Small Scale Continuous Flow Bioreactor for Enhanced Adherent Cell Culture

Major Qualifying Project Submitted to the Faculty of WPI in partial fulfillment of the requirements for the Degree of Bachelor of Science

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Abstract

The biomanufacturing industry is a highly in-demand field, being valued at $13.3 billion in 2018, as it is utilized for the production of biologics such as monoclonal antibodies and vaccines in the treatment of diseases. Most of these manufacturing processes utilize suspension bioreactors, whereas there has been a slower development in large scale manufacturing involving adherent cell cultures. The current standard for adherent bioreactors is the utilization of microcarriers to provide a substrate for cellular adherence. However, the culture start-up process is inefficient and time-consuming, characterized by low attachment efficiencies and the generation of shear forces capable of detaching cells. This project aims to design a novel bioreactor system which supports adherent cell culture, while improving upon cellular adherence and volumetric efficiency. The final design incorporated a packed bed of microcarriers within a continuous pumping system, thereby promoting bead-to-bead migration and the recycling of unattached cells. The system was tested for proper continuous fluid flow, and the microcarriers were optimized to maximize surface area and cellular attachment potential.

Acknowledgements

Executive Summary

I.   Introduction

      A bioreactor is a manufactured device that is able to support an environment that allows biological functions to occur. Bioreactors can be utilized for specialized applications such as microbial fermentation and the production of biological molecules, such as vaccines, which require the functionality of healthy cells in order to be produced. The most fundamental principle of bioreactors is promoting robust cell growth at various densities by offering the ability to scale up or scale down in terms of working volume of cell media. This scale up process grants greater control and functionality in large-scale commercial manufacturing as well as in clinical trials and process development [1]. Within the biomanufacturing industry, suspension cultures are typically utilized in bioreactor processes due to their ease of scalability, allowing large cell densities to be achieved. In comparison, the translation of adherent cell culture into these larger biomanufacturing sizes has been more difficult since the presence and amount of surface area for cellular attachment is the main limiting factor.

      Cellular adherence is the process in which cells are capable of interacting with external biomolecules through focal adhesion complexes (FACs), large transmembrane protein complexes that allows the cytoskeleton of a cell to connect to the extracellular matrix. These FACs typically involve integrins on the cell surface. Adherent cell culture aims to promote this attachment in order to support adherent cells. For example, a protein commonly found in cell culture media is fibronectin, which is capable of binding integrins and FACs. In culture, these proteins found in media are capable of adhering onto cell culture surfaces, enabling cells over time to adhere and slowly spread out through interactions with the proteins [2]. As such, adherent cell culture focuses on the utilization and optimization of substrates in order to maximize cellular adherence and achieve the highest product yields in the smallest volume possible. When characterizing adherent bioreactor systems, attachment efficiency and cellular viability are important criteria to consider.

      It has been highlighted that with current microcarrier systems in place, attachment efficiency is an issue due to the repeated shear forces applied during the culture setup which is

capable of ripping cells off of the microcarriers. This occurs in the current bioreactor technology which combines microcarriers with stirred tank bioreactor systems. The initial culturing process utilizes intermittent stirring; however, this stirring combined with the impeller is capable of generating repeated shear stresses. As demonstrated with rabbit mesenchymal stem cells, GE Cytodex microcarriers have had as low as 16.7% of the total amount of inoculated cells attached to the microcarriers, and even after further optimization, only 65% attachment efficiency was observed [3]. Improving these attachment efficiencies for adherent bioreactors would yield higher cell densities, and ultimately, increase the capabilities of biomanufacturing scalability. As such, the overall goals of this project are to break down the design and operation of current adherent cell culture technology, and fabricate a novel bioreactor design capable of increasing adherent efficiency and volumetric efficiency with the potential for pharmaceutical and industry translation.

II.   Device Design

Design Objectives
During the design process, the team broke down the initial design objectives into more in-depth needs and wants, and then used a pairwise comparison chart to produce the following ranked objectives for the bioreactor system:

1. Non-disruptive to cells
2. Volumetric Efficiency of the Bioreactor
3. Promotion of Maximum Confluency

Functions and Means
Afterwards, the team brainstormed various functions to address these objectives. Further research was done on each function to find different means of achieving each function. A Functions/Means table was created as displayed below in Figure ES1:

Design Functions	Potential Means		
Fluid flow & Nutrient Distribution	Pumping	Impeller	Vessel shape
Oxygenation	Continuous perfusion	Stirred system w/ oxygenator	Pumping
Homeostasis	Biocompatible materials	Biocompatible coating	Incubator Compatible
Seeding method	Static method	Intermittent method	Perfusion system
Sterility maintenance	O-rings	Filtration	Threading Connections
Adherent Cell Culture	Microcarriers	Scaffold	Packed Bed
Adherent material	Cytodex (Dextran)	Glass (borosilicate)	Polystyrene

Figure ES1. Functions-Means Table

Alternative Designs

The team generated several alternative designs that would be able to accomplish these objectives, as shown below.

Stackable Scaffold Bioreactor

This design consisted of a stackable scaffold which would be placed inside the bioreactor vessel. The stackability of the scaffold allows for different scalability options, as more or less scaffolding could be stacked depending on the desired size of the bioreactor. The impeller system which is typically found in stirred tank bioreactors is replaced with a pumping mechanism that would pump the culture media from the bottom of the bioreactor to the top, thereby passing media, nutrients, and oxygen through the scaffolds for the attached cells. This design can be seen below in Figure ES2.
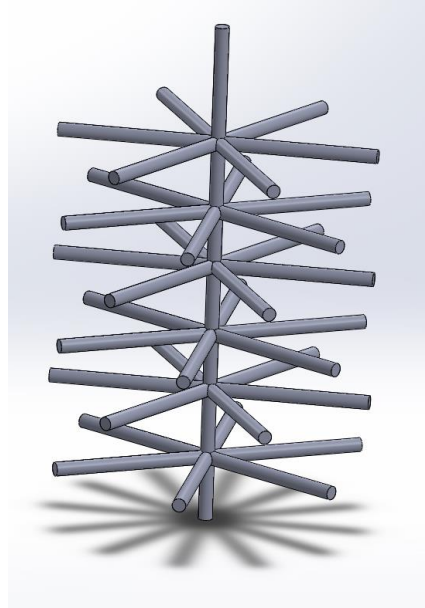
Figure ES2. Alternative Scaffold Bioreactor Design

Packed Bed Bioreactor

This design focuses on addressing cellular adherence in addition to volumetric efficiency with the utilization of a packed bed of microcarriers. The typical impeller system in the current Cytodex system is replaced with a continuous pumping mechanism, where the media continuously travels through this packed bed of microcarriers, so that cells that are unable to attach are looped back into the beginning of the system in an attempt to seed them onto the beads. The replacement of the impeller system helps to reduce shear forces and provides a method for nutrients and oxygen to be constantly delivered to cells seeded within the packed bed of microcarriers. The packed bed itself enables an increase in available surface area for cellular adherence since many microcarriers can be packed together within a smaller volume in comparison to the Cytodex system. In addition, cellular bead to bead migration is possible due to the dense packing of the microcarriers, further enabling confluency and cellular proliferation to achieve higher amounts of cell densities. This design can be seen below.
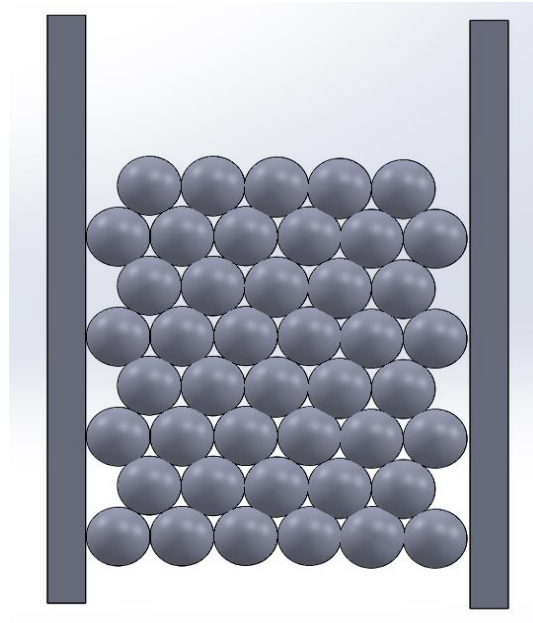
Figure ES3. Packed Bed Design

Interlocking Microcarriers

This design involved maintaining the current stirred tank bioreactor system that is utilized in the Cytodex system, while focusing on increasing the potential surface area for cellular adherence and proliferation through interlocking microcarriers. One main issue with the current Cytodex system is that there is a lack of bead to bead cellular migration, and as such microcarriers which were not seeded with cells initially will likely never be able to contain any cells since the attached cells are confined only to the bead they initially attached to. As such, this design incorporates the utilization of polarized glass beads, half of which are positively charged and the other half which are negatively charged. While the initial intermittent seeding method will still be utilized, as the microcarriers are mixed within the bioreactor, the polarized charges will enable the positively charged beads to become attracted to the negatively charged beads. This will increase the available surface area for the initial seeding phase, while also enabling cells to migrate between microcarriers, which will also increase the available surface area for cellular growth and confluency.

