specifically they have been shown to be effective in regenerating bone and cartilage tissues [17]. MSCs are very resilient and can be easily harvested from the human body. Their culturing and expansion *ex vivo* is also a fairly straightforward process increasing the interest of the clinical community. MSCs are fairly abundant in the human body and they have been isolated and harvested from adipose tissue, bone marrow and dental tissues, as well as from new-born umbilical cord blood [18].

MSCs are anchorage dependent stem cells, which means they cannot be cultured as single cell suspension in media but rather require a surface to attach and adhere to in order to proliferate [19].

2.2.1 Current Clinical Trials

Several clinical trials have sought to better understand mesenchymal stems cells and their behavior in order to maximize their potential therapeutic opportunities. A 2016 study conducted by Salzig investigated human MSCs derived from both bone and adipose tissue sources in regard to their attachment, growth and spreading behavior on particular surfaces. The researchers investigated the effects of collagen type IV and fibronectin on the behavior of the stem cells, as well as different media compositions including serum-containing media and chemically defined media. The study demonstrated the success of fibronectin as a coating only for the growth of

MSCs derived from adipose tissue, while demonstrating the effectiveness of FGF-2 and collagen IV for the growth of bone derived MSCs. The study also showed preliminary results that the protein/peptide-free CDM was sufficiently successful in expanding different primary hMSCs supporting a theory that fewer supplements are necessary in the medium and that the cells can survive in basic medium [7].

A 2016 study conducted by Tsai and colleagues sought to study hMSC expansion in a packed bed style bioreactor in contrast to the traditional microcarrier systems used to expand and grow mesenchymal stem cell populations while limiting cell differentiation. To do so, researchers seeded and grew cells in a bioreactor filled with Fibra-cel disks composed of polypropylene and non-woven polyester fibers. After nine days of culture, the cells had a 2.8 day population doubling time with homogeneous cell distribution in the fibrous bed. Notably as well, cells did not differentiate and maintained their properties as the packed bed system provided more ideal conditions than microcarriers. Cells experienced very low shear stress compared to microcarrier systems that have to continually be stirred to remain suspended and distribute oxygen and nutrients throughout the bioreactor system [20].

A 2018 exploratory study conducted by Athersys, Inc was done to understand the effects of their special and proprietary MultiStem treatment the goal of which was to treat individuals with acute onset of moderate to severe Acute Respiratory Distress Syndrome (ARDS). The study, conducted both in the United States of America and in the United Kingdom, included two parts. The first part was a small initial dose confirmation phase. The second part was a bigger double-blinded, placebo-controlled and randomized phase. Individuals began treatment within four days of their initial ARDS diagnosis although the average time between the diagnosis and the start of treatment was only two days. The study had a relatively small subject pool with only six subjects treated with MultiStem in the beginning stages and only 20 subjects were treated with an intravenous injection of 900 million MultiStem cells in the latter stages. The placebo group consisted of 10 subjects and also received an intravenous injection, however without any MultiStem cells. After their initial treatment, subjects were observed and monitored for a period of 28 days. Overall the results of the study showed that the individuals that received the MultiStem treatment had lower mortality (25%) in comparison to the mortality of the placebo group (40%). Patients treated with the MultiStem treatment also experienced fewer days on ventilators (40.2% lower) and in the intensive care unit (27.2% lower) [21].

2.3 Bioreactors

Bioreactors are an apparatus in which a biological reaction or process is carried out, especially on an industrial scale. Table 1 below lists several different types of bioreactors that are used for mass cell production as well as protein production. Each of the bioreactor type has advantages that make it useful for the production of specific cells and proteins. For example, there are bioreactors with minimal shear force in order not to stress the cells or induce differentiation. Also, some cell types need to adhere to a surface in order to proliferate. In order to better understand the different bioreactors that are currently on the market the table lists bioreactors types used, a brief description of how it works, the material that is used for the scaffold or walls, and finally the cell type that is typically used within that bioreactor.

Table 1: Current Bioreactors and Corresponding Material and Cell Type Supported

Bioreactor	Description	Material	Cell Type
Micro-carriers and scaffolds	Porous micro- carriers were developed for mammalian cell recombinant protein production in stirred vessels	Denatured type I collagen, negatively charged plastic particles, gellarin, cellulose, etc.	Large variety, anchorage dependent
Spinner flasks	Spinner flasks are glass or plastic vessels with a central magnetic stirrer shaft and side arms for the addition and removal of cells and medium, and gassing with CO_2- enriched air	Variety of scaffolds	MSCs with osteogenic differentiation
Rotating- wall bioreactors	A horizontally rotating cylinder which is completely filled with culture medium (no gas–liquid interface) rotates liquid inside at the same angular rate as the wall	Biopolymers	Osteogenic
Wave bioreactors	The wave bioreactor system provides a gentle wave motion for mixing, provides higher oxygen transfer than in spinner flasks, and has been shown to perform comparably with stirred- tank bioreactors for working volumes between 1 and 100 L	Polyethylene bags	Large variety
Column bioreactors	Hydrodynamic principles that govern the flow pattern inside columns are applied to achieve uniform (plug) flow through the column	Variety of scaffolds	Cartilage
Parallel plates	The bioreactor consists of two primary compartments: a gas compartment that is separated from the bottom compartment by a gas- permeable, liquid-impermeable membrane, and the liquid-filled bottom compartment with a tissue culture plastic surface for support of anchorage- dependent cells	Tissue culture plastic surface	Osteogenic and anchorage-dependent cells
Hollow- fibre	A hollow- fibre bioreactor is a two-compartment system consisting of intracapillary and extracapillary spaces.	Distribution of collagen, proteoglycan and glycosaminoglycan	mammalian cells

bioreactor	Intracapillary flow is distributed by headers to a hollow- fibre bundle that is potted in resin	content	
Microfluidic bioreactors	Microfluidic devices are fabricated using soft lithographic techniques originally developed by Whitesides and colleagues.122-125 Poly(dimethylsiloxane) (PDMS), which is biocompatible,126 optically transparent, permeable to respiratory gases and elastomeric, is cast onto silicon wafers that have been patterned and profiled by photolithography	Distribution of collagen, proteoglycan and glycosaminoglycan content silicon mold	Large variety

2.3.1 Current "Gold Standard" in MSC Expansion Systems

The current "gold standard" in 2-dimensional MSC expansion is the Xpansion® Multiplate Bioreactor System that is offered by the Pall Corporation. This system utilizes a single-use 2D design with multiple plates stacked horizontally on top of each other that is used to allow for larger-scale production of adherent cells using traditional 2D cell culture techniques, with a maximum surface area of 122,400 cm^2 [22]. The current solution to the limitations possessed by MSC expansion in 2-dimensional systems are microcarriers.

2.3.2 Impeller Types and Flow

For stirred tank reactors, the type of impeller used is directly related to the type of flow and shear stress the cell type can withstand for proper proliferation. Orientation of the impeller and agitation direction determines if the flow is either axial or radial. Axial flow is when the fluid is pushed up or down the shaft of the impeller while radial flow is when the fluid is pushed outwards from the shaft towards the bioreactor walls [23].

Rushton

The Rushton impeller is flat bladed with the blades vertical along the shaft. Position of the blades results in radial flow. The flat blades result in high shear forces on the cells and are best for non-shear sensitive cells such as yeast or bacteria [23].

Pitched Blade

A combination of radial and axial flow is achieved through the 45-degree angle of flat blades that are characteristic for pitched blade impellers. The blade angle allows for better mixing and higher oxygen mass transfer between the cells. The blades have lower shear force and are better for low shear cell lines or cells grown on microcarriers. The 45-degree angle and low shear force also makes viscous cultures achieve proper nutrient dispersion [23].

Gentle Marine Blade

Similar to pitched blades, gentle marine blades also produce a combination of axial and radial flow. Gentle marine blades produce a much slower flow for lower shear cells and gently mix the medium [23].

Spin Filters

Spin filters use marine blade impellers, but the impeller is enclosed in a screened cage. The cage design has small pores that prevent cells from entering the cage, keeping the cells closer to the bioreactor walls. A dip tube in the center of the reactor continuously withdraws media and recirculates it into the system. A media feed tube outside the reactor also provides a steady supply of fresh nutrients to the media as needed. While the mesh screen is a great way to keep cells separated from the media for media free harvest, the mesh can get clogged with cells and require replacement, making the system not ideal for repeated runs [23].

2.3.3 Gold Standard Bioreactors

There has been a growing interest in the use of disposable and single use technologies within the bioreactor systems. Studies utilized a single-use 3 L stirred tank bioreactor with collagen-coated microcarriers for expansion of MSC derived from bone marrow. The MSCs were left to propagate in the single-use 3 L bioreactor for 5 days. There was a 5.2 fold increase in total cell number from 30 to 150 million cells. Additionally, BM-MSC were propagated in a disposable stirred tank bioreactor achieving viable cell densities of 2.5-2.7x10^5 cells/mL during a 12 to14 day culture period [24].

Across the industry as a whole, there are several types of bioreactors that each serve their own specific need: rocking bed, stirred tank, rotating wall vessels, perfusion bioreactors, and insulation/expansion automated systems. Rocking bed bioreactors utilize single use bags that have limited scale up potential while some systems have a maximum volume of 500 L. Stirred tank bioreactors are the most versatile with a maximum volume of up to 1000 L, however the stirring systems are known to impact cell differentiation and viability. Rotating wall vessels, perfusion bioreactors, and insulation/expansion automated systems all fall into a class of ‘small scale’ bioreactors with volumes from 100 mL to as high as 10 L in the case of the rotating wall vessels. Rotating wall vessels and perfusion bioreactors each have low turbulence while perfusion bioreactors and insulation/expansion automated systems both utilize automation in their operation [25].

Current stir tank bioreactors are often single use due to the difficulty in reaching sterilization standards after a batch run. Common sellers of bioreactors have bioreactors with temperature control, robust industrial automation, scale-up ability and gas flow variability. Most bioreactors have the ability to have the control panel, gas flow, and pumps to be manipulated in order to adhere to a specific cell type's needs for growth. Bioreactors come in a range of sizes from benchtop bioreactors at 4.5L - 10L to scale- up bioreactors ranging from 50L - 2000L [26].

2.4 Microcarriers

Microcarriers most often come in the form of spheres or beads, manufactured from various materials including gelatin, cellulose, plastic and glass. They are most often ball-like scaffold structures that provide a necessary attachment surface for anchorage-dependent cells

suspended in culture, such as the culture used in bioreactors [27]. There are several types of microcarriers that have been developed over the past few years and although they are quite popular today and have been found to significantly increase the surface area available within a bioreactor they come with serious limitations and leave a lot of room for improvement.

2.4.1 Solid Spherical Microcarriers

One of the most popular microcarriers on the market is the Cytodex™ surface microcarriers. There are two types of Cytodex™ microcarriers, Cytodex™ 1 and Cytodex™ 3. Cytodex™ 1 microcarriers are made of a cross-linked dextran matrix combined with positively charged N, N-diethylaminoethyl groups. Cytodex™ 3 microcarriers are made of a matrix of cross-linked dextran and are covered in a thin layer of denatured collagen which is susceptible to the effects of trypsin. Cytodex™ microcarriers are advertised to have optimal size and density for cell growth and "high yields" of cultured cells. Dextran microcarriers are also advertised to possess advantages such as being biologically inert, strong but not rigid and transparent making microscopic examinations simple. A study comparing cell expansion on Cytodex™ microcarriers found that significantly high recovery rates were only achieved for cells grown on the collagen coated microcarriers. Cytodex™ 1 prices range from $391 for 25 gr to $3291 for 500 gr of dry powder microcarriers. Prices for the collagen coated Cytodex™ 3 microcarriers are higher with 500 gr of dry powder microcarriers costing $3554 [28].

Another type of microcarriers available on the market are Corning microcarriers. These microcarriers are made of Class VI polystyrene and are available with a wide variety of surface treatments. Advantages of using these polystyrene microcarriers include a shorter preparation process, without the need for swelling and washing [29]. A study using Corning's Synthemax II microcarriers for the expansion of MSCs found that the cultured cells had a cell attachment rate of between 80% and 90%, after a period of 24 hours from the initial seeding and a cell expansion of between 5-and 7-fold [30]. The price for 500 g of Synthemax II microcarriers is $6704 [31].

2.4.2 Porous Microcarriers

Porous microcarriers are designed to allow adherent cells to adhere within the structures by entering the pores along the structure shown in figure 1. The advantages of porous microcarriers include their ability to provide additional surface-area for cells to adhere to, while also providing a certain degree of protection from the hydrodynamic shear stress often created in stirred bioreactors [32]. Limitations of porous microcarriers include difficulty in oxygen and nutrients distribution within the microcarrier, as well as damaging waste accumulation within the pores [33,34].

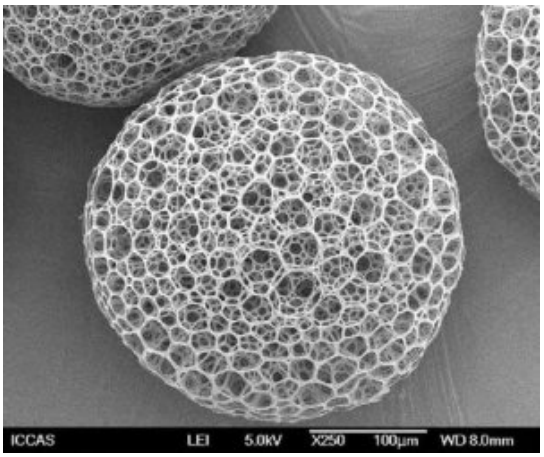

Figure 1: Porous Microcarrier

2.4.3 3D-shaped Hydrogel Cell Microcarriers

The advantages of this novel microcarrier design include localized cell culture, shear-stress shelter, flow alignment and ease of fine resolution high-speed imaging. However, these microcarriers are not very common and have yet to find application in the field of cell production [35].

2.4.4 Liquid Microcarriers

The main advantage of liquid microcarriers is their ability to create a special interface for anchorage-dependent animal cells. To overcome the limitations involved in culturing such cells on solid surfaces and the following collection of the cells, the liquid microcarrier system emerged. The liquid microcarriers provide the anchorage-dependent animal cells with a surface at the interface between the culture medium and a hydrophobic liquid to which they can adhere and then spread and proliferate on. Liquid microcarriers such as these possess additional advantages such as their simplicity and complete flexibility, thus allowing for the application within vessels of various shapes [33].

2.4.5 Common Microcarrier Materials

The majority of microcarriers are manufactured using both natural and synthetic polymers. Synthetic polymers used in microcarrier production include materials, such as polyhydroxyethylmethacrylate, polystyrene, polyacrylamide, polyurethane foam, polyethylene, polycarbonate, Perspex™, PVC, Teflon™, cellophane, and cellulose acetate. Natural polymers used in microcarrier production include gelatin, collagen, cellulose, glucose, chitin and chitosan, alginic acid and ECM [33].

2.5 2-D Static Culture Systems

The currently and commonly utilized 2-dimensional static culture systems used in labs across the United States come with important limitations, such as reduced cell numbers and compromised of cell functions. Specifically, the current scale of these 2-D systems in lab settings is limited with surface areas ranging from 12.5 cm^2 to 225 cm^2, and although they are simple to operate and cost effective, a very large number of these systems would be necessary to produce enough MSCs for clinical trials. The use of multiple systems would be more time consuming and require more labor, while working with a very low surface to volume ratio. The chances of

contamination would also be increased, resulting in higher risk of cell deformation and death [8]. Other more modern expansion systems, like microcarriers and the vertical wheel design, that will be described in more detail below also come with serious limitations and difficulties, that result in their inability to meet the demand currently present on the US biomedical market.