III.   Final Design

The final design of our bioreactor system consisted of three major components: The reactor, a one-liter media flask, and a pump. The reactor itself consists of the outer reactor shell and a middle insert in which the beads are packed. The shell is comprised of two end pieces into which a borosilicate glass tube is press-fit and sealed with rubber O-rings. The end pieces are internally tapered to allow for even fluid distribution, and are connected to the larger system through nipple ports on either end. The middle insert is a hollow, cylindrical, double threaded tube with two steel meshes of 200-micron porosity on either end, which are secured in place by threaded caps containing rubber O-rings to create a watertight seal. The beads are packed between the meshes, and the insert is then placed within the glass tube before it is fit into the end pieces. This packed bed design is a significant component of the bioreactor system as it allows for large amounts of surface area to be achieved in smaller bioreactor volumes, making the system more volumetrically efficient. Additionally, cellular bead to bead migration will be promoted, thereby allowing for a greater utilization of the available surface area for cellular adherence.

In order to allow for proper fluid exchange and media oxygenation, the one-liter media bottle is outfitted with a ChemGlass^{TM} three port assembly cap, to which the reactor tubing will be connected. The pump used is an E-series Manostat Peristaltic Pump, with a non-adjustable flow rate of 56.8 milliliters per minute. This pumping mechanism will allow for reduced shear stresses in the system since it replaces the impeller system used in the initial culture process. In practice, the system is assembled with the media bottle and reactor being placed within an incubator, with the pump being connected externally through rubber stoppers placed through the incubator walls. The entire system is displayed in Figure ES4 below.

Figure ES4. Final Bioreactor system design

IV.   Results and Discussion

We performed several verification tests including testing the fluid flow of our system and performing calculations to confirm that the shear stress created in the system is low and that the bioreactor is capable of supporting the oxygenation requirements of the cells. These results indicated that continuous flow is maintained within the system, and that the peristaltic pump with its constant flow rate of 56.8 mL/min is capable of delivering enough oxygen for the maximum possible number of cells in the system, while also generating shear stresses that are low enough that they will not negatively impact cellular morphology or behavior. After assembling the novel bioreactor system, a comparison study would have been run between a current state of the art adherent bioreactor and our novel bioreactor by running the two systems simultaneously for two weeks. Throughout the culture period, measurements of key nutrients and metabolites would have been performed to verify cell viability. A final cell density test would have involved image analysis to obtain comparative measurements of the cell concentration on the Cytodex and glass beads. The nutrient/metabolite assays would have determined the levels of Glucose, Glutamax, Glutamate, Lactate, Ammonia, and Lactate Dehydrogenase in the medium.

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From the results of the static culture experiment, it would be expected to see bead-to-bead migration in our bioreactor, which is not achieved in the current state of the art. This could have resulted in higher cell densities for the glass beads compared to the Cytodex. The expected result of the nutrient test is an exaggerated trend line for our novel bioreactor in comparison to the adherent intermittent stirring system. This variance would be due to faster nutrient depletion and waste generation that corresponds to the higher cell counts. Overall, we were able to achieve bead-to-bead migration in static culture, confirm continuous fluid flow of our system, and calculate a 6.5-fold increase of volumetric efficiency from our novel bioreactor system with the utilization of glass beads.

V.   Conclusions and Recommendations

Several objectives were met through the process of fabricating the final design and verifying its functions. First, bead to bead cellular migration was achieved within static culture, and as such this same result would be expected if translated into the packed bed bioreactor system. This would meet the objective of promoting maximum confluency by allowing cells to migrate onto microcarriers which previously did not contain any cells from the initial seeding process. Second, continuous flow was achieved within the bioreactor. This continuous flow is important because it would theoretically help to meet the objective of increasing attachment efficiency through the recycling of unattached cells within the closed-loop system. Third, volumetric efficiency was demonstrated with the novel bioreactor as calculations highlighted that our packed bed bioreactor achieves a 6.5-fold increase in volumetric efficiency in comparison to the Cytodex microcarrier system. This is significant as it would allow for higher cell densities to be achieved in lower amounts of volume.

Going forward, several recommendations can be made regarding the design and functionality of the bioreactor. First, due to COVID-19 the team was unable to perform the suggested comparison study between the novel bioreactor and the current microcarrier system, and so this should be performed in order to compare the achievable cell densities. Additionally, the middle insert could be modified so that there is a see-through window within the design, allowing users to directly visualize the packed bed and ensure that it is stable. In terms of

optimizing the bioreactor design, it would be ideal to make the reactor incubator-independent like most current benchtop bioreactors. This would involve adding a thermal jacket for temperature regulation, an oxygen supply for proper culture oxygenation, and nutrient supply for the system. The reactor may also be modified to interface with process control sensors like current benchtop bioreactors so that temperature, pH, and oxygen can be directly measured. Additionally, the scalability of the bioreactor should be confirmed with the manufacturing and testing of larger-sized models to ensure that in the end, this bioreactor design will be capable of supporting larger biomanufacturing processes.

References

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Chapter 3	**All**	**All**
Chapter 4	**All**	**All**
Chapter 5	**All**	**All**
Chapter 6	**All**	**All**
Chapter 7	**All**	**All**
Chapter 8	**All**	**All**

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Chapter 1: Introduction

The general need that is being addressed with this project is the design and functionality of bioreactors in the current biomedical industry, more specifically those engineered towards adherent cell culture. A bioreactor is a manufactured device that is able to support an environment that allows biological functions to occur. The main priorities are to support cell survival along with tissue structure, organization, mechanical properties, and function. The most fundamental principle of bioreactors is allowing robust cell growth at various densities by offering the ability to scale up or scale down in terms of working volume of cell media. With this, bioreactors can be utilized for specialized applications such as microbial fermentation and the production of biological molecules, such as vaccines, which require the functionality of healthy cells in order to be produced. This scale up process grants greater control and functionality in large-scale commercial manufacturing as well as in clinical trials and process development [1].

As such, the optimization of adherent bioreactors is a significant aspect in improving yield and productivity. In terms of current bioreactors on the market, the micro-reactor scale is anywhere from 15 mL to 10 L and the large scale is typically 10,000 to 25,000 L; however, most of this is applicable only for stirred-tank bioreactors aimed towards suspension cultures. In many cases, each aspect of the reactor must change in order to accommodate larger volumes. Such components include the impeller, which is the primary stirring mechanism of a reactor. It typically consists of a set of blades attached to a motor that is capable of exerting the necessary force and rpm to thoroughly mix the bioreactor medium. Other components include the sensory systems which monitor pH, oxygen levels, and other nutrients, as well as gas exchange components which bubble essential gasses into the reactor chamber, and fluid flow paths which allow for nutrient and substrate exchange. The most important aspects of a bioreactor are the ability to provide proper oxygenation and nutrient exchange for the cells in the environment in order to maintain homeostasis and optimize their growth. Current bioreactor systems for adherent cells utilize microcarriers, small beads or disks which provide an attachment surface for adherent cells to anchor on, helping to increase their viability [2]. As such, when characterizing adherent bioreactor systems, attachment efficiency and cell viability are important criteria to consider. Unfortunately, upon interviewing with a client in the biomanufacturing industry, it was

highlighted that with current microcarrier systems in place, attachment efficiency is an issue due to the repeated shear forces applied during the culture setup which is capable of ripping cells off of the microcarriers. In fact with rabbit mesenchymal stem cells, certain microcarrier products have had as low as 16.7% of the total amount of inoculated cells attached to the microcarriers. And even with optimization of the attachment conditions, only an attachment efficiency as high as 65% was observed [3]. Improving these attachment efficiencies for adherent bioreactors would help to yield higher cell densities, and ultimately, increase the capabilities of biomanufacturing scalability.

The overall goals of this project are to break down the design and operation of current adherent cell culture technology, and fabricate a novel design capable of increasing adherent efficiency and cell viability for the purposes of optimization with the potential for pharmaceutical and industry translation. In essence, this requires understanding the fundamental function of the bioreactor and its many physical and theoretical aspects that contribute to the fluid dynamics which govern mixing in the system. The design has to incorporate these multidisciplinary factors in order to generate a novel system capable of improving upon current microcarrier systems. After the creation of a specific design, this novel system will have to be tested in comparison to current microcarrier systems in order to determine whether the attachment efficiencies and total cell densities are improved in the experimental bioreactor. Another important aspect to consider as well is the potential for scalability. Many bioreactors in the biomanufacturing industry are designed so that operations on a bench-top bioreactor can be translated into a large-scale manufacturing process. As a result, the potential for scalability is a factor as well, as the design of this novel system must be streamlined enough so that it is reproducible on any scale.

In order to complete the overall goals for this project, a general strategy was utilized which encompasses several phases of design and ideation. First, essential background research into literature about the design and functionality of bioreactors was conducted, especially in the context of adherent cell cultures. This knowledge on the fundamentals of bioreactor operation was then translated into a better understanding of current microcarrier systems on the market and their mechanisms for cell attachment. One of the key aspects was the fact the success of adherent

cells is dependent on oxygen and nutrient transfer, fluid flow, and the mechanical forces imparted by the flow.

The next step was the design aspect of our project. Once thorough research was conducted, we started designing the bioreactor and its different components. This included prototyping with dimensions, materials, and the various components required to create a fully functional bioreactor capable of supporting cellular homeostasis.

Once a design was chosen for prototyping, various verification experiments were performed in order to test cellular attachment and fluid flow. These were essential components which must be integrated into the bioreactor system to ensure that the adherent cells were capable of attaching, growing, and maintaining homeostasis without any disruptions. Verification of attachment was important as this established the type of materials which was used within the bioreactor. Meanwhile, the verification of fluid flow helped to ensure that proper laminar flow is imparted by the system so that shear stresses did not mechanically disturb the cells in the system. Optimization of these aspects ensured that the final design of the bioreactor was fully optimized. In order to actually test the final product, there were several metrics and benchmarks that were compared. Cellular growth and nutrient depletion curves were compared between control bioreactors and the experimental bioreactor. This was done in order to ensure that cellular function and viability was improved, if not at the very least not negatively affected, by the novel design. For example, the doubling time for human dermal fibroblast cells is approximately 24 hours, and so it is expected that these numbers either remain the same or are improved between the control and test bioreactor [4]. By utilizing these various metrics and benchmarks, the quality and feasibility of the team's designed component could be assessed and analyzed.

The succeeding chapters of this report will attempt to provide a detailed account of the MQP project. It will begin with a literature review in which the current state of the art of bioreactors will be identified and evaluated, its limitations analyzed, and potential modifications noted as key focal points of the design process. The report will then provide an overview of the project strategy in which the methods and timeline for the project will be laid out. This will be followed by an in-depth account of the design process, including a concrete set of design constraints, associated parameters, preliminary ideas, and the prototyping process. It will then

lead into the final design and validation, before carrying onto a discussion of the methods, procedures, processes, and performance of the final design. The report will conclude with a retrospective on the entirety of the project, while providing recommendations for future efforts in related fields.

Chapter 2: Literature Review

2.1 Cell Culture and Bioreactors

Bioreactors are commonly defined as a device in which biochemical processes occur where the process can be closely monitored and the environment is able to be tightly controlled and observed. They are often translated from benchtop production to industry use due to their ease of reproducibility. This reproducibility is made possible because the monitoring and automation control technologies are similar in both scales and must simply be replicated to the desired scale [5]. The bioreactor plays a crucial role in cell culture in order to provide an enhanced environment for tissues to thrive and grow [6].

Bioreactors allow for an environment in which cells are able to be cultured, which is why it is important for the seeding of these cells to be accurate and successful. There are a variety of ways in order for cells to be seeded in a bioreactor and the technique can affect the properties of various materials that may be used in the bioreactor [5]. There are two main varieties of bioreactors, which are known as stirred-tank bioreactor and packed-bed bioreactors. Stirred-tank bioreactors provide an environment where the cells are cultured while in suspension with a constant agitation due to an impeller. This type of bioreactor is constantly in motion, which can lead to effects of shear stress on cells. The constant motion allows for cells to flow freely in the agitated media, creating a homogeneous mixture of cells and nutrients. This type of bioreactor is typically used for non-adherent cells. The utilization of an impeller within the system disrupts the bubbles that are released from the air sparger in order to control dissolved oxygen in the system. Additionally, the impeller is typically set at a 45-degree angle in order to provide both radial and axial mixing, while the pitched blade decreases the shear stress that could affect sensitive cells [7].

The packed-bed bioreactor is a system in which cells are placed in an environment that is comparable to *in vivo*. The size and shape is similar to that of the stirred-tank; however, there are different mechanics in order to produce a homogeneous environment. The packed-bed system is typically used for adherent or anchorage-dependent cells. The packed-bed is able to provide protection of the adherent cells from the shear forces experienced in a bioreactor while simultaneously allowing oxygenated media to flow through the bed [7].

There are three main categories of bioreactors in order for nutrients to be introduced and maintained in the environment. The first category is a batch system where cells remain in a fixed volume of media. In this system the nutrients introduced into the system are gradually consumed and as they are consumed, by-products begin to form. The environment is continuously changing due to the formation of by-products, and the media is not changed until the end of the entire run. This system is easy to operate and there is a low risk of contamination because there is no disturbance to the environment throughout the entirety of the process. However, the disadvantage is that these systems produce the lowest cell densities compared to the other two systems [8].

Fed-batch is slightly modified from the batch system and is very common in the bioprocess industry. In this system there is a set time for cells to grow and there are various increments of time where nutrients are added throughout the process. The culture is not collected until the entirety of the run similar to the batch system. Due to the fresh nutrients being added continuously, there is a large amount of biomass at the end of the run; however, because the cells are not being overfed there is not much by-product [8].

Continuous bioreactor systems have fresh medium continuously added, while in parallel, used medium as well as cells are harvested. The volume is able to stay constant because the addition and subtraction of the same volume of medium occurs at the same time. Scale-up of this system is simple because the working volume remains constant. However, this system may introduce complications with sterility due to the constant addition and harvesting that may lead to exposure to the outside environment [8].

Although there are various types of bioreactors, in the end the bioreactor system needs to be optimized so that shear effects are minimal, sterility is not compromised and cell densities are increased in order to achieve the highest possible efficacies.

2.2 Adherent Cells - Process and Criteria

Adherent cell culture is a rapidly expanding field for both research and commercial purposes. Adherent cells are especially important for tissue engineering applications, as the vast majority of organic tissue is composed of adherent cells. In nature, adherent cells grow and proliferate on complex biomaterial scaffolds composed of compounds and proteins which form the extracellular matrix. In culture conditions, the cells must be given an artificial, stable surface upon which to grow and proliferate.

In the biomanufacturing industry today, the vast majority of cell cultures involve non-adherent cells that are cultured within suspension systems. This is due to the fact that the yield and survivability of adherent cells is limited by the available solid surface area within a culture or reactor. This introduces significant issues in terms of scalability and production efficiency. Additionally, many of the adherent cells desired for research and commercial purposes are very delicate and difficult to seed, culture, and harvest. Such cell types include endothelial, skeletal muscle, and stem cells. Much of the current industrial and academic adherent cell culture interest revolves around stem cells, as they are self-renewing, have remarkable ability to differentiate into many different cell types, and have become a significant part in the generation of therapies for many diseases/illnesses [9]. Stem cells show promise in major advancements in the cell culture industry, however, they are associated with several difficulties. They are fragile, costly, and difficult to maintain in high volumes. With this said, a high cell viability and yield is a necessity in taking advantage of the cells' shorter lifespans.

 In order to increase cell viability and efficiency in the bioreactor, cells are attached to small microcarriers, which increases the total surface area cells are allowed to grow on. Cell adhesion in these systems is achieved through a long, tedious process. In this process, cells and microcarriers are mixed together into a homogeneous mixture. The mixing inside the bioreactor is then turned off, allowing all products inside the bioreactor to settle to the bottom. Cells then are given time to adhere to the microcarriers. The time given to the cells to allow them to adhere to the microcarriers differs depending on cell type.

The difference in adhesion time can be contributed to the extracellular matrix (ECM) of the cell. The ECM of a cell contains many different adherent molecules and peptides. These molecules are typically negatively charged, allowing them to adhere easily to positively charged surfaces [10]. Cellular adherence is the process in which cells are capable of interacting with these external biomolecules through focal adhesion complexes (FACs), large transmembrane protein complexes that allows the cytoskeleton of a cell to connect to the extracellular matrix. These FACs typically involve integrins on the cell surface. Adherent cell culture aims to promote this attachment in order to support adherent cells. For example, a protein commonly found in cell culture media is fibronectin, which is capable of binding integrins and FACs. In media, these proteins are capable of coating cell culture surfaces, enabling cells over time to adhere and

slowly spread out [11]. The timing of adhesion depends on how quick the cell can extend their ECM and adherent molecules onto the point of attachment.

Once the cells are given time to adhere, the mixing is turned back on and the process is repeated many times in order to cover as many microcarriers as possible with cells. During re-agitation, and mixing, cells experience shear stresses from the media being passed by. These shear stresses can cause cells to detach from the microcarriers rendering the cells useless during after that time. This is a widely used process and has been said to be inefficient by researchers due to the time it takes and the overall low cell adhesion associated with it.

2.3 Current Microcarrier Systems and Materials

In today's market, one of the largest trends in the biotechnology industry is the development and optimization of cell culture technologies in order to produce the highest possible yield, whether this is cells or products made from cells, at the lowest possible labor and financial costs. In terms of adherent cells, mammalian cells are commonly used in industry, however, it should be noted that most animal cells typically have lower yields in comparison to other types of cells. As such, larger culture volumes are typically required in order to produce higher amounts of product, while scale up is difficult for adherent cells in traditional culture plates. Due to this, the utilization of microcarriers have been developed, creating a practical, high-yield culture of adherent cells, enabling higher cell densities to be accommodated in lower amounts of volume [12]. By increasing the capabilities for production while requiring less space than typical monolayer cell cultures, microcarriers provide a more efficient adherent cell system and enable lower labor costs.