2.5.1 Current Scaffolds

The current "gold standard" in 2-dimensional MSC expansion is the Xpansion® Multiplate Bioreactor System that is offered by the Pall Corporation. This system utilizes a single-use 2D design with multiple hydrophilized polystyrene plates stacked vertically on top of each other that is used to allow for larger-scale production of adherent cells using traditional 2D cell culture techniques, with a maximum surface area of 122,400 cm^2. The stacked plates are placed within a stirred tank bioreactor. The compact design of the plates allows for a unique gas exchange system. Tubing runs down the center column of the plates and gas is exchanged along the plate channels. Each plate has 16 radial channels grooved into each plate which allows for media exchange along each plate. The impeller in the stirred tank bioreactor pushed the media upwards in axial flow and the media runs along the channels in the plate radially. The media moves up and over each plate running along the radial channels. Figure 2 shows the media flow route across multiple plates and channels. The channel design allows for equivalent media, nutrient and gas dispersion across all the plates and all the cells resulting in a more optimal yield [22].

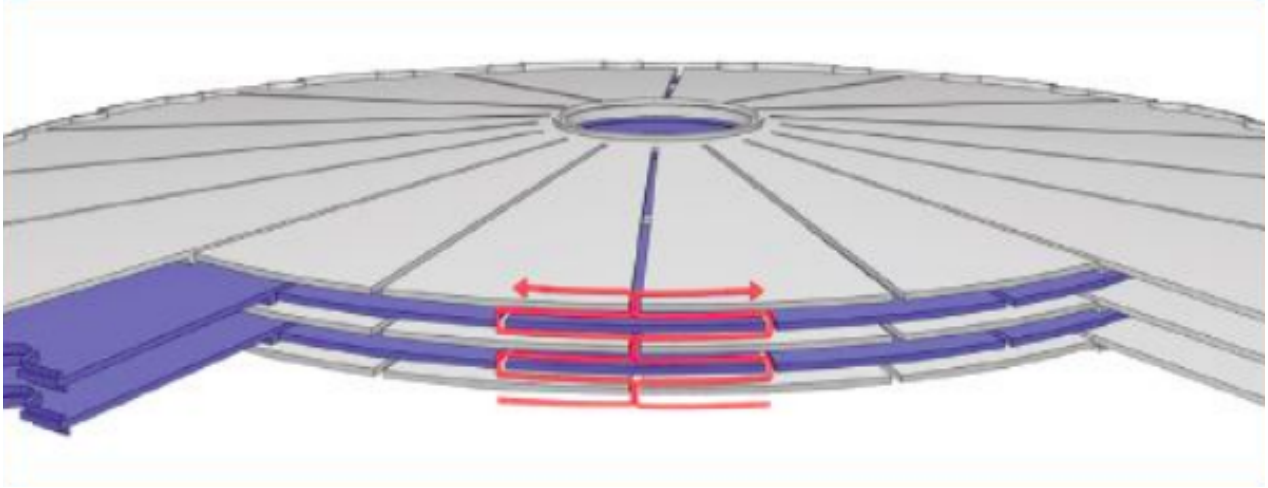

Figure 2: Pall Xpansion® Multiplate Bioreactor System

2.5.2 Wheel Bioreactors

The wheel bioreactor is a new form of technology created by PBS Biotech. The wheel bioreactor is a vertically-oriented wheel which differs from traditional horizontal impellers. It is enclosed inside of the bioreactor vessel and rotates around a stationary shaft. It has unique features that separate it from today's standard bioreactor systems. These features include a long wheel radius, peripheral paddles, and the rounded vessel bottom which contributes to the gentle, tangential fluid flow (Figure 3). It is composed of oppositely oriented internal vanes. These vanes impact the "cutting and folding" axial fluid flow pattern throughout the center of the wheel. It is a quick and efficient form of mixing with uniform particle suspension and dispersion. It generates low shear stresses in 50 mL to 500 L volumes. Additionally, it is scalable, using the same mixing parameters in the 50 mL and being able to apply them to a 500 L volume to achieve

a similar micro-environment.

Their innovation Vertical-Wheel™ Technology is driven by two different forms of power, MagDrive Technology and AirDrive Technology. In MagDrive, the Vertical-Wheel™ motion is powered by coupling between magnets on the wheel and the housing unit. This system has a number of advantages including; eliminating the need for anti-foaming agents or shear protectants, allowing for lower working volumes for in situ harvesting, optional secondary heating system for static seeding without temperature drop, and it is optimal for culturing shear sensitive cell aggregates or anchorage dependent cells on microcarriers.

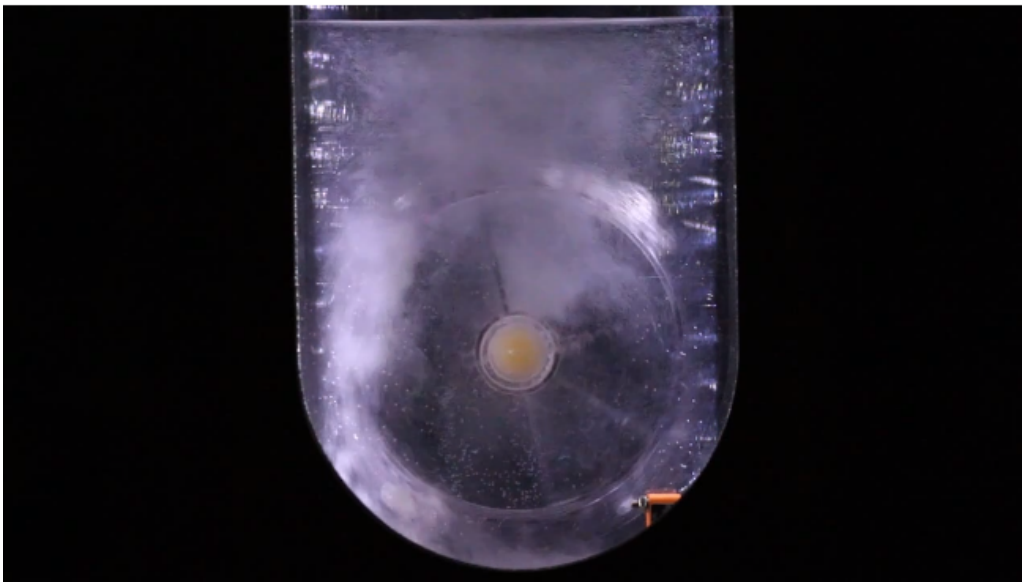

Figure 3: Wheel Bioreactor

AirDrive Technology operates a little differently. The Vertical-Wheel™ motion is propelled by the buoyant force of gas bubbles (Figure 4). These gas bubbles are introduced from the sparger at the bottom of the disposable bag and are captured in the air cups on the impeller wheel periphery. The advantages of this driven system include; independent control of pH, dissolved oxygen (DO), and agitation through main gas sparger. There is inclusion of a microsparger which has customizable pore sizes in order to achieve high mass transfer rates, development of efficient CO_2 stripping, and optimal high cell density suspension culture processes [36].

Figure 4: Wheel Bioreactor Propelled by Gas Bubbles

2.5.3 Packed Bed Bioreactor

Another new and advancing form of bioreactors are packed bed bioreactors. These types of bioreactors are completely filled with a material, such as polystyrene pellets, and are pushed together so that media can still flow through the system delivering the needed nutrients to the cells, and are close enough that cells can proliferate and expand across pellets. They are expanding in the world of medicine as a promising tool for tissue engineering applications. Some of the advantages of packed bed bioreactor over different types of bioreactors is the reuse of enzymes, continuous mode of operations, low substrate and product inhibition, high yield of desired product. Another advantage of packed bed bioreactors is their capability to support various cell lines for long culture periods, all while keeping shear conditions to a minimum. Low shear conditions are present in these packed bed bioreactors due to the immobilization of cells within macroporous matrices [37].

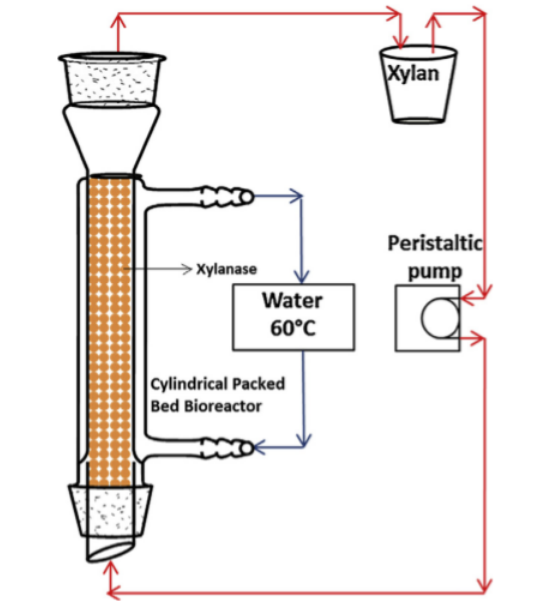

Figure 5: Packed Bed Bioreactor Schematic

Fibra - Cel Disks

A microcarrier-like structure used in packed bed bioreactors are Fibra-Cel® Disks. For anchorage dependent cells, these disk structures act as a structure for adherence as well as increase the surface area within the reactor. The disks are made of non-woven polyester fiber and polypropylene mesh shown in figure 6. The mesh is electrostatically pre-treated to improve cell adherence onto the material. The matrix pattern on each disk not only provides a structure for the cells to adhere too, but also protects cells from damaging shear forces from the impeller fluid flow or gas bubbles [38]. Fibra-Cel® disks have a higher transfer of nutrients and oxygen versus standard microcarriers due to the matrix [39].

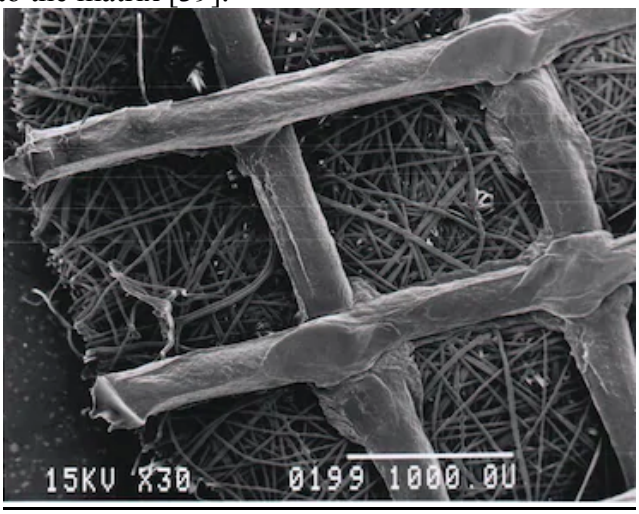

Figure 6: Fibra-Cel Texture

Nutrient Distribution and Waste Product Removal

The main difference between the nutrient distribution and waste removal of perfusion bioreactors and stirred bioreactors is that perfusion has cells inside the bioreactor and continually removes cell waste products and media depleted of nutrients by cell metabolism, and adds fresh media to the cells at the same rate as the spent media is removed. Through this method new fresh media is constantly being supplied so the cells do not get caught in dead spots ensuring they get the proper nutrients and the waste is removed. In a stirred bioreactor however, the nutrients in the system are being constantly stirred around to make sure the entire media does not have spots where there are higher concentrations of nutrients or waste.

2.6 Limitations of Microcarriers

While microcarriers help slow down the shear forces on the MSC's during growth in a stirred tank bioreactor, microcarriers can also pose a danger to the cells. The microcarriers move freely in the media, improving nutrient dispersion and media flow, but the spheres will often collide with one another damaging the adhered cells. Further damage can come from the spherical beads colliding with the impeller, bioreactor wall, or impeller shaft as well shearing the cells off the bead or damaging them [19,40].

2.7 MSC Limitations

As multipotent stromal cells, Mesenchymal Stem Cells have the ability to differentiate into numerous different cell lines, such as osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes. For each cell line there are different conditions that can induce the differentiation. The induction of osteoblast can be done by using human fibronectin, 20 µg/mL or a 0.1% gelatin and coating a tissue culture plate and seeding cells with normal MSC growth media. After an overnight incubation and replacing the culture media with OsteoMAX-XF™ Differentiation Media the cells will differentiate after 14-21 days. Also, other external forces can induce differentiation such as turbulence and fluid flow. Within a bioreactor there must be enough fluid flow to allow for proper distribution of nutrients within the system, however, the shear forces of the fluid cannot exceed 9 mL/min or else the MSC will begin to differentiate or die [41].

2.8 Harvesting Techniques

Several methods are currently available for harvesting cells from microcarriers, the current gold standard in MSC production. The most common method is the use of proteolytic enzymes, such as trypsin or collagenase. Before washing the microcarriers in either of these enzymes, rinsing with PBS greatly increases the efficiency of the harvest. Aside from these standard procedures, dextranase can also be used for Cytodex™ microcarriers. Dextranase is able to completely digest these microcarriers while leaving the MSCs unharmed. Other less popular methods of harvest include sonication, chelating agents, exposure to hypotonic or cold conditions, and alteration of the surface tension of the culture media [42]. A 2014 study looked to enhance the efficiency of the manufacturers' generally suggested protocols by using trypsinization alongside agitation by increasing the rate of the impeller of the bioreactor for a short period of time. This adjustment increased the harvesting efficiency from as low as 5% to 95% in some tests. The increased agitation did not impact the viability or differentiation of the MSCs, a concern when exposing MSCs to increased shear stresses [43].

Chapter 3: Project Strategy

3.1 Initial Client Statement

The project statement for the project was to develop novel coatings or systems to enable adherent cell growth within a bioreactor on microcarriers.

3.2 Objectives and Constraints

3.2.1 Overall Project Objectives

The overall goal of this novel system is to maximize cell growth within a bioreactor by utilizing an increase in surface area to volume ratio. The chosen cells to which we want to increase cell growth are mesenchymal stem cells (MSC) due to the increasing prevalence of chronic diseases and the demand for new and effective cell-based treatments and therapies. In order to meet this demand, there is a need for modern and advanced production techniques of the required cells with the greatest clinical potential, MSCs. By increasing the surface area to volume ratios within a bioreactor system, we can increase the overall production of MSCs for clinical trials and future applications. Along with maximizing the surface area to volume ratio, we want to allow for proper flow and mixing of the media within the bioreactor. However, the flow speeds must be low enough not to damage or initiate differentiation of the cells with shear stresses, but also high enough to allow for nutrient dispersion throughout the system. Nutrient dispersion within the media is a key aspect when it comes to increased cellular growth. Additionally, we will need to incorporate some means of regulations of the media and the bioreactor system as a whole. This will include regulation of temperature, oxygen, CO_2, etc, levels and sensor systems to track the levels of cell growth and nutrients within the media and alert and monitoring systems to notify the users when it is the accurate time to harvest.

3.2.2 Design Requirements

In order to ensure compatibility and minimum performance of our design, standards must be put into place. These technical requirements can be seen listed in Tables 2 and 3 show the chosen material of our design and the architecture of our design.

Table 2: Material Technical Design Requirements

Material

Design Requirement	Value or Attribute
Withstand Temperature	37° Celsius
Maintain pH	7.0 - 7.4
Allow for Cellular Adherence	Greater than 50% surface utilization
Inert Surface Chemistry	Non-toxic and non-corrosive surface

Table 3: Architecture Technical Design Requirements

Architecture	
Design Requirement	**Value or Attribute**
High Surface Area to Volume Ratio	Greater than 13,200 cm^2 per liter
Sufficient Nutrient Distribution	Sufficient O_2 distribution and fluid flow
Effective Harvest	Greater than 5% recovery efficiency
Scalable	Scalable to commercial application

3.2.3 MSC Growth Requirements

One of the main functions of this design includes all aspects that keep the MSC healthy and proliferating. This will be accomplished through various design requirements. In order to maintain proper regulations for the development of MSC, the temperature must be maintained at 37° Celsius and 5% CO_2 level. This will be done through a means of an incubator incorporated into our design. A pH must also be maintained at 7.0-7.4. This will be done through our chosen media suspension. Additionally, in order to sustain MSC growth, there must be sufficient nutrient distribution throughout the chosen system. This will be accomplished through rotating propellers at the bottom of the bioreactor and an architecture that will allow for proper fluid flow and nutrient dispersion throughout the system [44].

3.2.4 Mechanical Requirements

There are mechanical aspects within the design that must be accomplished through our requirements. This includes maximizing the surface area to volume ratio to allow for the most MSC adhesion within our system. This will be expressed through our chosen design. Maximum adherence will not only be accomplished through a high surface area to volume ratio, but also through the chosen type of material. This material must allow for cellular adhesion of MSCs.

Minimal turbulence and fluid flow rate are a very strict requirements that were put into place. There must be enough fluid flow to allow for proper distribution of nutrients within the system, however, the shear forces of the fluid can-not exceed 9 mL/min or else the MSC will begin to differentiate or die which is not wanted within this design system [41].

3.2.5 Feasibility Requirements

There are requirements within our design that were met in order to ensure the feasibility of our design. There must be an effective harvesting technique performed within 2 hours to preserve cell viability and to avoid differentiation [45]. The design must be able to become scalable to a commercial application.

3.2.6 Ethical Standards

The ethical concerns associated with this project are with the methods, materials, and process used to enhance cell attachment. Throughout the process there must be assurance that any operators must not be exposed to or potential risk of hazardous or hazardous substances. Residues used for enhanced coatings on a scaffold must also not leach into the cell product for example polystyrene is not toxic to MSCs however are toxic within mammals. There are also considerations to take into account with the future clinical application for our scaffold design for cultured stem cells. There are many ethical concerns in today's society when it comes to using stem cells. For this particular design, we will be focusing on providing the necessary surface area for the growth of adult mesenchymal stem cells. Therefore, the ethical considerations and concerns associated with embryonic stem cells are not relevant in this discussion. The main ethical concerns for the clinical application of adult MSCs include unwanted differentiation of the MSC and their possibility to suppress anti-tumor immune responses and create new blood vessels that may lead to tumor growth [46, 47]. While we are unable to address the concern of anti-tumor immune response suppression within our system, we are able to control the unwanted differentiation. As mentioned before, this will be regulated through our fluid flow systems. A shear stress of 5 dynes/cm^2 will not be exceeded within our system, in order to enable MSC proliferation, without forcing differentiation [41].

3.3 Revised Client Statement

The revised client statement is to develop a novel design to enable adherent cell growth of mesenchymal stem cells by maximizing surface to volume ratio within bioreactors, considering proper nutrient dispersion and keeping the shear stress on the cells below the minimum stress for differentiation while creating a cost-effective system.

3.4 Management Approach

3.4.1 Major Milestones

1. Defining the scope of the project (9/18/19)
2. Completing Background Research (11/8/19)
3. Draft 1 of Report (9/18/19)
4. Draft 2 of Report (9/25/19)
5. Draft 3 of Report (10/2/19)
6. Interviewing Stakeholders (11/30/19)

7. Selecting feasible designs from original brainstorms (12/6/19)
8. Prototyping selected designs (1/24/20)
9. Testing mechanical stresses on designs (shear forces, elastic modulus etc) (2/14/20)
10. Cell culturing on designs (3/20/20)
11. Analysis of cell growth over time (4/2/20)
12. Analysis of harvesting ease/time of MSC off of the design (4/2/20)
13. Final Report (4/16/20)
14. Final Presentation of findings (4/16/20)

3.4.2 Gantt Chart

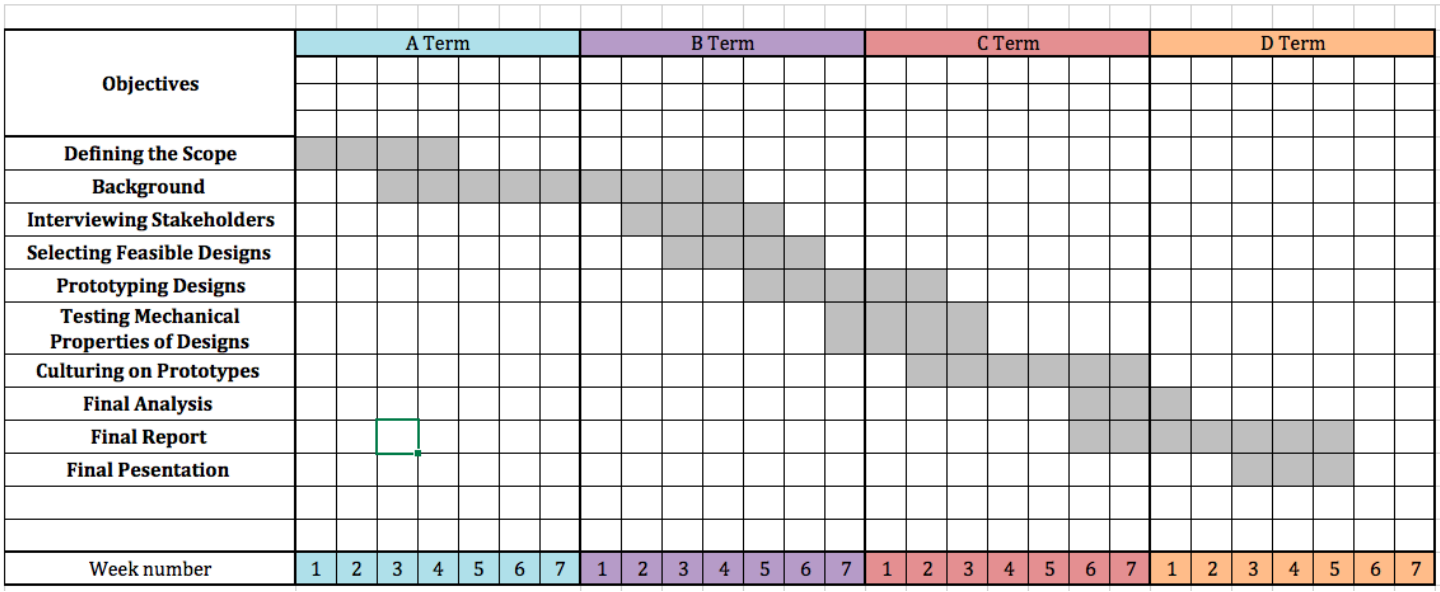

Figure 7: Gantt Chart

3.4.3 Financial Statement

Throughout the duration of this project, funding will be based on $250 per person, and will, therefore, have a total of $1,250. This will be put towards the acquisition of MSC's as well as different materials for testing and lab fees. We would like to keep our initial testing at a small scale no larger than a 100 sq. cm range to keep within our financial budget. Overall, we would like to create a microcarrier system that is low cost, in order to maintain feasibility of possible commercial use in the future. Ideally, our system would be produced and sold at a lower cost than our competitors' products. For example, General Electric's Cytopore® Microcarriers Beads or Xpansion® Multiplate Bioreactor System that is offered by the Pall Corporation.

Table 4: Financial Statement

Item	Cost

Lab Fees	$250
Steel Mesh	$15.99
Fiberglass	$17.99
Filter Paper 100 μm	$15.99
Filter Paper 75 μm	$7.99
Total	$307.96

Chapter 4: Design Process

4.1 Needs Analysis

4.1.1 Need for MSC

As has been previously stated, the need that the scaffold design will address is the production of more MSCs per volume in a bioreactor through the increasing of the surface area to volume ratio within the bioreactor. While there are various specific diseases and conditions that require MSCs for treatment and therapies, it was decided to quantify the need for osteoarthritis treatments specifically.

Osteoarthritis was selected for treatment quantification because therapies and treatments of this condition based on application of MSCs have been proven to be both safe and successful in phase I clinical trials [15].

In one clinical trial it was found that the injection of MSCs with a count between 20 and 24 million cells during one round of treatment produced positive results. Not only was the treatment able to reduce pain in the joints affected with osteoarthritis, but the effects of the treatment lasted for up to 6 months. The treatment was also able to increase the thickness of cartilage at the affected sites, reversing some of the effects of osteoarthritis [6].

In another clinical trial it was shown that a single injection of 2 million MSCs derived from adult adipose, or fat, tissue was able to reduce pain significantly by clinical standards. Furthermore, the one injection of MSCs increased the overall function of the arthritic joints that were treated using the MSCs [15].

Using the data from the clinical studies mentioned above in combination with estimates of 14 million people in the US suffering from osteoarthritis of the knee joint, an estimation for the clinical demand of MSCs for patients with osteoarthritic knees can be made [48]. By multiplying the number of cells used successfully per treatment by the number of patients that require this treatment, the calculated demand of MSCs for the treatment of this condition ranges between 28 and 308 million MSCs.

4.1.2 Need for New Suspended Media Expansion System

There is a great need in the biomedical industry for new designs and systems for MSC expansion in suspended media. The currently and commonly utilized 2-dimensional static culture systems come with important limitations, such as reduced cell numbers and comprised cell functions. Specifically, the current scale of these 2-D systems in lab settings is limited with

surface areas ranging from 12.5 cm^2 to 225 cm^2, and although they are simple to operate and cost effective, a very large number of these systems would be necessary to produce enough MSCs for clinical trials. The use of multiple systems would be more time consuming and require more labor, while working with a very low surface to volume ratio. The chances of contamination would also be increased, resulting in higher risk of cell deformation and death [8].

Microcarriers, which are the current gold standard for MSC growth, have their own shortcomings and limitations. The seeding process onto the microcarriers is a major limitation as the process is both time-consuming as well as inefficient. In an attempt to seed all microcarriers with cells, the bioreactor must be stirred and seeded multiple times, but even then, there will be many numerous microcarriers with no cells attached. In general, around a 50% seeding efficiency can be obtained through this process without dedicating large amounts of time for the process. An important aspect of this microcarrier efficiency is that if a microcarrier only has one or two cells adhere to it, then the cells will not have enough cell to cell interactions to properly proliferate and will therefore have that microcarrier end up with no cells attached. After the seeding process is complete and the cells are being cultured in the bioreactor, it is common for only around 50% of the surface area to be utilized, as can be seen in Figure 8 below. Another problem microcarriers face is that while the bioreactor is being stirred, the microcarriers are constantly colliding with one another, making it possible for the cells to completely shear off, again leaving the microcarrier with no cells attached. The final limitation associated with microcarriers is the inefficient harvest process, which under standard microcarrier harvest procedures only harvesting around 5% of the cells [43].

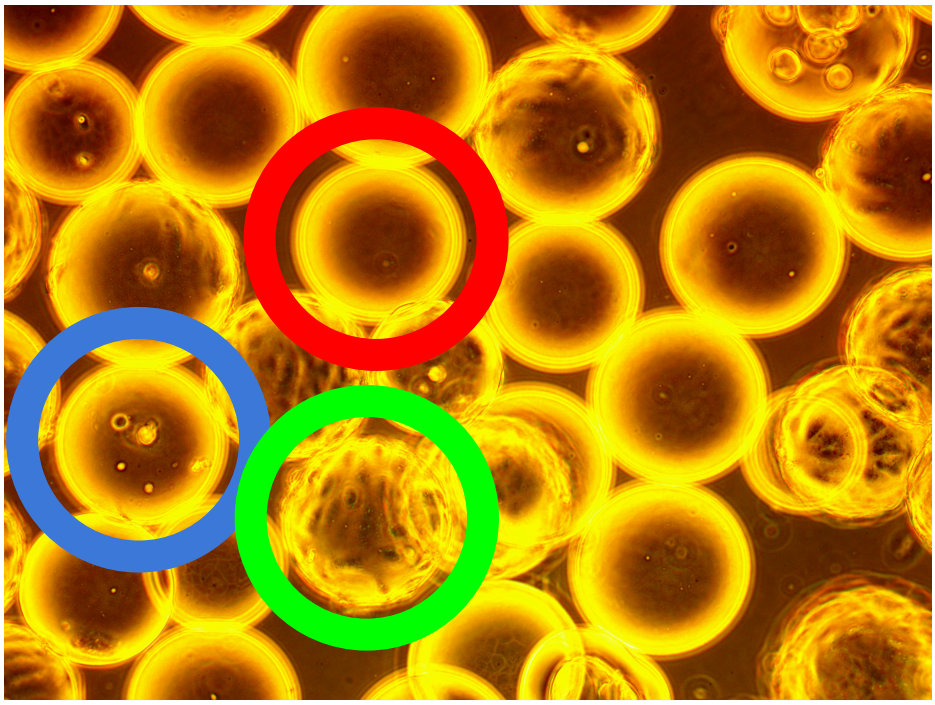

Figure 8: Microcarriers post seeding with the green circle showing an efficiently seeded fully confluent microcarrier, the red circle showing a microcarrier with no cells, and the blue circle showing a microcarrier with very few cells that will most likely not have enough cell to cell interactions to become confluent

4.1.3 Need for Operational Ranges

There are several "needs" that our design must fulfill to be able to accomplish the greater goal of our project. The first mandatory criteria that our design must fulfill is to be functional under the predetermined operational ranges. Since most expansion processes of human MSCs have taken place in fully controlled rocking and stirred tank bioreactors with volumes ranging from 250 mL to 5 L, those are the bioreactor sizes that our scaffold design must be scalable for. The design should be able to support starting densities of MSC of approximately 15,000 cells per mL, while also being able to support harvesting densities of approximately 200,000 MSCs per mL. It needs to be able to function under agitation rates of approximately 64 rpm [49].

Our design also needs to be able to ensure the necessary oxygen concentrations in all locations of the scaffold as oxygen concentration is another factor contributing to human MSC differentiation. 20% oxygen levels have induced adipogenic differentiation of human MSCs, while 5% oxygen content has greatly influenced chondrogenic differentiation. It has also been recorded that even short-term lack of the appropriate oxygen levels, as little as 48 hours under 1% oxygen, can lead to irreversible damage to the differentiation potential of human MSCs, specifically into bone [10]. However, this significant lack of oxygen, known as hypoxia when fallen below 2%, can induce the formation and expression of certain vasculogenic characteristics [50]. Our design needs to make sure there are no "dead spots" within the scaffold where cells will experience hypoxia.

Another criterion that our design must fulfill is a cost or price of no more than the budget we have allocated for this project, which is $1,250.

4.1.4 Prioritizing Tasks

In order to prioritize the tasks our system needs to perform, a pairwise comparison was done shown in Table 5. Seven main goals were identified in order to create a successful system to improve production of anchorage dependent cells: maximizing cell growth, maximizing surface area to volume ratio, maximizing seeding efficiency, maximizing cell to cell interaction, maximizing harvesting efficiency, maximizing ease of manufacture, and minimizing cost.