Microcarriers are designed as spherical, micrometer-sized carriers wherein adherent cells are capable of adhering and forming monolayers. These carriers typically range in diameter from 10 μm to 5 mm, with the ideal size being considered as 100 to 300 μm [12]. Other forms of microcarriers come designed with pores, thereby enabling cellular penetration and theoretically a higher surface area for more cell adherence. There are some challenges associated with porous microcarriers, however, such as the increased difficulty of cell harvesting [13]. Microcarriers are often utilized in stirred suspension bioreactor or micro reactor systems, as stirring enables a homogeneous culture environment around the carriers, ensuring that cells are uniformly getting proper aeration and nutrients while also disrupting any microcarrier aggregation that may occur.

These culture systems can include cultures with a constantly stirring impeller, shaker flasks on rocker platforms, or with smaller flasks, a magnetic stirrer system to drive the impeller [13]. One limitation with microcarrier systems is the fact that it is slightly harder to quantify the cell density and the number of viable cells, as this would involve having to detach the attached cells, which would not be ideal until the end of the culture process. As such, there has been the development of indirect quantification techniques which involve measuring metabolic activity markers, such as glucose consumption or lactic acid production, or the concentration of enzymes such as lactate dehydrogenase [13].

Various materials are utilized for microcarriers, ranging from natural polymers such as collagen and dextran to synthetic plastics and silicone rubber or even glass. Currently on the market, one of the biggest sources of cell culture microcarriers is GE Healthcare with their Cytodex microcarriers. One product, the Cytodex 1 microcarrier, is made from cross-linked dextran, is positively charged, and has an average diameter of 190 μm [12]. One promising alternative to the utilization of natural polymers is glass, as much research has focused on demonstrating comparable results between glass carriers and dextran or plastic microcarriers. Glass has already been researched as a promising cell culture substrate, with the material now being utilized in various cell characterization processes such as centrifugation assays to determine attachment strengths [14]. As such, the transition to microcarriers is not an unexpected development. Although there is currently not a glass microcarrier product out on the market, much research is being conducted to examine its feasibility and potential advantages in comparison to polymer-based systems, with one study demonstrating the biocompatibility and feasibility of facilitating controlled cell growth on glass microcarriers [15].

In terms of the optimization of microcarrier culture, one of the most important aspects is the beginning stirring process involving the attachment of the cells onto the carriers. The typical process utilized in the industry currently is an intermittent stirring process, which involves culturing the cells and microcarriers at a slightly lower volume than the final intended volume. Afterwards, stirring through the impeller will occur over a small period of time at frequent intervals, with "down periods" in between each stirring session where the microcarriers and cells will settle to the bottom of the culture. These "down periods" enable the cells to physically attach to the microcarriers, while the stirring phases help to ensure homogeneity in the environment [13]. Once the attachment process is completed, continuous stirring will occur as normal in a

stirred culture system, although the specific culture process and impeller parameters will depend on the cell line.

2.4 Current Limitations

Several limitations exist with the current adherent bioreactor system that utilizes Cytodex microcarriers in conjunction with an intermittent stirring process. First, research has found that the utilization of these microcarriers generally leads to low attachment efficiencies. For example, research with rabbit mesenchymal stem cells demonstrated that GE Cytodex microcarriers have had as low as 16.7% of the total amount of inoculated cells attached to the microcarriers. Even after further optimization of the attachment conditions, only an attachment efficiency as high as 65% was observed [3]. Attachment efficiency is an extremely important parameter within adherent cell culture because it is desirable to maximize the amount of cells capable of attaching and proliferating within the bioreactor system when the system is inoculated. Having low efficiencies will result in a waste of resources due to large amounts of cells dying and not being utilized due to the inability to adhere onto the substrate within the system. In the end, this will result in economic loss as well as the need to utilize larger bioreactor systems to achieve certain final density yields when smaller systems could potentially be used instead if the attachment efficiency was higher.

This limitation is corroborated when interviewing Chris Bellerive from WPI's Biomanufacturing and Education and Training Center. Our interview with Chris can be found in Appendix A: Interviews with Chris Bellerive. Essentially, Chris highlighted that low attachment efficiencies are typically observed, and a large reason for this is the amount of shear stresses generated during the intermittent stirring process. Additionally, although the stirred tank system enables homogenous mixing of microcarriers in the media, this constant stirring creates dead space within the system so that it is extremely difficult for microcarriers to come into contact with one another. Due to this, there is a lack of cellular migration between microcarriers, and so microcarriers that were not initially seeded with cells will not be able to support any cells since they are all confined to the initial microcarriers they were seeded on. This is a reason why the current adherent bioreactor system is volumetrically inefficient. Chris highlighted as well the long process of the initial culturing stage, where the intermittent stirring process is time-consuming and requires constant manpower to operate and monitor. As such, a potential need to

be examined is the development of an enhanced bioreactor system aimed at increasing the overall attachment efficiency in order to better optimize the cell culture process. Doing so will allow for larger cell densities to be achieved with smaller bioreactor volumes, helping to decrease the time and labor costs associated with bioreactor operations.

2.5 Surface Modifications

When examining and optimizing adherent cell culture, an important factor to consider is the promotion of cellular attachment within the system. Various techniques have been studied and well-characterized in the optimization of cellular adherence in order to improve cell densities. One such technique is the utilization of oxygen plasma treatment in order to increase cell adherence. Plasma treatment is considered a "cleaning" technique where a gas is electrically charged with electrons moving freely, resulting in a partially ionized state. The mixture of atomic and molecular ions, neutral atoms, and electrons can interact with a surface, thereby initiating the plasma treatment. Studies have focused on the utilization of oxygen plasma treatment in the promotion of cellular adherence. For example, this technique has demonstrated the ability to modify the surface properties of Polydimethylsiloxane (PDMS), a common silicone-based polymer that is commonly found in microfluidic cell culture. More specifically, the application of oxygen plasma treatment changed the surface properties of PDMS to become more hydrophilic when its surface is typically hydrophobic [16]. This surface modification is important as it has demonstrated the promotion of increased cellular adherence and growth on silicone surfaces, making it a potential technique to be considered when optimizing adherent cell cultures [17].

Another surface modification that has been characterized is the utilization of collagen as a surface coating to promote cellular adherence. Collagen is one of the most abundant proteins associated with the extracellular matrix *in vivo*, making it an excellent candidate to be examined due to its natural role in cellular adherence through cell-surface interactions. More specifically, it has been demonstrated how collagen surface coatings can be utilized in the modification of surfaces in order to promote high levels of cell attachment, proliferation, and even survival when the cells are subjected to stress [18]. The utilization of ECM protein coatings is an important modification to be considered when optimizing culture conditions for maximal cell adherence.

Furthermore, gelatin coating is another cost-effective method to increase cell adherence, as it binds ECM proteins such as fibronectin quite effectively, as well as mimics the pliable surfaces that adherent cells grow on in nature [19]. Additionally, gelatin is an abundant, naturally occurring material, making it highly biocompatible, and as a result, it is commonly used for surface treatment in laboratory adherent cell culture. Its most common applications typically involve endothelial cell culture; however, it is expected that it can facilitate the growth of a wide variety of adherent cells [20].

Chapter 3: Project Strategy

The goal for this project is to design a novel bioreactor system capable of increasing adherent efficiency and final cell densities. In this chapter, the client statement will be broken down into various objectives, design requirements, and specifications to guide the fabrication process of the novel system. Afterwards, the project strategy and timeline will be discussed.

3.1 Initial Client Statement

The initial client statement that was given to the MQP team was to design one aspect of a micro-bioreactor that will be used for rapid screening and process optimization and development activities. As shown, this initial statement is open-ended and vague, with no specifics in terms of what aspects or components of the bioreactor to focus on for technological improvement. In addition, there is no specification on the end objective of the team's design besides potentially being utilized in optimization and process development. As such, this will require the team to conduct background research into the fundamental bioreactor processes and their utilization within industry in order to gain a better perspective and scope in developing project objectives.

3.2 Design Requirements

This section will discuss the design objectives, constraints, and specifications regarding the goal to increase the cellular attachment and final cell densities of adherent bioreactors.

3.2.1 Design and Project Objectives

Given the initial client statement, literature review and background research with industry experts was conducted in order to understand the current limitations within bioreactor systems. The team decided to focus on adherent bioreactor systems and their limitations, as discussed in Chapter 2: Literature Review. More specifically, the overall goals for this project are now to fabricate a bioreactor design capable of increasing adherent efficiency and optimizing the necessary bioreactor system so that smaller volumes can be utilized. A revised client statement embodies these new goals, as detailed in Section 3.4 Revised Client Statement.

As such, several design objectives can be identified which captures the general scope of the project and its goal to contribute to the technical improvement of adherent bioreactor processing. These main objectives are listed below.