Table 5: Pairwise Comparison

Task		A	B	C	D	E	F	G	Score
Cell Growth	A	X	1	1	1	1	1	1	6
Surface Area to Volume Ratio	B	0	X	1	1	1	1	1	5
Seeding Efficiency	C	0	0	X	1	1	1	1	4
Cell to Cell Interactions	D	0	0	0	X	1	1	1	3
Harvesting Efficiency	E	0	0	0	0	X	1	1	2
Ease of Manufacture	F	0	0	0	0	0	X	1	1
Cost	G	0	0	0	0	0	0	X	0

4.2 Design Requirements: Industry Standards

The system, its operation, and the process used to design it must be in accordance with applicable international standards established by the International Organization of Standardization. Standards identified to be applicable are ISO 11737-2:2009 (Sterilization of Medical Devices), ISO 13485:2016 (Quality Management of Medical Devices), and ISO 13022:2012 (Medical products containing viable human cells). ISO 11737-2:2009 gives criteria for tests to be used to define, validate, and maintain a sterilization process. These criteria will be critical for the design as the system will need to be sterilized in order to be used many times over. ISO 13022:2012 is designed to reduce the risk of hazards when designing, manufacturing, and testing medical devices utilizing human material. Abiding by this standard will guide the design of the system and testing of the system. Tests used to evaluate and validate the design must closely follow this standard to ensure the safety of all team members. Beyond testing, appropriate considerations must be made to ensure that the system is user-friendly, safe for an operator, and consistent in producing a safe product. Accordance with ISO 13485:2016 is specifically intended to ensure the production of consistent and high-quality medical products developed with a customer-centered focus and an emphasis on continual improvement. The standard identifies criteria for a quality management that spans the lifecycle of the product and its development, from design and development to servicing and maintenance. Accordance with these standards is critical in ensuring the safety of all stakeholders.

Aside from the standards listed above, there are additional standards that are designed to mitigate and eliminate hazards for operators of a bioreactor. Firstly, special precautions must be taken in order to reduce the risk of fire when working with potentially flammable additives, such as isopropyl alcohol. To reduce this risk, flammable resources must be stored in accordance with the requirements of 29 CFR 1910.106 and all wiring and equipment must be in accordance with EM 385-1-1, Section 11.G and NFPA 70 which includes, but is not limited to ground-fault protection. Some chemical hazards are also present while operating a bioreactor, specifically, exposure to carbon dioxide as a by-product of the system and exposure to waste and highly concentrated additives. OSHA standard 29 CFR 1910.146 specifies testing for carbon dioxide in confined spaces. Exposure to other waste products and hazardous additives is reduced or eliminated with the proper use of approved personal protective equipment (PPE), for example, nitrile gloves, face shields, splash aprons, and HEPA respirator filters [51].

4.3 Concept Map

The below concept map outlines the overall goal of this project and means of accomplishing that goal (Figure 9). There are four methods that may be used to carry out the overarching goal to increase efficiency of MSC production: increase surface area to volume ratio, increase surface area utilization, increase seeding efficiency, and improve harvesting efficiency. This work did not directly work to ease harvesting efficiency, and instead, will focus on the three other methods. In order to increase surface area to volume ratio, novel structures including wrapped cylinders, anemone-inspired structures, and fibrous brush structures were explored. Methods for easing the seeding process include utilizing a single, rigid body structure and/or using a novel material that is faster/more adherent for MSCs. Lastly, to increase surface area utilization, a bioreactor system that compresses microcarriers creating a packed bed styled system was tested.

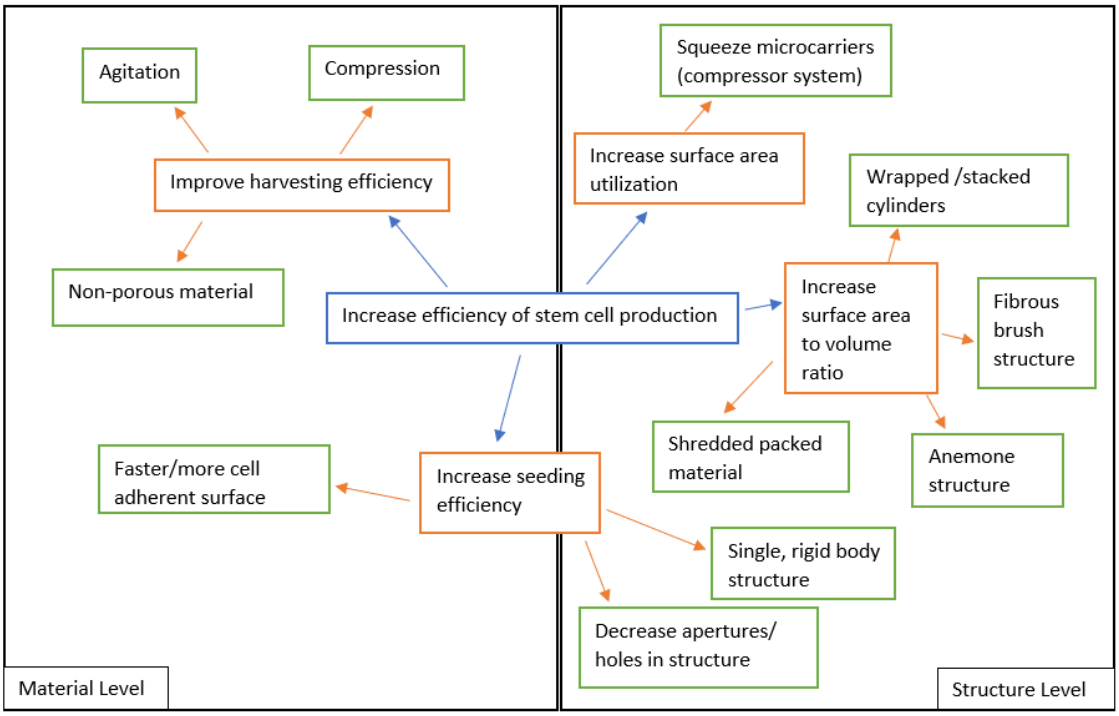

Figure 9: Initial Concept Map of Project Goals

4.4 Conceptual Designs

Specific materials were chosen and selected to be run in each of the studies for individual reasons pertaining to nontoxicity, malleability (shaping the material into a desired construct), compression capability, surface area to volume ratio cellular adhesion abilities and cost.

Polystyrene is commonly used for all cell culture plates and flasks. Although, for our particular studies it was initially considered because of its ability to be spin coated onto any desired surface as well as its capabilities to be manipulated in order to form and hold pores in its structure through the addition of zinc oxide. This creates a porous surface of the spin coated polystyrene and increased surface area and textured surface for cells to adhere to. Pore sizes of 50 nm and 200 nm were created to test their ability in cell attachment and proliferation. Glass slides were prepared and cleaned thoroughly by sonication then washed in acetone, isopropyl alcohol and deionized water for 10 minutes, repeated 3 times each. The glass slides of approximately 6.45 cm^2 in area were then coated with a ZnO seed layer by spin-coating 5 mm zinc acetate dihydrate in ethanol at 2000 rpm for 30s and annealed at 350°C for half an hour in air. The ZnO seed determined the pore size, which were 50 nm and 200 nm as mentioned previously.

Polyurethane foam was chosen as a tested material because it has proven to allow for cell growth in multiple studies. However, one of the main reasons this material was chosen is because of this capability to be compressed or compacted into a packed bed. Furthermore, the foam is also porous, allowing for a large surface area for the cells to sink into and attach to. Polyurethane foam is also inexpensive and can be purchased and produced in large quantities.

Another testing material that was chosen for the studies was 304 Stainless Steel Woven Wire 120 Mesh. Stainless steel is a common material used in medical devices, including bioreactors. Because of this, it proved to be a promising material when testing cellular adhesion and proliferation. The stainless steel mesh also held other reasons for being chosen such as its

malleable capabilities. It has a wire diameter of 0.09 mm and apertures of 0.13 mm. This 120 mesh design allowed for fluid flow through the scaffold, however also it was able to be shaped into any scaffold design needed. The woven mesh design also provided an increased surface area to volume ratio when compared among other design materials due to its woven construction format. Stainless steel mesh is mass produced and can be purchased at a cost of $0.25 per 100 cm^2.

Lastly, fiberglass was chosen as a tested material because of the proven effectiveness of culturing cells on glass slides. It has been known that cells adhere and grow on certain types of glass. Fiberglass was chosen because of its cell growth capabilities and its ability to be packed together in shreds to allow for a greater surface area to volume ratio. Fiberglass is inexpensive and can be produced or purchased in large amounts.

4.4.1 Material Cell Loading

Human fibroblast cells were obtained from Professor Page and were cultured to confluence in a T75 flask.

The materials were seeded with 70 μL of cell suspension. The seeded materials were then incubated for 15 minutes, removed from the incubator and additional media was added to prevent drying out. After 3 repetitions the materials were viewed under the microscope to verify that cells were attached and then the wells were filled, and cells were incubated for a time period between 4 and 7 days and medium was changed every other day.

4.4.2 Harvesting and Cell Counting

A procedure designed to test overall seeding potential, proliferation, and effective harvesting of cells on potential materials was utilized. These new materials identified through research, as well as control substrates already used by the industry, were seeded with the same quantity of MSCs from the same cell line and given 15 minutes to attach to the new substrate. After given time to double several times, approximately 7 full days the cells on all samples were trypsinized, harvested, and counted using a hemocytometer. The protocol is found in Appendix D.

4.4.3 BrdU Assay

A BrdU assay is used to test and verify proliferation of cells on novel materials that may pose challenges while harvesting. BrdU, or Bromodeoxyuridine, is an analog of the nucleoside thymidine that is incorporated into the cells' DNA during the DNA synthesis phase of the cell cycle. BrdU is detected by first incubating methanol fixed cells with a mouse anti-BrdU antibody followed by incubation with an Alexafluor-488 conjugated anti-mouse IgG1 antibody. Lastly, Hoechst 33342, a fluorescent-blue nucleoside stain, is incorporated and binds to the DNA of each cell in the culture (shown in Figure 10). Therefore, under fluorescent light, green fluorescence will show each cell that had undergone DNA synthesis during the BrdU incubation period while blue fluorescence shows all cells in that culture (shown in Figure 11). From this, proliferation of cells on materials can be verified based on the proportion of newly divided cells in a culture and the total number of cells. The protocol is found in Appendix E.

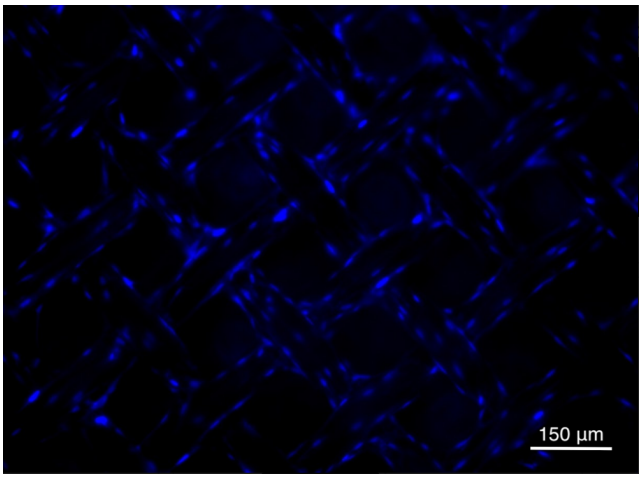

Figure 10: Collagen Coated Mesh Fluorescent-Blue Stain of Fibroblast Cells

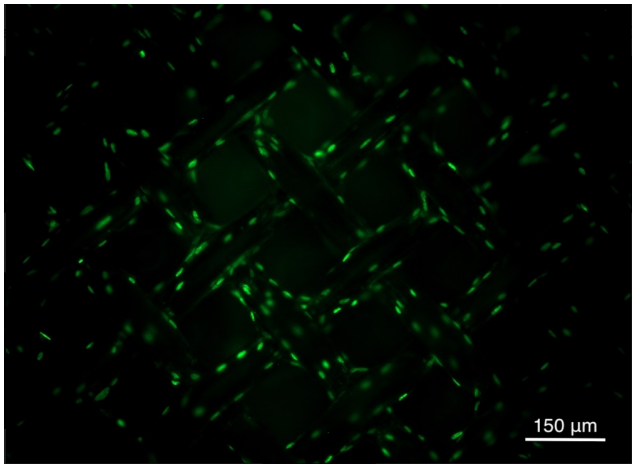

Figure 11: Collagen Coated Mesh Fluorescent-Green Stain of New Fibroblast Cells

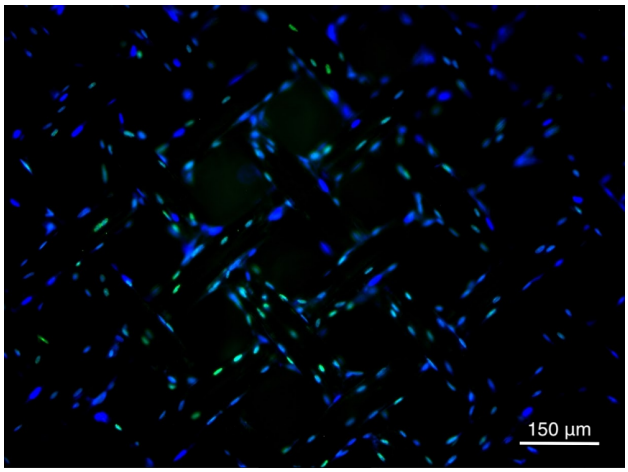

Figure 12: Collagen Coated Mesh Image Overlay of Original Cells and Newly Proliferated Fibroblasts

4.4.4 Relevant Design Calculations For Experimental Parameters

Relevant equations consist of those around the quantification of cells in order to prove that the material is beneficial. Some of these equations include cell recovery which will tell us what percentage of the cells are still alive at harvest as compared to those that were alive at seeding. Another equation is viability, which is the percent of live cells versus dead cells.

$$\square\square\square\square\square\square\square\ \square\square\square\square\square\square\square\square\square\square = \frac{(\square\square\square\square\square\ \square\square\square\square\square\square\square\square\square\square\square\ \square\square\square\square\square\square\square)}{(\square\square\square\square\square\ \square\square\square\square\square\square\square\square\ \square\square\ \square\square\square\square\square)} \times 100$$

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Figure 13: Relevant Equations

4.5 Preliminary Experiments

Our initial material testing involved seeding 20,000 umbilical cord hMSCS in a volume of 150 µl. This experiment was designed to give us initial insight on what materials were most promising for adherent growth and should be continued on further. Materials chosen for this experiment were polyurethane foam, polystyrene 50 nm pore size, polystyrene 200 nm pore size, polystyrene nonporous, collagen coated mesh, plain glass and collagen coated mesh. This selection of materials encompasses materials from our conceptual designs as well as using industry standard materials of collagen and glass to promote cell adhesion which we will use as a baseline to compare to our materials. Each sample of cells was dropped on a single piece of each material. The seeded materials were incubated for fifteen minutes to allow the cells to attach to the material substrate. Then the sample was removed from the incubator and 100 µl of complete media was added to the surface. The sample was then placed back in the incubator for another 15 mins. This procedure was repeated 3 times. After 45 minutes, the well with the sample was filled with media and the seeded surface was submerged in media.

There were 3 pieces of each in order to calculate the average growth rate and doubling time between samples of the material (Figure 14). After 4 full days for the cells to grow, the materials were removed from the media and placed in a fresh dish. The sample was then rinsed in DPBS ($Ca^{2+/}Mg^{2+}$-free) and the sample was submerged in 3ml of Trypsin and incubated for 10 minutes. After adequate trypsinization, media was added, and the cells were counted using a hemocytometer. Cell yield, growth rate, and doubling time are presented below in Table 6.