List of Design Objectives

1. Improvement of cellular attachment on the bioreactor substrate
2. Improvement of the bioreactor's volumetric efficiency

To further expand on these, the first main objective details how the design must be able to improve seeding efficiencies in comparison to the current microcarrier technology, which in turn will allow for higher final cellular yields. The second main objective is to address the issue of surface area being the limiting factor within adherent bioreactors, thereby creating a system that is volumetrically efficient so that higher cell densities can be achieved in smaller bioreactor volumes.

The process for meeting the needs of the client and achieving the design objectives can be accomplished through several project objectives which encompass the entirety of the project from ideation to fabrication and experimental verification. A list of the project objectives is presented below.

List of Project Objectives

1. Determine best microcarrier material for cell attachment
2. Verify shear stresses, oxygenation mechanisms, and volumetric efficiency
3. Develop strategies to fabricate a functional device
4. Test functionality and compatibility of novel design in comparison to current adherent system

First, the team determined the best microcarrier material on which the cells inside the bioreactor system will adhere to throughout the entirety of the process. This aspect consisted of testing various materials as surfaces for the cells to adhere to, as well as various surface treatments on the material to obtain successful cell seeding. This objective is important because the adherent cells require proper attachment to a surface during the cell culture process in order

to avoid cell death. In order for the cells to grow in the environment they must be adhered to some material and not free-floating in suspension, and so optimization of the microcarrier technology helps to improve cellular attachment and the maximum number of cells supported by the system. Additionally, the current bioreactor process involves an intermittent stirring process that requires manpower and must be monitored. The process is not only time-consuming, but could potentially cause problems due to the inherent shear stresses of the intermittent stirring that could rip off any adherent cells from the microcarriers, thereby reducing the attachment efficiency and the number of cells capable of growing and proliferating in the bioreactor. Increasing the attachment efficiency involved understanding the intermittent stirring mechanism and potential alternatives that would help to achieve proper nutrient and oxygen distribution while mitigating the potential for cell detachment during the culture process.

Afterwards, oxygenation within the novel system needed to be verified using various equations that proved proper oxygenation of the cells is achieved in this environment. In addition, the shear stresses imparted within the system needed to be verified to ensure that they are not large enough to negatively impact the cells or detach them from their surface. These systems needed to be validated before the system was built in order to ensure that it would function properly. Additionally, the bioreactor system needed to be characterized in terms of the possible number of cells supported per unit volume, as this is indicative of volumetric efficiency.

Third, strategies needed to be developed in order to fabricate a functional device. This involved research into the types of materials typically utilized in bioreactor systems and those which are biocompatible, meaning that they are not cytotoxic and do not induce any cellular death upon interaction with the cell culture. Finally, the final design will be tested within the bioreactor setting in comparison to the current adherent system in order to test its functionality and gain quantitative data on its impact on cellular growth or viability. This is an important objective because it will highlight if the novel design created by the team was capable of improving the adherent cell culture process in the context of bioreactors by streamlining the culturing process and enhancing attachment efficiency. These objectives provided a general framework for the team to work within, as accomplishing each goal put the team one step closer towards the final design and validation of the final product.

3.2.2 Design Constraints

When designing our novel bioreactor there are a few constraints that we must abide by that alter the process of our project. These can be broken down into two separate categories: incubator-based constraints and pump-based constraints. In terms of incubator-based constraints, this reactor must be able to function within an incubator as the incubator is the main source for maintaining the environmental conditions which support cellular homeostasis. Although the bioreactor may be designed in the future to be incubator-independent, currently the design will be incubator-dependent in order to forgo the utilization of a bioreactor process controller. As such, the bioreactor must be able to fit within the 18 inches of shelf space in the incubator provided to the team in Goddard Hall 006. Additionally, this incubator must have specific conditions in order to support fibroblast cells in the reactor. This includes maintaining environmental conditions at a temperature at 37°C ± 0.5°C, 5% ± 0.5% carbon dioxide levels, and a humidity greater than 90%.

Additionally, the bioreactor design will have constraints due to the pump which will be utilized in the designs (this pumping mechanism and rationale is expanded upon in Section 4.2.1 Conceptual Design Modifications). The pump that is used must be maintained outside of the bioreactor due to the high humidity which may be capable of interfering with the pumping system. Additionally, the pump to be provided is non-adjustable and set to a constant flow rate of 56.8 mL/min. Therefore, the final design must work within these constraints in order to ensure that the novel system is capable of running and ensuring the cells in the bioreactor are able to grow and proliferate.

According to the Worcester Polytechnic Institute MQP guidelines, each team receives a budget of two-hundred and fifty dollars per team member. This makes our total budget one thousand dollars to complete our project task. Another constraint that we have is time. All MQPs must be completed by the end of the academic year so we must finish our project by May 2020.

3.2.3 Functional Blocks

The components of an adherent bioreactor can be broken down into various functional blocks, each representing a specific operational aspect of the bioreactor cell process which contributes to its success. First is the aspect of cell attachment, which is essential for any

adherent culture since these specific cells require a substrate to adhere onto in order to properly grow and proliferate. In the current adherent systems, microcarriers accomplish this by providing a potential surface for cellular anchorage. Another important facet of cell attachment is the process with which this occurs as well. As described in the literature review, the current process entails intermittent stirring to allow cells to settle onto a bottom layer of microcarriers before being mixed. This process is essential to cellular attachment, and thus modifications to this will potentially impact the efficiency of cellular adherence.

Another functional block to be considered is cellular confluency. Once the cells are seeded onto the microcarriers, their confluence on the beads will help to determine final cell densities and viabilities. Ideally, all microcarriers within the system would be 100% confluent, allowing for the maximum possible number of cells based on surface area. Unfortunately, not all beads once harvested achieve 100% confluency in the current Cytodex system. In fact, many beads do not contain any cells at all due to the fact that cells were unable to be seeded onto them during the initial seeding process. With confluency being inherently related to the final cell densities, modifications can be made to help improve this, such as increasing potential surface area for cellular attachment or enabling a process of bead to bead migration, where cells are capable of growing and dividing from one microcarrier onto another.

Both of these functional blocks will be considered during the initial ideation process since modifications can be made to any of these components in an attempt to improve overall adherent cell culture performance.

3.2.4 Design Functions and Means

When creating our bioreactor, it is important to consider a variety of design functions inherent to the functional blocks described above in Section 3.2.3 Functional Blocks to ensure the success of the product. Table 1 presents a Design Functions-Means table and the content of this chart will be discussed in the following paragraphs. Since bioreactors directly interact with living cells, the materials and design of its components need to be biocompatible and promote the growth of these cells. In order to do this, our design must be able to replicate the many functions of a bioreactor without interfering with the typical systems in place that ensure cell homeostasis.

One function our design must be able to achieve is the delivery of nutrients to the cells in the system. This can be achieved by making sure the mixing process produces a homogeneous mixture where the medium can flow through all the cells constantly which ensures the proper distribution of nutrients and gases. This type of mixture and flow can be made possible by utilizing an impeller like the current Cytodex system, introducing a pumping mechanism that will pump the media through the bioreactor, or modifying the bioreactor vessel shape to enhance homogeneous mixing. Oxygenation is another function that needs to be considered, as enough oxygen must be provided to support the growth of the cells. Currently this is accomplished in the Cytodex system with the combination of an oxygenator and an impeller to distribute the oxygen. However, other methods to accomplish this may include a pumping mechanism where media is pumped into the bioreactor already containing the dissolved oxygen, or a continuous perfusion system can be used instead where fresh media containing dissolved oxygen is constantly supplied into the system.

Another function is the maintenance of the proper biological conditions for the cells in the bioreactors. In order to achieve this, a sterile environment must be maintained during all times. Many sterilization processes can be utilized to sterilize the environment before actual operation, such as autoclaving the bioreactor and its components, using radiation, or chemical means. In order to keep the device sterile over a period of time during its operation, O-rings and filtration systems can be utilized, in addition to using sterilizable materials in the design of the bioreactor system. Another function that the design must accomplish is to avoid introducing any toxicity to the cells in the bioreactor. This can be achieved by using biocompatible materials or by using biocompatible coatings on the materials chosen. Another aspect that needs to be taken into account is the stress imparted on the cells during the seeding process. Our bioreactor must be able to properly seed cells without inducing any additional stresses as this can negatively affect cellular behavior or cause the cells to lyse. The current Cytodex system utilizes an intermittent method, while other potential alternatives include a static method where the bioreactor system is shut down for a time period so that cells will be allowed to slowly settle onto the substrate, or a perfusion method that involves continuous movement of cells in media over the cell culture surface.

Lastly, our bioreactor system must be able to support cellular adherence. This can be accomplished with the utilization of microcarriers, although other alternatives which may

increase surface area include a packed bed design and the use of scaffolds within the reactor. The substrate material can also be modified, with potential materials including Dextran like the current Cytodex microcarriers, borosilicate glass and polystyrene.