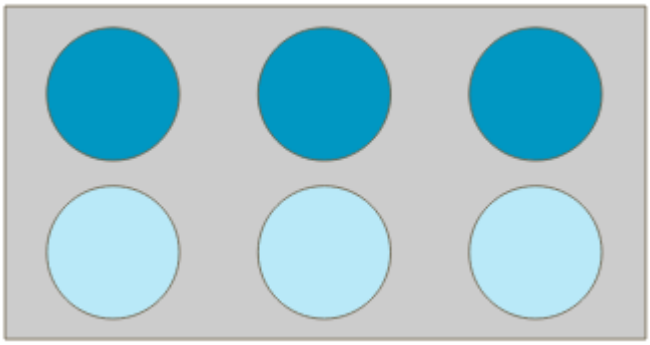


Figure 14: Material Testing 1 Schematic

Table 6: Material Results Test 1: 4 Days

Material	Cells Seeded	Average Yield (After 4 Days)	Growth Rate (% Daily)	Doubling Time (Days)
Collagen Coated Glass Slide	20,000	60,000	50	2.52
Plain Glass Slide	20,000	40,000	25	4.000
Polystyrene (50 nm)	20,000	34,375	17.97	5.119
Polystyrene (200 nm)	20,000	28,125	10.16	8.132
Collagen Coated Mesh	20,000	18,750	-1.56	-0.395
Polyurethane	20,000	4,150	-19.81	-1.763
Polystyrene (Nonporous)	20,000	-	-	-

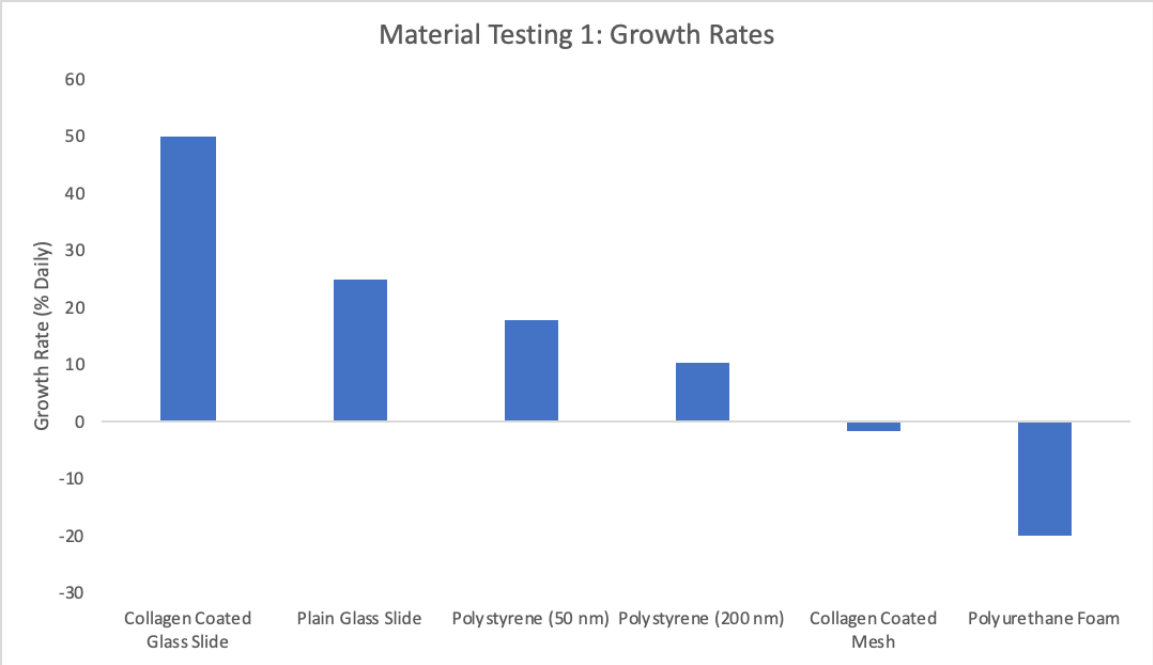


Figure 15: Material Testing 1 Growth Rates Bar Chart

A second experiment was performed to mimic and improve results of the first experiment. This experiment used human fibroblast cells. The schematic of the 6 well plates are shown below in Figure 16. Two samples of each material were used and seeded with 100,000 fibroblast cells each. The materials selected for this experiment were collagen coated stainless steel mesh, 200 nm pore sized polystyrene, 50 nm sized pore polystyrene, glass, a control of the bottom of the well and polyurethane foam. These materials encompass promising materials from experiment 1 as well as incorporating collagen to improve stainless steel mesh results and a control result from the plain well plate. 15 minutes were given to allow cells to adhere 2 mL of media was placed in each well in order to fully submerge the sample in the media. The media was changed every other day and the cells were given 7 full days to grow. Each sample was removed from the media and placed in a fresh dish. The sample was then rinsed in DPBS ($Ca^{2+/}Mg^{2+}$-free) and the sample was submerged in 3 ml of Trypsin and incubated for 10 minutes. After adequate trypsinization, media was added, and the cells were counted using a hemocytometer. Cell yield, growth rate, and doubling time are presented below in Table 7.

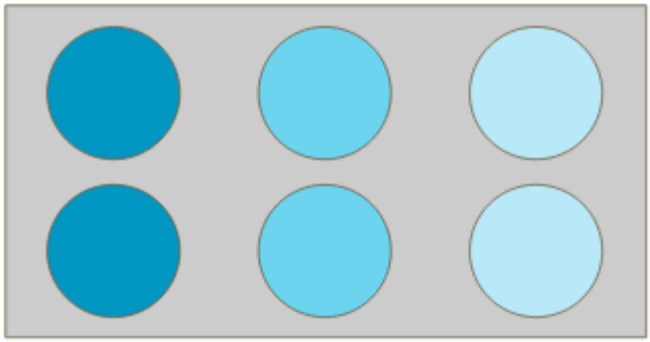


Figure 16: Material Testing Culture Dish Schematic

Table 7: Material Testing 2: 7 Days

Material	Cells Seeded	Sample 1	Sample 2	Average	Growth Rate (% Daily)	Doubling Time (Days)
Cell Culture Dish	100,000	435,000.00	405,000.00	420,000.00	45.7	3.381
Glass	100,000	262,500.00	502,500.00	382,500.00	40.36	3.617
Porous Polystyrene (200 nm)	100,000	480,000.00	247,500.00	363,750.00	37.68	3.757
Porous Polystyrene (50 nm)	100,000	382,500.00	142,500.00	262,500.00	23.21	5.027
Collagen Coated Mesh	100,000	7,500.00	60,000.00	33,750.00	-9.46	-4.467
Polyurethane	100,000	-	-	-	-	-

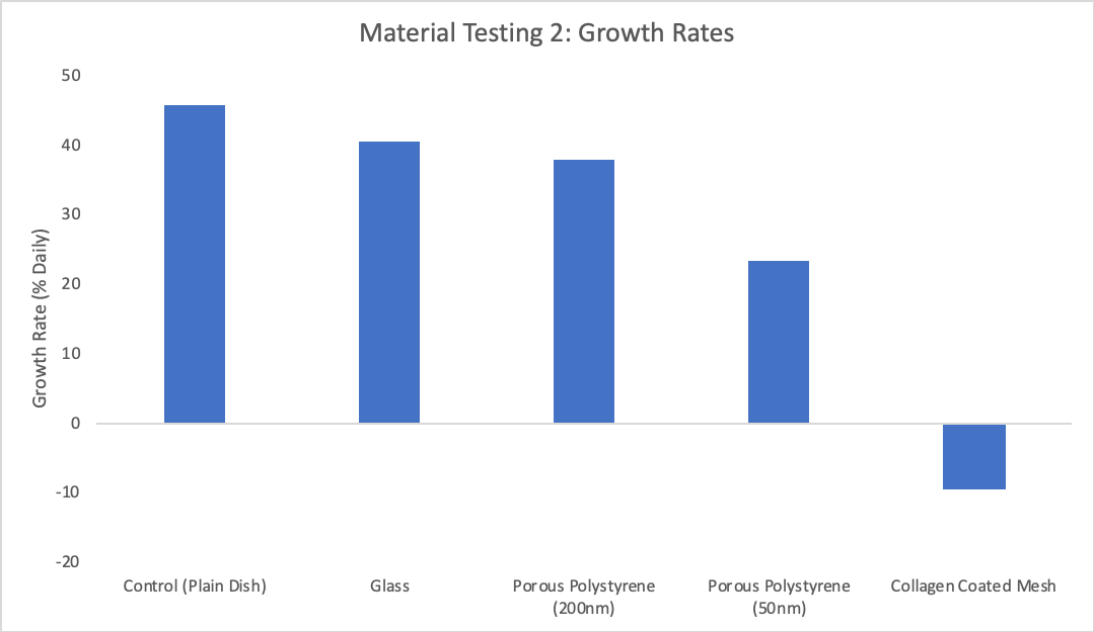


Figure 17: Material Testing 2 Growth Rates Bar Chart

After concluding the experiment and analyzing the results of the polyurethane foam it was decided the harvesting of this material was too difficult and impractical for industry use. Stainless steel mesh continued to be pursued as a viable option in further experiments because the flexibility of the structure and high surface to volume ratio would be beneficial for large scale manufacturing of MSCs.

A third experiment was performed in order to quantify newly proliferated cells on 3-dimensional materials. 100,000 cells were seeded on the seven material samples and given 72 hours to adjust to the new environment. Materials for experiment 3 were collagen coated mesh, polystyrene 50 nm pore size, polystyrene 200 nm pore size, mesh soaked in culture medium, plain mesh, and fiberglass. These materials encompass the new materials for a structure that we believe are most promising based on the previous 2 experiments as well as the addition of fiberglass as an alternative option for a structure design. Specifically, this experiment will be used to validate the 3 types of mesh since the cells falling through the pores will not be counted and the adhesion to the structure and proliferation on the structure is the focus of the experiment. The BrdU was then added to the samples 24 hours prior to imaging in order to stain the newly proliferated cells DNA for imaging. After images were collected showing the seeded cells, newly proliferated, and an overlay image of both stains ImageJ was used in order to count and quantify results. Table 8 shows the comparison between original and newly proliferated cells at the sample site on each sample. The results are present in Figure 18 for easier visualization. The control, collagen coated mesh, soaked mesh and plain mesh all had more newly proliferated cells on the material than original cells. This data is promising in pursuing mesh as a viable architecture as it is presenting to be a suitable environment for large amounts of cell growth.

Table 8: BrdU Assay Results of Seeded Cells Versus Proliferated Cells

	Control	CCM	Polystyrene 50 nm	Polystyrene 200 nm	Soaked Mesh	Plain Mesh	Fiberglass
Original Number of Cells	1037	350	239	614	370	177	337
Newly Proliferated Number of Cells	1539	353	103	546	567	197	n/a

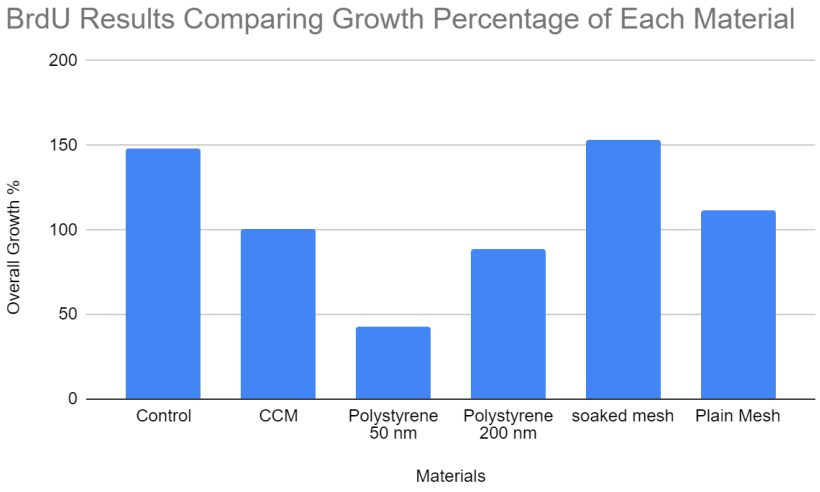


Figure 18: Results of BrdU Assay Bar Chart

Soaking the mesh in cell medium (Appendix F) resulted in improvement between initial cell adhesion on the mesh during seeding as well as large improvement on the number of newly proliferated cells. The initial seeding on the soaked mesh was approximately double of the plain mesh and the newly proliferated cells on the soaked mesh was approximately triple that on the plain mesh. Improving proliferation on the mesh led to a further experiment that aimed to eliminate cells falling through the pores without limiting flow between pores.

A fourth experiment was performed after analyzing the results of the previous study. It was decided to continue testing on mesh that was not coated in collagen as it appeared to clog pores and limit cell motility on the material. Alternatively, mesh was soaked in complete growth medium for 4 days prior to cell seeding. Plain non-soaked mesh was also seeded along with a new chosen material, fiberglass. The 5 layers of mesh were laid over one another in order to mitigate cells falling through the mesh holes. The fiberglass was also layered over one another in order to enhance cell seeding efficiency. All material samples were seeded with 100,000 fibroblast cells. The cells were allowed to grow for 6 days before being trypsinized and counted using a hemocytometer. Overall, soaked mesh showed the best results with the highest growth rate shown in Table 9. This matched the observed data seen in the BrdU study of mesh being a suitable environment for adherent cell growth.

Table 9: Architecture Material Testing 3: 6 Days

Material	Cells Seeded	Sample 1	Sample 2	Average	Growth Rate (% Daily)	Doubling Time (Days)
Soaked Mesh	100,000	1,775,000	725,000	1,250,000	191.66	1.647
Plain Mesh	100,000	1,575,000	900,000	1,237,500	189.58	1.653
Fiberglass	100,000	435,000	405,000	420,000	53.33	2.898

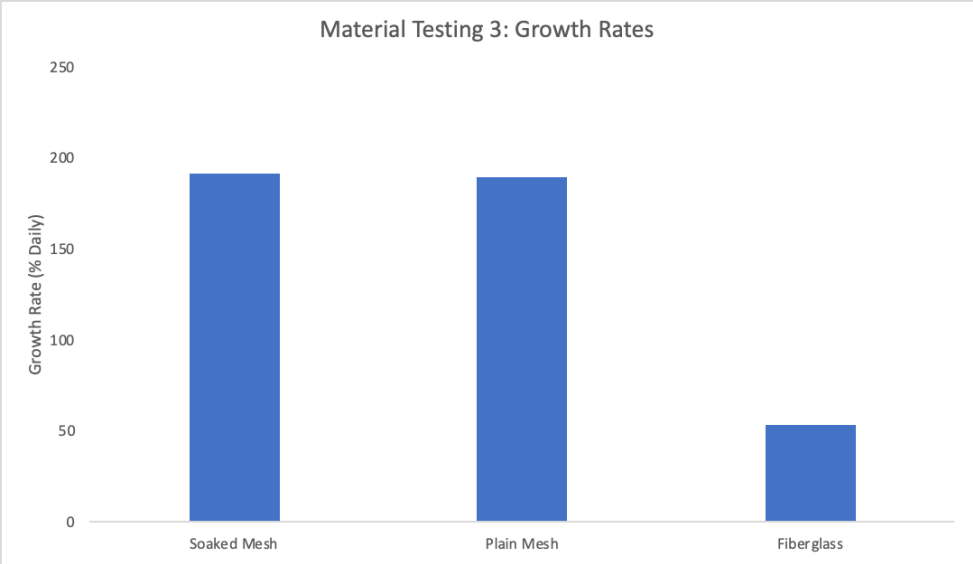


Figure 19: Material Testing Growth Rates Bar Chart

After performing architecture material testing, structural calculations and pressure drop experiments were performed in order to assess the viability of nutrient dispersion and surface area to volume ratios of each of the chosen materials. A miniature chamber was developed in order to recreate a bioreactor simulation. Each of our materials were packed within the tubing shown in Figures 21 and 22 and were tested or fluid flow rates and pressure drops. The results can be seen in Table 10.