Table 1. Design Function-Means Table

Design Functions	Potential Means		
Fluid flow & Nutrient Distribution	Pumping	Impeller	Vessel shape
Oxygenation	Continuous perfusion	Stirred system w/ oxygenator	Pumping
Homeostasis	Biocompatible materials	Biocompatible coating	Incubator Compatible
Seeding method	Static method	Intermittent method	Perfusion system
Sterility maintenance	O-rings	Filtration	Threading Connections
Adherent Cell Culture	Microcarriers	Scaffold	Packed Bed
Adherent material	Cytodex (Dextran)	Glass (borosilicate)	Polystyrene

3.2.5 Design Requirements and Specifications

The novel bioreactor designed by the group must meet certain design criteria and specifications in order to function and fulfill the client's needs. All the design requirements are listed in Table 2 below and will be discussed in more detail in the succeeding paragraphs.

First and foremost, since a bioreactor system is meant to maintain an optimal cellular environment for homeostasis, the novel bioreactor must be able to fit within an incubator capable of providing the necessary temperature and oxygenation for the cells in the bioreactor. Current bioreactor systems utilize their own self-modulating temperature and oxygenation functionalities

to fulfill this requirement, however the novel bioreactor design will likely be unable to incorporate these same functionalities, and thus must rely on an incubator for temperature and oxygen control. As a result, the bioreactor must work within the confines of the incubator. The current incubator in Goddard Hall 006 was measured to have approximately 18 inches of working space, and so the bioreactor must not exceed this height in order to properly fit within the incubator environment. Additionally, it cannot introduce unintended mechanical forces on a large scale that would disrupt the attachment process of the adherent cells and rip them off of the microcarriers, or affect their morphology or health. Studies have indicated that with Human Endothelial Kidney (HEK) cells, a common adherent cell line, shears above 6.5 dynes/cm^2 may have an effect on orientation and morphology, while higher levels of shear such as 26 dynes/cm^2 will detach the cells from their surface and negatively impact cellular viability. Additionally, it has been found that the metabolism of HEK cells are affected by shear stresses as high as 6.5 dynes/cm^2, while the metabolism of human umbilical vein endothelial cells (HUVECs) are influenced by stresses at 10 dynes/cm^2 [21]. As such, this range of 6.5-10 dynes/cm^2 will be the upper limit for the shear forces imparted by the novel bioreactor design.

Another key design requirement is reusability. The bioreactor must be reusable, and therefore sterilizable with ease. It must withstand a variety of sterilization methods, from chemical to high temperature (autoclave) sterilization. For this reason, the bioreactor and its components must be designed from a relatively inert material that is able to maintain its structure at temperatures well over 121°C, which is the temperature at which autoclaving processes operate. These specifications will allow the bioreactor to withstand multiple cycles of use and sterilization before they may need to be replaced. Afterwards, this sterility must also be maintained. This involves creating an air-tight, closed environment so that contamination can be avoided.

Furthermore, the component must be biocompatible with a wide variety of cell types in order to maintain a high degree of versatility. It cannot be toxic, or otherwise chemically harmful to the cells in the bioreactor, while remaining non-degradable as to avoid contaminating a cell culture with material particles. In addition, the design should not interfere with the typical maintenance of homeostasis the bioreactor provides for the cell culture. For example, mammalian cell lines are generally maintained at temperatures of 37 ± 0.5 °C, while providing dissolved oxygen levels of $5\% \pm 0.5\%$ CO_2 and cell viabilities greater than 90%. In the end, the

most important condition that needs to be considered is the biological conditions of the cells and their productivities, which is influenced by many bioreactor aspects such as fluid flow and process control. These design requirements are summarized in Table 2 below.

Table 2. Design Requirements

Design Requirement	Value or Attribute
Work within incubator	18 inches in height
Must not introduce significant mechanical forces	Low shear stresses on microcarriers (limit of 6.5-10 dynes/cm^2)
Compatible with adherent cell culture	Observed cellular attachment within system
Must be reusable	Easy to sterilize (must be able to withstand autoclave temperatures of at least 121 °C)
Maintain biological conditions for cells	Mammalian cell lines - 37 ± 0.5 °C, 5% ± 0.5 % CO_2 , > 90% humidity
Scalability	Design allows for functional fabrication of different-sized bioreactors
Sterility Maintenance	Air tight, Avoid contaminations
Compatible materials	Non-toxic, non-degradable, biocompatible

3.3 Design Standards

When designing the bioreactor system, it was important to consider the following standards/regulations when it comes to working with bioreactors and living cells. These standards are essential in determining the possible ways to achieve our goal and also in determining the success of the final design. A summary of the standards considered with the fabrication of the novel bioreactor are shown in Table 3 below:

Table 3. Summary of Standards Considered in Design

ISO 11737	Sterilization of Medical Devices: Microbiological Methods, Part 1 — Determination of a Population of Microorganisms on Products
ASTM F1980:2007	Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices
ISO 13845:2016	Medical devices - Quality management systems - requirements for regulatory purposes
ISO 10993-5:2009	Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity
FDA 21CFR866:2018	Title 21, Subchapter H, Part 866 - Cell and Tissue Products

The first standard that should be considered is ISO 11737 (Sterilization of Medical Devices: Microbiological Methods, Part 1 — Determination of a Population of Microorganisms on Products) [22]. Sterility is extremely fundamental for the operation of a bioreactor as contamination must be avoided before the bioreactor is inoculated with cells. As such, the sterility of our novel bioreactor will be in accordance with this standard [22]. The team plans to design the bioreactor system so that it is reusable, and so it must be sterilized in between uses. This standard will be used to determine the sterility of the bioreactor from use to use. The standard establishes the requirements for validation and control of sterilization, such as how to determine whether or not the reactor is sterile. If the bioreactor contains the same amount of microorganisms on it as before sterilization it is considered not sterile and must be re-sterilized and checked again.

The second standard that should be considered is ASTM F1980:2007 (Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices). This standard will be used in terms of the sterility of the barrier system in the bioreactor [23]. It is essential that sterility is maintained over a prolonged period of time, as bioreactor operations can range from days to weeks. This standard addresses the accelerated aging theory, in which properties may change over time, affecting the proper function of a sterile barrier system. As stated previously, it is essential to ensure that the inside environment of the bioreactor is sterile when in use and no contamination will occur.

The third standard that should be considered is ISO 13845:2016 (Medical devices -

Quality management systems - requirements for regulatory purposes). The design of the novel bioreactor must be in accordance with this standard [24]. This standard provides guidelines throughout the entire design process, from ideation to prototyping and final design fabrication, in order to ensure that the product's function and quality sufficiently meets the client's requirements. The product must function the way it is intended and accomplish all parts of the revised client statement. As such, this standard is relevant to the design process.

The fourth standard that should be considered is ISO 10993-5:2009 (Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity) [25]. This standard will be useful in determining the toxicity of a material, as it specifies various types of tests that can be performed in order to determine whether or not a material is toxic to cells when in use. This standard is important because the bioreactor cannot introduce toxicity to the cells, as this would compromise the function of the cells and the operation of the bioreactor.

The fifth standard that should be considered is FDA 21CFR866:2018 (Title 21, Subchapter H, Part 866 - Cell and Tissue Products) [26]. These standards/regulations will be used when it comes to testing the bioreactor with live cells. These regulations help to define and specify many parts of the cell culture process such as culture medium, quality control kits, and microbial monitoring, which will be important in the operation and testing of the bioreactor system.

3.4 Revised Client Statement

After conducting further research and developing the objectives for this project so that it is more reflective of the issues relating to cellular yields in biomanufacturing, the client statement was revised. The updated statement now states to design a novel bioreactor system, which allows increased cell viability and attachment efficiency compared to those from current adherent bioreactor designs. As shown, the project will now focus specifically on creating a novel bioreactor system and how cell viability can be increased, specifically through the use of a packed bed microcarrier system. In the end, this novel reactor will help increase cell viability and attachment efficiency of cells in microcarrier systems.

3.5 Management Approach

During a project, it is important to set a variety of different milestones. Creating such milestones will help us understand the important steps needed in order to design and create a final product and to ensure the completion of the project in a timely manner. The major project milestones for this project are as follows:

➔ Undergo and complete necessary background research into bioreactors (August 2019 - October 2019)

➔ Define and state clear project objectives (September 2019 - October 2019)

➔ Complete the first four chapters of the MQP report (September 2019 - October 2019)

➔ Conduct a series of client interviews in order to gain a clearer understanding of the practical industrial applications of bioreactors (October 2019 - November 2019)

➔ Continue conducting client interviews to focus on specifics of adherent bioreactors and current industrial needs (November - December 2019)

➔ Begin designing initial concepts of novel bioreactor system (January 2019 - February 2020)

➔ Conduct initial validation tests before manufacturing final design (February - March 2020)

➔ Finalize manufacturing of final novel bioreactor design (March 2020)

➔ Conduct necessary at-home experiments to validate functions of experimental bioreactor (March - April 2020)

➔ Finalize MQP report and Present the results of the MQP Small Scale Bioreactor project (May 2020)