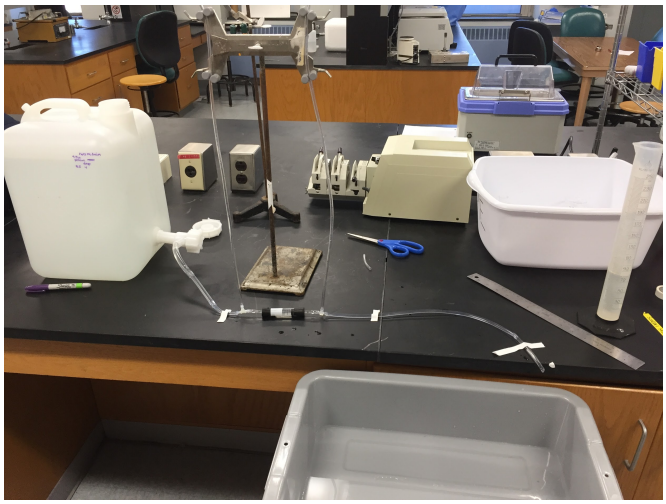


Figure 20: Pressure Drop and Fluid Flow Setup

Figure 21: Packed Bioreactor Setup

Table 10: Flow Rate and Pressure Drop Results

	Control	Packed Mesh Disc Design	Rolled Mesh Cylinder Design	Packed Fiberglass Design	Packed Foam Polyurethane	Packed Microcarriers
Flow Rate (mL/min)	160	140	170	120	N/A	N/A
Pressure Drop (in)	4.5	4.5	3.0	6.0	10	10

Calculations were also performed in order to find the surface area to volume ratio of the fiberglass and stacked mesh disks. These surface areas were compared against the industry golden standard which is microcarriers. These results can be seen in the bar chart shown below (Figure 22).

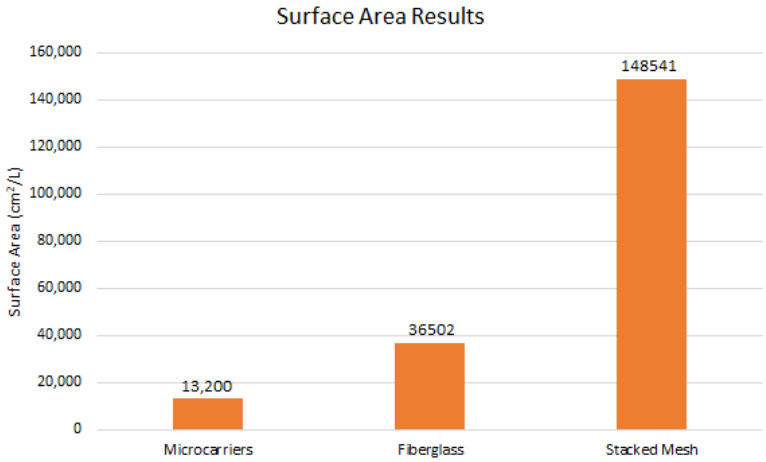


Figure 22: Surface Area Results

4.6 Final Design Selection

From preliminary material and architecture testing, three alternative designs have been identified: Stainless Steel Mesh Stacked Disks, Stainless Steel Mesh Cylindrical Roll, and Porous Polystyrene Packed Shards. This section will assess each design against key requirements and attributes and identify a final design.

The Stainless Steel Mesh Stacked Disks and Stainless Steel Mesh Cylindrical Roll were chosen because they offer the greatest improvement in surface area-to-volume ratio comparing to microcarriers, as they can provide 148,451 cm^2/L while microcarriers can only provide 13,200 cm^2/L. These two designs were also selected because they allowed for sufficient fluid flow when packed together, indicating that they would not obstruct appropriate nutrient dispersion and waste elimination. The stainless steel mesh material used in these designs was chosen due to its ability to support cell adherence and proliferation, as well as non-toxic and non-corrosive nature. The other reasons stainless steel mesh was chosen as the base material for this design is because of its flexible and moldable nature, which allowed us to create the desired architectures. Although other materials such as plain glass and cell culture plastic have a definite advantage in supporting cell adhesion and proliferation, they are not viable options for a scaffold design within a bioreactor precisely because they lack the flexibility and compliance provided by stainless steel.

The Porous Polystyrene Packed Shard design was selected because throughout our tests the polystyrene coating with a pore size of 200 nm showed the highest cell attachment and proliferation. As this material was the most successful in our tests it was chosen as a coating for our scaffold design. There were several limitations due to the coating technology, so based on that we made a decision to stick with glass as the sub-substrate and use a packed glass shard design with polystyrene coating.

4.6.1 Alternative Designs

The first of the alternative designs (Design #1) is the Stainless Steel Mesh Stacked Disks. This design consists of circular cut disks from the woven mesh. The circles can be cut to fit any desired size the bioreactor needs. The stainless steel wire is approximately 0.09 mm in diameter. The wires are separated with an aperture of 0.13 mm. The mesh is 304 stainless steel material which is non-corrosive, sturdy and durable. This design is representative of a packed bed chemical reactor design. The disks are stacked over each other in a fanned set-up in order to decrease the openings throughout the stacked bed. It is stacked for this reason in order to limit the number of cells falling through the packed bed when initial seeding takes place. These circular mesh cut-outs were to be soaked in culture medium for 24 hours prior to culturing within the bioreactor. From preliminary material testing, stainless steel mesh soaked in culture medium is shown to support adherent cell growth at a similar efficiency as collagen coated stainless steel mesh at a fraction of the cost. The packed system also allows for a high surface area to volume ratio with plenty of contact between neighboring disks, allowing for increased cell to cell communication and migration. The stainless steel mesh material also provides little resistance to media and nutrient flow, enabling sufficient nutrient and oxygen distribution throughout the packed bed.

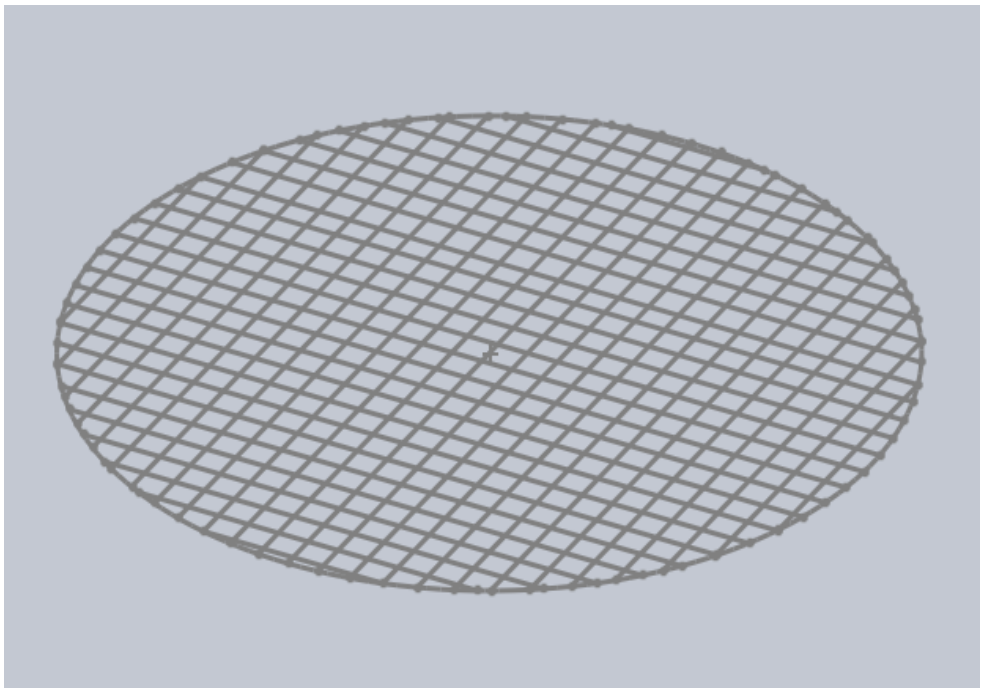


Figure 23: Stainless Steel Mesh Disk

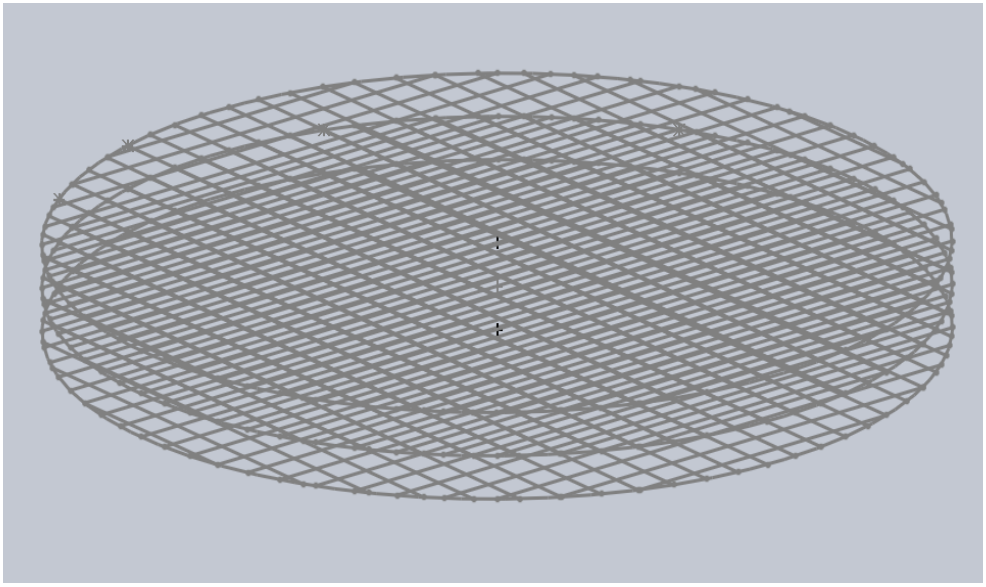


Figure 24: Design #1: Stainless Steel Mesh Disks Stacked

The second of the alternative designs (Design #2) is the Stainless Steel Mesh Cylindrical Roll. This design consists of the same material as the woven mesh circular disks in Design #1, however the mesh is spun into a cylinder shape as shown above. The length of the cylindrical roll can be cut to fit any desired size the bioreactor needs. The stainless steel wire is approximately 0.09 mm in diameter. The wires are separated with an aperture of 0.13 mm. The mesh is 304 stainless steel material which is not easy to rust, sturdy and durable. Additionally, it is very bendable and can maintain the curved shapes as shown below. The stainless steel is rolled loosely to create space for media, oxygen, and nutrients to flow vertically through the system.

Therefore, there is a lack of contact between surfaces within the system, limiting cell to cell communication and migration to only a single plane of mesh. This stainless steel mesh is soaked in culture media prior to culture similar to Design #1. The architecture of this design provides less surface area to volume ratio compared to Design #1, but also offers ease of manufacture and a lesser cost of goods to produce.

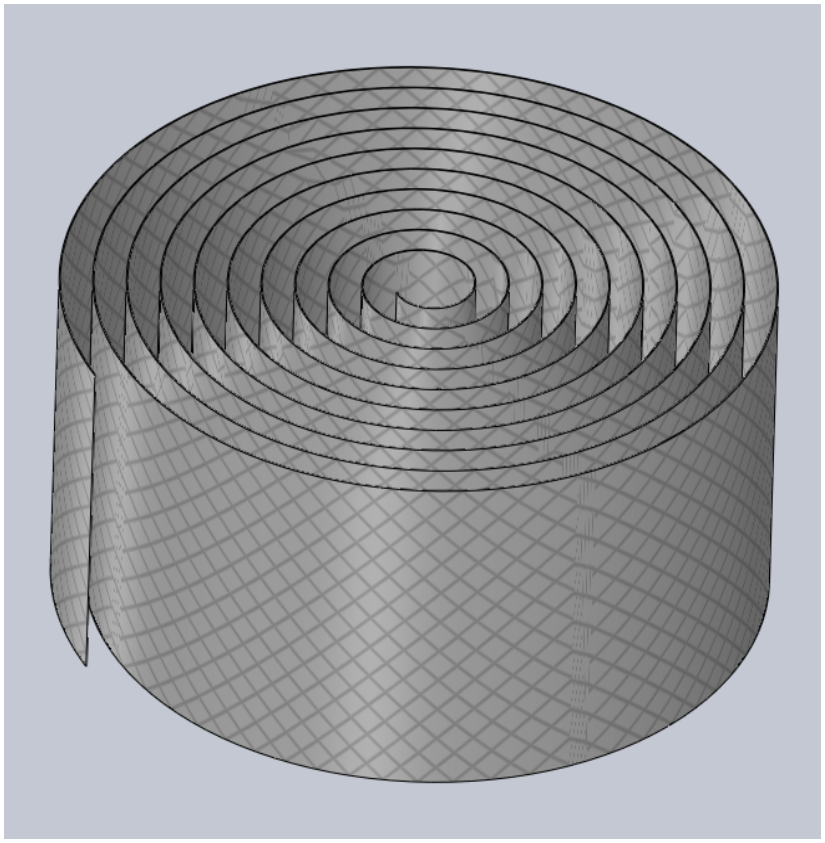


Figure 25: Design #2: Stainless Steel Mesh Cylindrical Rolls

The third of the alternative designs (Design #3) is the Porous Polystyrene Coated Fiberglass. This design, similar to Design #1, is representative of a packed bed design. However, in this design the packed material is made of glass shards coated with polystyrene with 200 nm pours. In preliminary material testing, porous polystyrene was able to support adherent cell growth with efficiency competitive with industry standard materials. The packed bed design offers similar cell to cell advantages as Design #1, however, the porous polystyrene coated glass offers less overall surface area compared to the stainless steel mesh material. Additionally, porous polystyrene coated glass has a more intensive and more expensive manufacturing process compared to the soaked mesh material in alternative Designs #1 and #2.

4.6.2 Final Design

Based on the below pairwise analysis, Design #1 (Stainless Steel Mesh Stacked Disks) was selected as the final design.

Table 11: Final Design Pairwise Analysis

Attribute	Weight	Design #1	Design #2	Design #3
Cell Growth	7	0	0	1
Surface Area to Volume Ratio	6	1	0	-1
Seeding Efficiency	5	0	1	1
Cell to Cell Interactions	4	1	0	0
Harvesting Efficiency	3	0	1	-1
Ease of Manufacture	2	1	1	-1
Cost	1	1	1	-1
Total		13	11	0

Chapter 5: Design Verification

5.1 Proof of Concept Methodology

To verify the final design, a small-scale bioreactor containing the final design: Stainless Steel Mesh Stacked Disks. This small-scale bioreactor will be attached to a continuous flow pump that will induce continuous flow and fresh media into the bioreactor, a separate oxygenation pump will provide constant oxygen input to the system. The stainless steel mesh disks will be cut to the same diameter as the bioreactor interior and soaked in media for at least 24 hours prior to culturing. Disks will be stacked to half the height bioreactor. The system during the culturing process will be placed in an incubator maintaining 37℃and 5% CO_2.