These milestones and events can be summarized utilizing a Gantt Chart as shown in Figure 1:
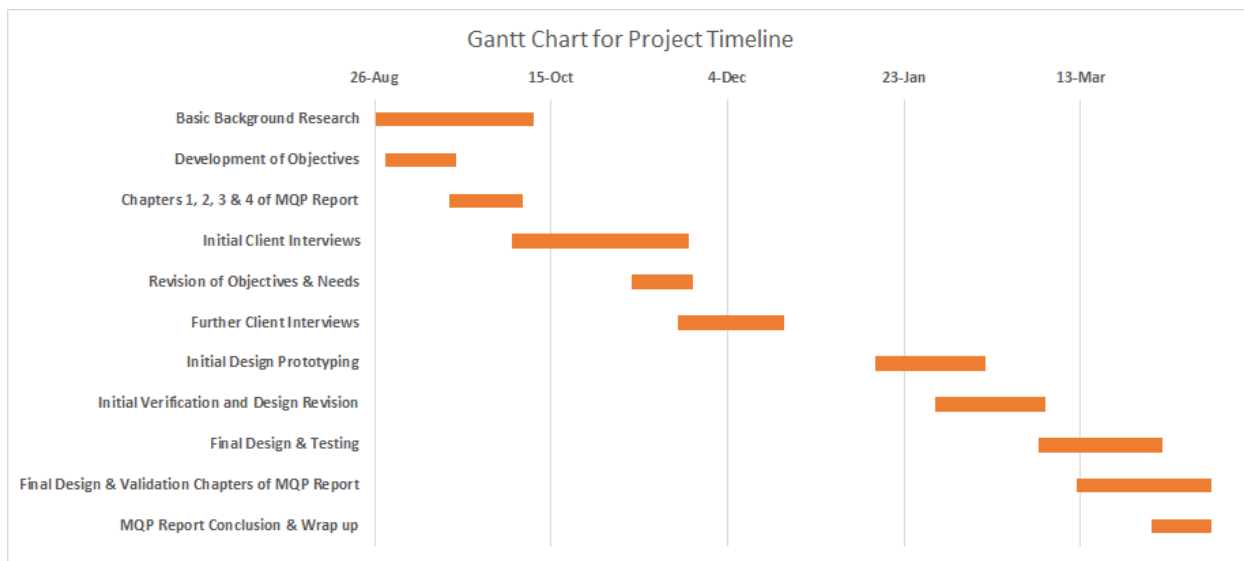
Figure 1. Gantt Chart for Project Timeline

Major Qualifying Project teams at WPI receive a set budget for their projects. This project has a set budget of $250 per student on the team. Since our team has 4 members, the total budget for this project is $1,000. Any amount over this has to be paid for by the team itself. When designing the bioreactor system, it is important to remember this budget when buying materials, creating the bioreactor and its components, and testing it.

When designing our bioreactor, we must consider the possible materials to be used and the cost of those materials. Some of the possible materials we plan to use are listed in Table 4: Cost of Potential Materials. The potential costs of these items were found using the CES Edupack software provided to us by WPI.

Table 4. Cost of Potential Materials

Material	Cost (USD)
316L Stainless Steel	5.77-6.35 per kg
Borosilicate glass	4.49-7.48 per kg
Low Density Polyethylene	1.61-1.65 per kg

Chapter 4: Design Process

Chapter 3 specified the design objectives, constraints, functions-means, and specifications of the final design in order to fabricate the system according to the requirements and goals. This chapter will break down the design needs and evaluate the initial designs to determine which design best satisfies these requirements.

4.1 Initial Concept Map

When the team first received the initial client statement, we began to brainstorm ways in which we would be able to improve bioreactor usability in terms of streamlining the operations to improve efficiency in the context of resources and time. Below is the concept map that summarizes the initial concepts thought of by the team that would be able to achieve improving efficiency, such as by reducing the instances of contamination, improving the longevity of the bioreactor when it is in use, reducing the set up process so that it is less time-consuming, creating a system that would be able to reduce the shear forces in the bioreactor, and increasing cellular adherence.
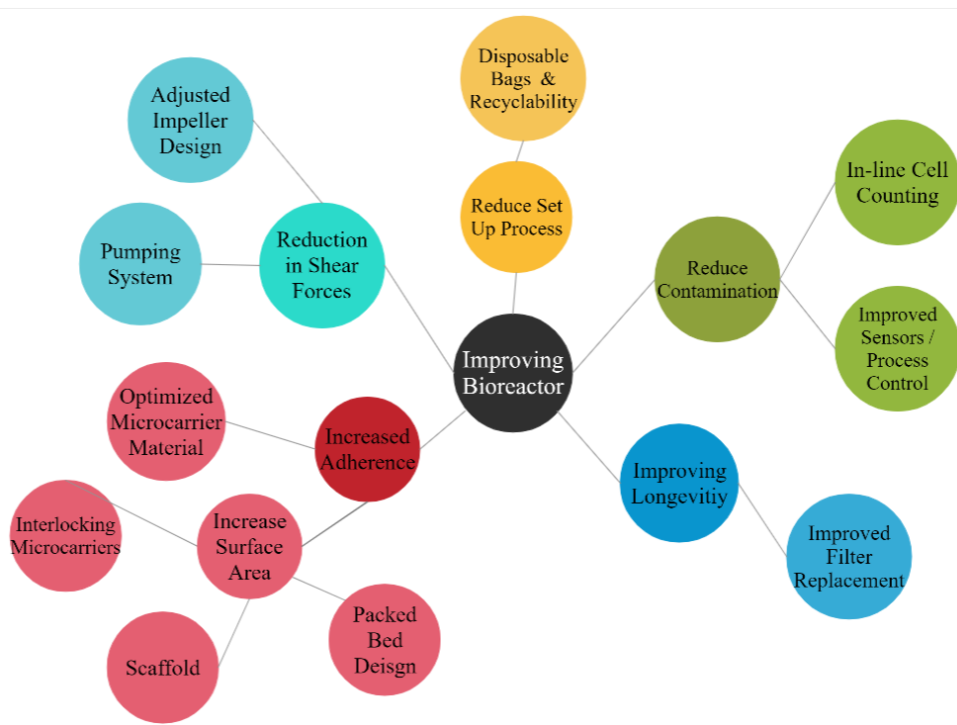
Figure 2. Concept Map

Once the team further consulted Chris Bellerive, we had learned that a major issue with bioreactors is the efficiency of adherent bioreactors in the context of cellular adherence and volumetric efficiency. This resulted in the team brainstorming various functions that the bioreactor must have, as well as means to achieve these functions, as described in Section 3.2.4 Functions and Means, and design requirements and specifications, as described in Section 3.2.5 Design Requirements and Specifications. Afterwards, we broke down the design requirements into specific needs and wants, as described in the next section.

4.2 Needs Analysis

The design requirements for the improved bioreactor system can be broken down into "needs" and "wants". These needs and wants can be found in Table 5 below:

Table 5. Summary of Needs and Wants

Needs	Wants
Non-disruptive to cells	Ease of Manufacturing
Volumetric Efficiency	Scalability
Sterilizable	Ease of Culture
Promotion of Maximum Confluency	

The first need involves the mechanical forces imparted on the cells during the culture process. This need encompasses analyzing the potential seeding methods and fabricating an improved system over the intermittent impeller stirring method that does not detach cells or negatively affect their behavior. As such, in terms of maintaining negligible impact on cell attachment and viability, the fluid flow should remain homogeneous, with laminar flow dominating rather than turbulent flow which could impart shear stresses on the cells. Although Reynold's number may be utilized to characterize the fluid flow, a more significant characterization is the shear force that is imparted on the cells. The designs specification outlined in Section 3.2.5 Design Requirements and Specifications provides an upper limit of 6.5-10 dynes/cm^2 that must be incorporated into the design.

Another need is that the design must be volumetrically efficient, meaning that it is capable of supporting larger numbers of cells in a smaller volume. As such, the final design would ideally be small enough to be portable, and would not require extensive manpower or time to start up and run the system. As mentioned previously, the current Cytodex system requires extensive labor and time due to the fact that it is not volumetrically efficient and requires an intermittent stirring system. Our design must consider this aspect so that in the end the bioreactor operations are physically streamlined.

Additionally, the materials chosen for the bioreactor system must be sterilizable in order to support homeostasis of the cells. Inherently, the materials chosen must also be biocompatible, so that after being sterilized a completely sterile environment is created that enables cells to properly grow and proliferate without being negatively affected. This signifies that in accordance to ISO 10993, the material that is utilized for the bioreactor should be classified with a cytotoxic grade of 0, indicating that there is no cell lysis or reduction in growth [25]. Since autoclaving has been decided as the method for sterilization, the material must be able to withstand temperatures up to 121°C. This sterilizability is important as it will enable the system to be reused multiple times without the need to constantly manufacture another system after each operation. Reusability will be economically beneficial as well since there will be only a single-time cost in manufacturing the system.

In the context of adherent bioreactor systems, one of the most fundamental aspects of design is the ability for cells to adhere to whatever scaffolding or surface is available within the system, and then properly proliferating to achieve maximum confluency. As such, the promotion of maximum confluency is extremely significant as this will enable the system to achieve the highest possible cell densities. This aspect in addition with volumetric efficiency is the main focus of optimization within the industry currently, where research is focused on generating the highest possible biological yield in the smallest volumes possible. The promotion of maximum confluency will thereby incorporate various aspects, such as providing a compatible surface capable of facilitating cellular attachment and proliferation, while also ensuring that the correct amount of oxygen and nutrients are supplied to the cells.