To start the culture, mesenchymal stem cells will be seeded to the top disk. The holes within the mesh would allow for cells to pass through to the lower disks and seed through the structure. The culture will last for 4 days, after which cells will be trypsinized, agitated, harvested, and counted. After the conclusion of this experiment, adjustments will be made to eliminate any shortcomings identified within the small-scale bioreactor, such as nutrient flow rate, seeding strategy. The same experiment will be repeated; however, it will have a duration of 7 days. After the conclusion of the two experiments, results will be analyzed to determine the overall output of the system. This output will be compared to the output of a system with the same volume that utilized microcarriers.

5.2 Anticipated Results

For the initial culture proof of concept testing that lasted for 4 days, results were anticipated to verify the viability of this design as a method for high mesenchymal stem cell production. Over the course of the 4 day culture, the cells, on average, would double every 30 hours after an initial lag period for adjustment to the new surface as has been seen throughout the initial material proliferation testing. Over the course of the 7 day culture, the cells would have a lower doubling time of 24 hours as the lag period present has less of a statistical impact over the longer time frame. Inspection of the mesh disks after the harvesting process would reveal that some cells were not successfully trypsinized and harvested. After the harvesting process was repeated, it would show that about 20% of the total cells remained on the structure and an 80% harvesting efficiency. These anticipated results translate to overall efficiency and production output greater than the industry standard microcarriers.

Chapter 6: Final Design and Validation

6.1 Economics

Economically, the successful implementation of our project would positively impact the economy of everyday living by lowering the cost of drug treatments for patients with chronic illnesses. The average American with a lifelong chronic disease spends 5,300 USD per year on drugs, therapies, and hospital visits. This yearly cost can accumulate upwards of 150,000 USD for a patient living with a chronic disease for 30 or more years [52]. The ability to mass produce mesenchymal stem cells for patient specific therapies would alleviate the need for expensive drugs as well as possible elimination of the need for drugs for treatment. A MSC treatment for chronic disease would be roughly 10,000 USD [53]. Initially, a patient specific therapy would be costly, but overtime the treatments will be less expensive and ultimately more cost effective as a treatment then years on other lifelong drug therapies.

Overtime, as our product became more streamline in the pharmaceutical industry and more labs adopted our scaffold for large scale production of MSCs, total cost for treatment would also reduce. As larger batch runs yield large densities of cells, one single run could in theory treat multiple patients for the same chronic disease or smaller patient specific runs could be conducted at a much lower cost to the patient.

6.2 Environmental Impact

Environmentally, there most likely will not be any major impacts on the natural environment. Our novel stainless mesh structure would allow the possibility for the single use scaffold to be sterilized and then recycled. Since there is no coating on the mesh there is no possibility for biological spreading to the ecosystem if recycled. There would be a reduction in biohazard decontamination due to the recyclability of our mesh however, our spent cell culture media would need to be properly disposed of. The system will have less of an environmental impact due to the generation of less potentially hazardous waste due to the efficiency of cell production compared to other, more complicated systems.

Reduction in overall medication consumption due to MSC therapies would reduce the amount of medications improperly disposed into the environment. Exposure to such medications in the ecosystem can damage populations and subsequently impact the ecosystem.

6.3 Societal Influence

The societal impact of production and marketing of our product would be a reduction in the stigma behind chronic diseases. Ordinary people would be impacted by our product because chronic disease impacts ordinary people. Fear of dealing with symptoms for the rest of one's life would be alleviated by the sales and marketing of our product by the treatment it provides. Alleviation of symptoms would reduce the use of powerful pain medication to deal with pain from chronic illness such as opioids, thus reducing the possibility of addiction from long term use.

There would be the societal influence of hope now for people with chronic diseases. Ordinary people may also be more in favor of stem cell treatments because through our marketing and production more and more ordinary people with “ordinary” chronic diseases would be treated. Our product would help a large population of people

in turn persuade the people impacted by knowing someone with a chronic disease into accepting patient specific and novel treatments such as our product.

6.4 Political Ramifications

On the global market our product has the ability to lower some pharmaceutical sales of large companies that manufacture drugs for chronic illnesses. This may influence politically wise, big pharmaceutical companies monopolizing the manufacturing of certain drugs and price gauging them to ensure a high profit. The effect on other countries would be further research into chronic illness therapies via our MSC scaffold for patient specific therapies. Globally, the stigma around a chronic illness being life long and debilitating on quality of life would also be lowered with the MSC therapies being an option for treatment to alleviate and treat chronic illnesses.

6.5 Ethical Concerns

Ethically, our product has the patient's quality of life at the forefront of the design. The MSC scaffold is designed to efficiently and quickly manufacture MSCs for patient specific treatments. Our product is designed to ensure no toxicity is in the cells when implanted in the patient and the cells will either be autologous or allogeneic cells to ensure ease of implantation in the body. Our product addresses not only alleviating symptoms of a chronic illness that cause pain and a lesser quality of life, but also has the promise of possibly curing chronic illness.

6.6 Health and Safety Issues

Our project will influence the health of people by providing a viable and long term treatment for chronically ill patients. Successful implementation of our project into the pharmaceutical field would positively influence the health of people as patients with chronic diseases recover from their illness or symptoms are relieved. Relief of symptoms from our design would put people with chronic illnesses at less risk for injuries from symptoms that hinder mobility or sight.

For the people using our product to produce MSC's in a lab setting our project will not require additional personal protective equipment than already necessary by the industry. Our project has no non byproducts that could be harmful to someone using or producing our scaffold. Our scaffold does not influence the personal safety of the user or the patients receiving therapies in a negative way. The project only poses the influence to positively affect health and safety.

6.7 Manufacturability

The subject matter of our MQP project, the MSCs on the mesh scaffold, will initially be challenging to reproduce. Challenges are in obtaining patient specific or allogeneic MSCs for manufacturing for treatments. After initial manufacturing of the mesh scaffolds, seeding and harvesting techniques for the MSCs to get the highest yield will be fine-tuned resulting in high reproducibility of techniques for further production.

The subject matter of our project can easily be reproduced by manufacturing stainless steel mesh and tailoring the sizes for any scale of bioreactor. Stainless steel mesh is already very reproducible therefore, the simple design of the layer meshed scaffold will be easy to reproduce on a large scale. Specifically, cutout molds of varying

sizes based on bioreactor volumes of the mesh discs can be created in order to increase manufacturing efficiency, repeatable quality of the product and to reduce manufacturing time. These circular mesh cutouts can then be stacked based on bioreactor volume on an assembly line. These techniques can be used when recreating the product in a scaled-up scenario.

In order to reproduce our product, circular cutouts of the 304 stainless steel mesh will be cut out into equal sizes of equal diameters. The mesh cutouts should be placed in cell medium for 24 hours in order to allow the mesh to be fully soaked before finishing the scaffold. After 24 hours, 5 disks should be placed on top of one another and fanned out in a design where the pores do not fully overlap. After the 5 disks are arranged they should be secured in place. After sets of the soaked fan disks have been secured the fanned disks can be placed in a bioreactor for cell seeding and culturing. This product has the ability to be scaled for any size bioreactor by simply changing the diameter of the disks and following the rest of the protocol.

6.8 Sustainability

Our project is sustainable because it does not have any known byproducts that could harm the environment. Stainless steel mesh is currently produced therefore there would not need to be the construction of any new factories or the harvesting of new raw materials that could influence the ecosystem. Less material needs to be used for a MSC therapy than currently used as well as an increased efficiency in production directly translates to a more energy efficient production. While our product is single use it has the ability to be recycled through proper sterilization since there is no biological coating on the mesh preventing it from being recycled with other stainless steel scraps.

Chapter 7: Discussion

The project was designed in order to create a novel scalable scaffold for adherent stem cell culture within bioreactors in order to increase the ease and efficiency of stem cell production and harvesting in the pharmaceutical industry. The novel scaffold needed to be made of non-corrosive, non-cytotoxic material that supported stem cell attachment and proliferation, while possessing advantages over the current gold-standard material. Such potential advantages included ease of production and manufacturing, having a reduced cost, requiring less treatment and preparation prior to use and enhancing ease of stem cell migration. The novel scaffold also needed a novel structure and architecture that would increase surface area to volume ratio within the bioreactor and surpass the current golden standard of 13,200 cm^2/L, while also allowing for media flow, proper nutrient distribution and sufficient oxygen transport within the scaffold system. The scaffold also needed to ease the cell seeding process, increase seeding efficiency, surface utilization and harvesting efficiency (recovery rate of cells). Through the design of the scaffold and six tests, the scaffold was proven to meet several of the objectives listed above and be a superior alternative to the current gold-standard in stem cell culturing and production.

7.1 Material Selection and Results

The first step of the material selection process was guided by extensive literature review, specifically looking into materials that support cell attachment without inducing cell differentiation, are non-cytotoxic and can remain corrosion-free after being submerged within media suspension for an extended period of time. The literature review focused on materials that have been experimentally proven to fulfil the criteria listed above and to be suitable as substrates for adherent cell culture but have not been widely adopted within industry. Due to limitations posed by a limited access to sterilization methods, the material used in the scaffold design had to either be purchased pre-sterilized or withstand sterilization by autoclaving and isopropyl alcohol baths. This limitation in sterilization methods eliminated the choice of many polymers as substrate materials due to their low melting temperatures. The final material choices consisted of 4 distinct materials including two polymers. The first polymer was polyurethane foam obtained in pre-sterilized condition. The use and testing of this material faced several major limitations. The first limitation was that the only means of cell retrieval was submerging the material in trypsin. The pore size and foam structure did not allow for proper trypsinization and resulted in -19.81% daily growth rate. As a result of such a poor yield and the impossibility of cell retrieval this material couldn't be used for the scaffold substrate. The second limitation was the opaqueness and porous nature of the material which impeded proper imaging under both a light and fluorescent microscope. The second polymer was liquid polystyrene made within the sterile conditions of the lab and coated onto glass slides. Three different types of polystyrene coatings were tested, solid polystyrene, porous polystyrene with a pore size of 50 nm and porous polystyrene with a pore size of 200 nm. Solid polystyrene had a 0% attachment and as a result yielded no cell count. The limitation of polystyrene was the inconsistent process of creating pores through the process of zinc oxide etching. Very often the zinc oxide particles that were used to create pores were not completely removed by 5% hydrochloric acid resulting in inconsistent surface topography across samples. However, this limitation and inconsistencies in the porous polystyrene samples are negligible, as the values of yielded cells and the doubling times on the porous polystyrene surfaces were often within an acceptable range of differentiation. The yielded cells and the doubling times on the porous polystyrene surfaces were

also significantly different, with the 50 nm porous polystyrene having an average yield of 262,500 cells after 7 days and the 200 nm porous polystyrene having an average yield of 363,750 cells after 7 days of culture.

7.2 Structure Selection and Results

The first step in the selection of potential structure and architectures for the novel scaffold was a literature review of structures in nature, various industries and everyday objects that possess a high surface area while not occupying a large volume. These structures were inspired by sea anemones, beehives and honeycombs, spider webs, shell and tube heat exchanger reactor, construction scaffolds, fiberglass insulation, hair-brushes and toilet paper rolls. Structures were first eliminated based on the moldability of the materials that could be used to create the selected architectures. As none of the materials that were available and accessible could be reasonably shaped in the form of flexible anemone tentacles or rigid and uniform honeycombs those structures were eliminated. Structures were then selected based on the greatest amount of surface area in the smallest volume that they could provide, which led to the selection of a cylinder design (much like a toilet paper roll) created out of mesh (much like a spider web) and a more simple design of stacked mesh cut outs. The stacked mesh cut outs yielded a surface area-to-volume ratio of 148,451 cm^2/L which is more than 11 times greater than the ratio provided by microcarriers, the current gold standard. Another structure that was selected was the packed fiberglass design. The surface area-to-volume ratio for packed fiberglass was calculated to be 36,502 cm^2/L, which is 2.8 times greater than the ratio provided by microcarriers. The measurement and calculation of surface area for the mesh and fiberglass came with accuracy limitations and assumptions of perfectly uniform wire diameters and constant rectangular size fiberglass pieces. These limitations and assumptions are acceptable and can be neglected for the sake of the design project as the average values of surface area for the mesh and fiberglass designs are significantly greater than those documented for microcarriers.

7.3 Final Design Verification

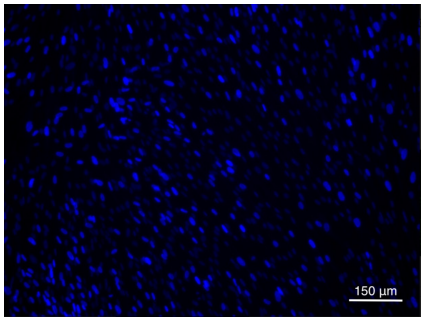
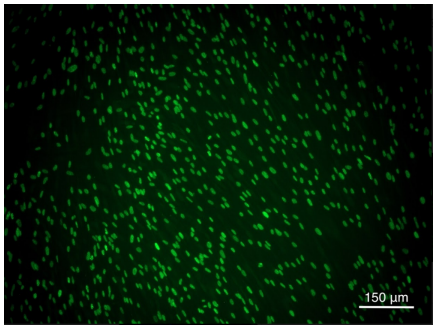
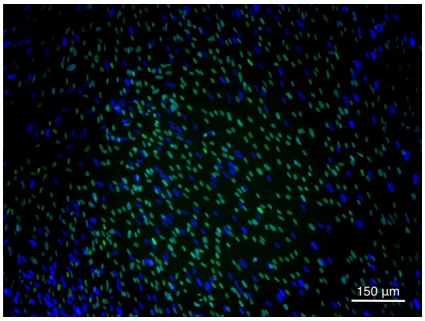
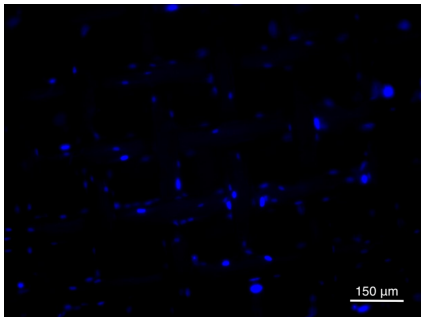
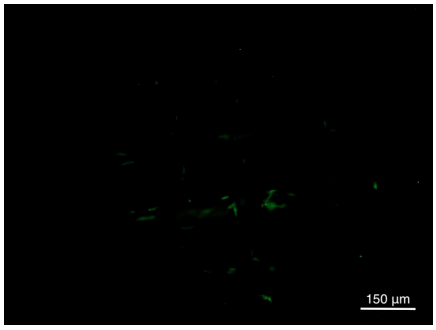
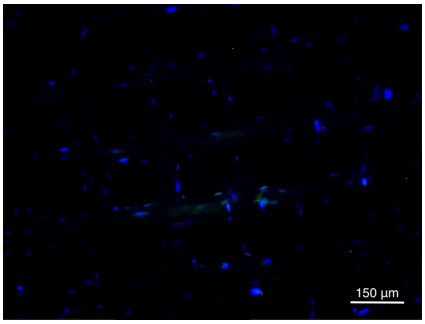
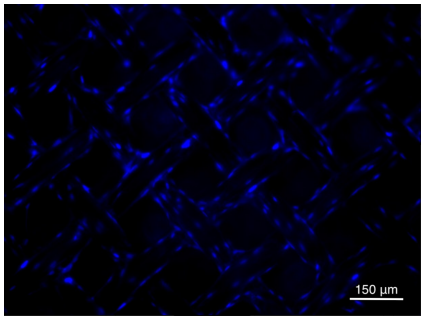
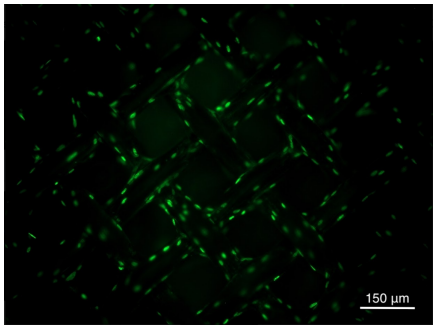
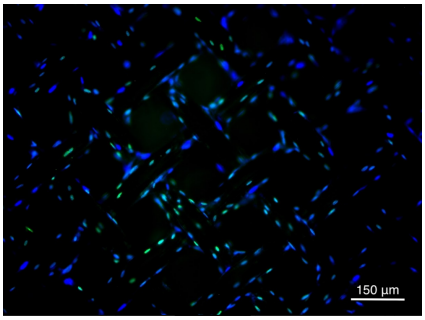
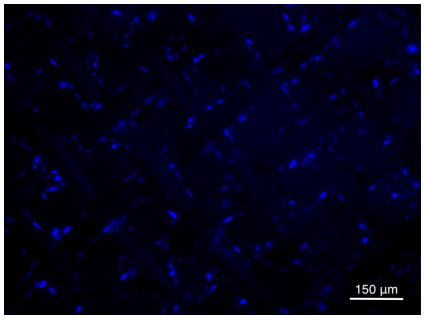
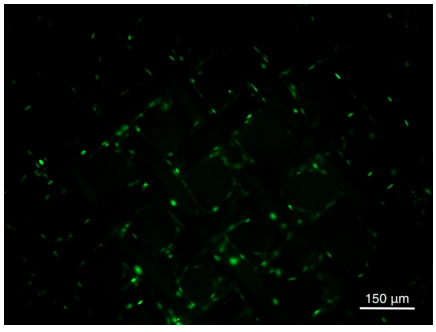
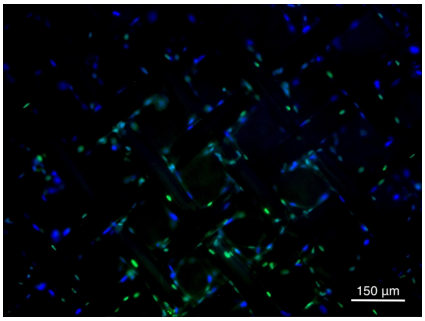
The final design verification process was cut short by the closure of the lab due to the COVID-19 pandemic. The preliminary closure of the lab resulted in the inability to continue any of the prior tests and conduct verification tests for the final scaffold design. The loss of two months of testing time resulted in a limited number of preformed tests and limited test results. None of the scheduled tests could be performed outside of the sanitary lab environment and without access to the incubators needed for cell culture. Thus, although the performed tests and calculations prove that the stainless steel mesh in cylindrical arrangement and in stacked disk arrangement provides more surface area per a unit of volume than the current golden standard of microcarriers and allows sufficient flow for the necessary nutrient distribution. Similarly, the fiberglass has proven to have more surface area per unit of volume than the current golden standard and still allow for sufficient media flow. Although not all of the verification testing was performed, the testing and the results that were obtained support the claim that our design possesses significant advantages over microcarriers and has the potential to significantly increase stem cell production in industry.

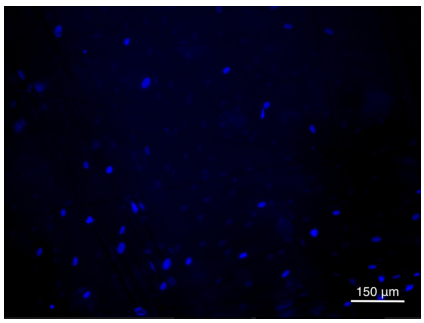
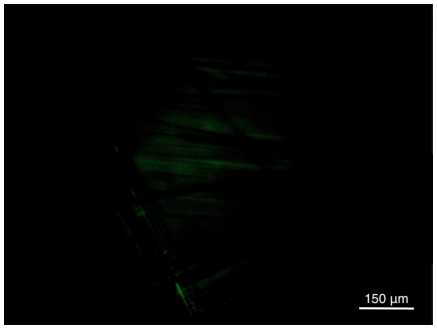
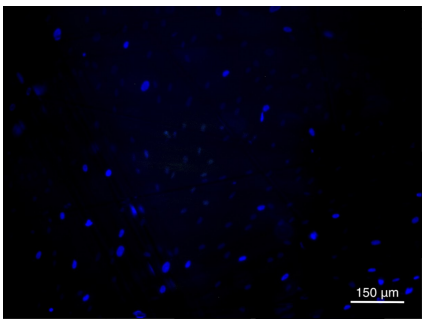
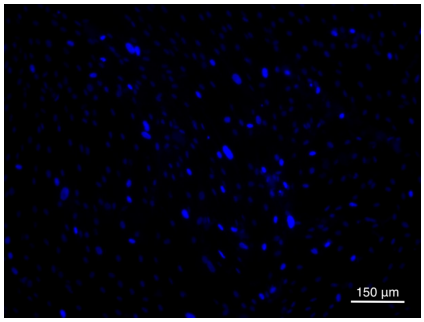
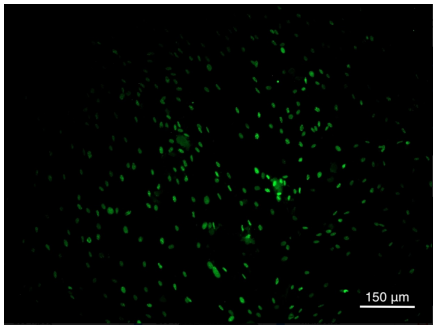
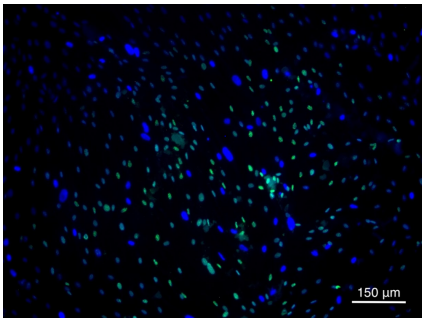
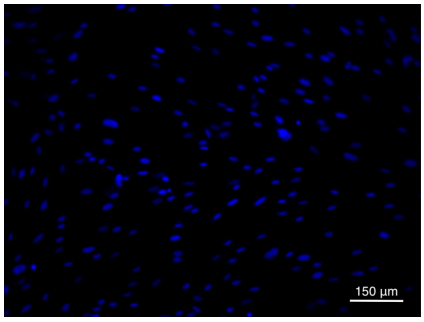
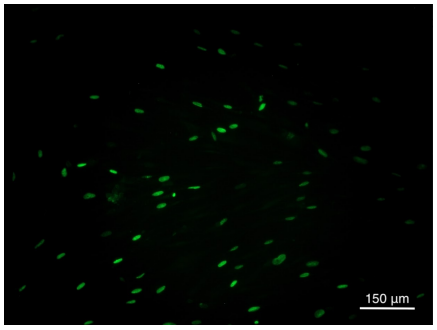
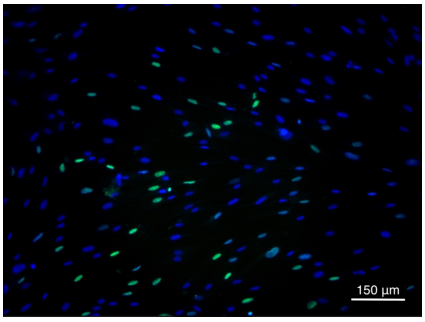
Chapter 8: Conclusions/ Recommendations

Current protocols and materials used for the mass production of Mesenchymal Stem Cells within a bioreactor is not as optimal as it can be. In the coming years with projected use of MSCs for research and medicine getting higher and higher, a new and improved scaffold within a bioreactor is needed. Current state of the art microcarriers are inefficient for seeding as well as harvesting at MSCs, leaving a need in the market for a new scaffold that could help reduce or remove these problems. In an attempt to improve upon these systems, this study looked at potential materials as well as scaffold designs that increase proliferation and numbers of MSCs. These were then combined and narrowed down to the three best candidates showing the most potential to be further studied: the Stainless Steel Mesh Packed Disks, the Stainless Steel Mesh Cylindrical Roll, and the Stainless Steel Mesh Cylindrical Roll. Through culturing cells on materials of similarly sized pieces, cell proliferation after several days could be both calculated, as well as visualized. Based on the results gathered it is believed that due to a higher surface area as well as capabilities for the cells to grow throughout the entire scaffold with more ease, this design has the potential to be more efficient at seeding and harvesting MSCs.

Multiple studies were planned to go more in depth and be able to better determine the most ideal scaffold structure, but due to COVID-19 causing labs to be closed down, these could not be completed. With this information it is believed that a design could have been backed up by data to truly show an efficiency at MSC proliferation that can-not be seen in microcarriers. Future recommendations would be to further study potential scaffold structures in order to determine a more ideal shape for seeding and harvesting. Although this study managed to look at fluid flow across the final three designs, being able to study the cell growth within a flowing system on these scaffolds would have supplied the most important numbers in showing their efficiency over microcarriers. This could also show the ease of harvest from these scaffolds providing more information on how it could be an overall improvement.

Appendix A: BrdU Photos

Sample	Existing Cells	Proliferating Cells	Existing & Proliferating Cells
Control			
Plain Mesh			
Collagen Coated Mesh			
Soaked Mesh			

Fiberglass			
200 nm Pore Polystyrene			
50 nm Pore Polystyrene			

Appendix B: Preparation of Coated Plates

Tissue culture plastic or glassware plates should be coated with 0.1% gelatin as follows:

1. Add sufficient 0.1% gelatin solution to cover the entire surface of the cultureware plate. Use 10 mL volume for 10-cm plates and T75 flasks. Incubate for at least 30 minutes at room temperature.
2. Just before use, aspirate the gelatin solution from the coated plate or flask.

Appendix C: Thawing of Mesenchymal Stem Cells

1. Do not thaw the cells until the recommended medium and appropriately coated 0.1% gelatin plasticware and/or glassware are on hand.
2. Remove the vial of Human Mesenchymal Stem Cells from liquid nitrogen and incubate in a 37°C water bath. Closely monitor until the cells are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells.
   IMPORTANT: Do not vortex the cells.
3. As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol. Proceed immediately to the next step.
4. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful not to introduce any bubbles during the transfer process.
5. Using a 10 mL pipette, slowly add dropwise 9 mL of Mesenchymal Stem Cell Expansion Medium or a suitable alternative of choice, pre-warmed to 37°C, to the 15 mL conical tube.
   IMPORTANT: Do not add the entire volume of media all at once to the cells. This may result in decreased cell viability due to osmotic shock.
6. Gently mix the cell suspension by slowly pipetting up and down twice. Be careful to not introduce any bubbles.
   IMPORTANT: Do not vortex the cells.
7. Centrifuge the tube at 300 x g for 2-3 minutes to pellet the cells.
8. Decant as much of the supernatant as possible. Steps 5-8 are necessary to remove residual cryopreservative (DMSO).
9. Resuspend the cells in a total volume of 10 mL of Mesenchymal Stem Cell Expansion Medium or a suitable alternative of choice, pre-warmed to 37°C, containing freshly added 8 ng/mL FGF-2.
10. Plate the cell suspension onto a 10-cm tissue culture plate or a T75 tissue culture flask.
    IMPORTANT: Seeding density should be 5,000-6,000 cells/cm^2
11. Maintain the cells at 37°C in a humidified incubator equilibrated with 5% CO_2.
12. The next day, exchange the medium with fresh Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37°C) containing 8 ng/mL FGF-2*. Replace with fresh medium containing FGF-2 every two to three days thereafter.
13. When the cells are approximately 80% confluent, they can be dissociated with Trypsin-EDTA and passaged further or frozen for later use.
    NOTE: Depending on seeding density and passage number (i.e. later passages), cells may take longer to reach 80% confluency.

Appendix D: Expansion of Mesenchymal Stem Cells

1. Subculture cells once they have reached approximately 80% confluence and are actively proliferating.
   IMPORTANT: Subculture cells before reaching 100% confluency.
2. Carefully remove the medium from the 10-cm tissue culture plate containing the confluent layer of human mesenchymal stem cells. Apply 3-5 mL of Trypsin-EDTA Solution and incubate in a 37°C incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.
4. Add 5 mL Mesenchymal Stem Cell Expansion Medium to the plate.
5. Gently rotate the plate to mix the cell suspension. Transfer the dissociated cells to a 15 mL conical tube.
6. Centrifuge the tube at 300 x g for 3-5 minutes to pellet the cells.
7. Discard the supernatant
8. Apply 2 mL Mesenchymal Stem Cell Expansion Medium (pre-warmed to 37°C) containing 8 ng/mL FGF-2 to the conical tube and resuspend the cells thoroughly.
   IMPORTANT: Do not vortex the cells.
9. Count the number of cells using a hemocytometer.
10. Plate the cells at a density of 5,000-6,000 cells per cm^2 into the appropriate flasks, plates, or wells in Mesenchymal Stem Cell Expansion Medium containing 8 ng/mL FGF-2.
11. Cells can be frozen in MSC growth media plus 10% DMSO at a density of 2X106 cells/vial.

Appendix E: BrdU Assay Protocol

1. Add 1.0 µl of BrdU stock solution per mL of culture medium to cells being assayed and incubate for 4 hours or the time required by the experimental protocol.
2. Aspirate culture medium and wash cells in 2X in DPBS+.
3. Aspirate DPBS+ and add ice cold (-20°C) methanol (1.0 mL/well for 24-well plate). Incubate for 10 minutes at -20°C.
4. Aspirate methanol and wash with 1.0 mL PBS for 10 minutes (plates can be stored at 4°C with PBS in wells if analysis is not to be done right away.
5. Aspirate PBS and add 1.5 N HCl (0.5 mL/well for 24-well or 0.25 ml/well for 48-well plate) and incubate at room temperature for 20 minutes.
6. Wash 3X with PBS, 5 minutes each.
7. If cells were cultured with serum, blocking is not necessary. If cultured in a serum-free system, block at room temperature for at least 15 minutes with 5% FBS in PBS+0.05% Tween-20.
8. Dilute anti-BrdU antibody 1:100 in PBS +0.05% Tween-20.
9. Add antibody solution at 150 µL/well for 24-well plate or 75 µL/well for 48-well plate) and incubate at room temperature for 30 minutes.
10. Aspirate antibody solution and wash 3X with PBS for 5 minutes each.
11. Add fluorescent dye conjugated secondary antibody diluted 1:500 in PBS+0.05% Tween-20 (150 µL/well for 24-well plate or 75 µL/well for 48-well plate) and incubate at room temperature for 30 minutes.
12. Wash 3X with PBS (without Tween).
13. Add 0.5 µg/mL Hoechst 33342 to the last wash (stock is 1 mg/mL) and incubate for 10 min at room temperature.
14. Aspirate Hoechst solution, wash with PBS and add PBS (1.0 mL/well for 24-well or 0.5 mL/well for 48-well plate).
15. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4°C wrapped in foil to protect from light.

Appendix F: Cell Media Composition

For 50 ml of Cell Media:

1. DMEM Basal Media - 44.0 mL
2. Feligro (FBS) 10% - 5 mL
3. Glulimax [100x] - 0.5 mL
4. Pennstripe [100x] - 0.5 mL

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