In terms of wants, there are several criteria which would be ideal for the team to incorporate into their design, although with the large amount of requirements and constraints this may not be as feasible. The first want is the ease of manufacturing the system. The ease will be

linked to the cost of the design, as both are related to the materials that are chosen as well as the complexity of the design. Manufacturing will also be related to the manufacturing method chosen by the team as well, as the team has a variety of options such as additive manufacturing or laser cutting. These are inherently connected to the final design of the product, and so as the team begins to prototype and devise the design, the manufacturing options will need to be assessed.

Another want for the design is that it must be scalable. Many bioreactor processes require a scale up process from benchtop reactors to large-scale industrial bioreactors, and as such, although this is not necessary, it would be ideal to incorporate the potential of scalability within the design. This would mean a streamlined design that is capable of being scaled up or scaled down to account for a large range of seeding densities. This is considered more of a want than a need by the team because we believe that the current focus should be on creating a system first that is capable of demonstrating enhanced attachment and volumetric efficiency. Ideally, once this system is created, we will then be able to demonstrate scalability with the fabrication of different-sized models, as in the end scalability will be important for any potential industry translation.

The final want for the design is the ease of the initial culturing process. It may be entirely possible that the culturing process is not modified at all with the current Cytodex systems, or it is modified but is still potentially as time-consuming, however another modification within the system is made which lowers the shear forces imparted on the cells. Ideally, this culturing process should be streamlined to contribute to the streamlining of the bioreactor operations, however this is considered more of a want than a need as the team acknowledges it may be possible to maintain the current seeding process if the final design is able to incorporate another modification that mitigates the detachment of cells and improves the attachment efficiency.

In order to determine the important aspects of our primary design objectives, we compared the design needs and wants to each other using a Pairwise Comparison chart, as shown in Table 6: Pairwise Chart Comparison. The chart gives a ranking to each factor on a scale of 0 or 1 to determine which factor is more important than the other. A ranking of 1 means that the factor on the left of the chart is more important to the team than the factor on the top of the chart and a ranking of 0 means the top factor was more important. A ranking of 0.5 means that the two factors being compared are equally important. The "X" in the chart denotes where the factor is

being compared to itself, therefore resulting in no ranking [27]. Table 7 is the total score of each factor and that determines the weight of the factors in order of importance. The pairwise comparison chart determined that the novel bioreactor being non-disruptive to cells and being volumetrically efficient are the most important factors, while scalability and ease of manufacturing were the least important factors. These top-ranked needs for the bioreactor system are important because ultimately, the bioreactor will be functioning as an environment for cells and therefore should not inherently cause cell death or impart a negative effect on cell function, while functioning within the smallest possible volume for maximum functional and economic efficiency.

Table 6. Pairwise Comparison Chart

	Non-disruptive to cells	Sterilizable	Ease of Culture	Ease of Manufacturing	Promotion of Maximum Confluency	Volumetric Efficiency	Scalability	Total
Non-disruptive to cells	X	1	1	1	0.5	0	1	4.5
Sterilizable	0	X	1	1	0.5	0.5	1	4
Ease of Culture	0	0	X	1	0	0	1	2
Ease of Manufacturing	0	0	0	X	0	0	0.5	0.5
Promotion of Maximum Confluency	0.5	0.5	1	0	X	0.5	1	3.5
Volumetric Efficiency	1	0.5	1	0	0.5	X	1	4.5
Scalability	0	0	0	0.5	0	0	X	0.5

Table 7. Total weight from pairwise chart

Design objective	Weight
Non-disruptive to cells	4.5
Volumetric Efficiency	4.5
Sterilizable	4
Promotion of Maximum Confluency	3.5
Ease of Culture	2
Scalability	0.5
Ease of Manufacturing	0.5

4.3 Concept Designs

4.3.1 Conceptual Design Modifications

The modifications to be considered for the initial conceptual designs for fabricating an improved adherent bioreactor system are shown below in Figure 3: Initial Concept Design Modifications. These design modifications are broken up and categorized by two distinct functional aspects of the bioreactor, cellular attachment and confluency within the system, as previously detailed in Section 3.2.3: Functional Blocks. These modifications were previously brainstormed in the broader context of improving bioreactor operations, as shown in the concept map in Section 4.1: Initial Concept Map, however here they will be further discussed and developed in the specific context of improving adherent bioreactor cultures. Analyzing the bioreactor by these two functional groups gives a wide range of aspects to analyze and dissect as it allows the team to understand each components' role and their interaction with other components in the optimization of the bioreactor operation.
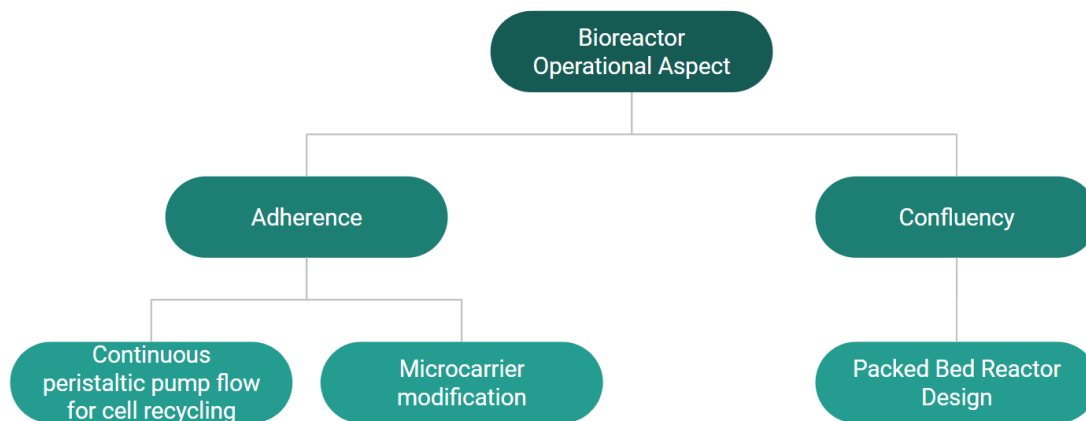
Figure 3. Initial Concept Design Modifications

As shown, three initial conceptual modifications have been brainstormed. In order to address the issue of cell attachment, two proposed modifications have been proposed: the usage of a continuous flow system and modifications to the microcarriers. The utilization of a continuous flow system would enable the recycling of unattached cells so that they are continuously in contact with the microcarriers. In the current systems, which utilize the Cytodex beads, cells that are unattached are simply left to float and die in suspension since they are unable to make sufficient contact with any microcarrier. The idea of continuous flow would enable the unattached cells to continuously travel through the system so that they can attempt to make contact again with the microcarriers. Additionally, a continuous flow achieves the bioreactor need for nutrient and oxygen exposure to the cells, effectively replacing the stirred-tank system which utilized an impeller for homogeneous distribution. This will also allow for greater modifications over the shear stresses imparted since the impeller system will now be replaced. The modification to the microcarriers would entail potential surface modifications, such as oxygen plasma treatment or gelatin coatings, which have previously been shown to help enhance cell adherence to surfaces. Furthermore, it is possible to completely change the material of the microcarrier itself, so that a different substrate can be utilized, such as glass or plastic. Ideally, changing the surface material would enable improved attachment.

Additionally, in the context of improving confluency in the system, another proposed modification is the utilization of a packed bed system. This would entail packing the microcarriers so that they are in close contact with one another, and then media containing the

cells, oxygen, and nutrients would flow through this packed bed. Ideally, cells would make sufficient contact with a microcarrier and then attach. The most important aspect of this design is that due to the closeness of all the microcarriers, cellular bead-to-bead migration would be possible. According to the client, one issue with the current Cytodex system is that due to the constant mixing of the stirred reactor, none of the microcarriers necessarily make sufficient contact with one another. As a result, there are many beads which are empty and do not contain cells. With the potential of bead-to-bead migration in this design modification, microcarriers which initially did not contain any cells are capable of providing an extra surface onto which cells from other beads can grow and attach onto. This would help to increase viable cell densities by effectively increasing the potential surface area for cell attachment and growth.

In the end, the team decided to utilize all three modifications to fabricate a closed-loop packed bed bioreactor system. This system integrates a packed bed system containing modified microcarriers, in addition to a continuous pumping mechanism. This design is discussed in the next section, while the design is further broken down into each individual component and detailed in Section 6.1 Final Design Overview. The materials utilized for the full system can be found in Appendix B: Bill of Materials, while a comprehensive list of each component of the bioreactor is found in Appendix C: Bioreactor Components.

The potential for scalability has been considered as well. If a streamlined design is created, this would allow for potential manufacturing at any scale required, thus allowing for potential translation into the biomanufacturing industry. The other design needs, such as being easily sterilizable and non-disruptive to the cells will be met to similar degrees across all concepts through the use of specific materials and sterilization procedures that will suit the overall design and intended function.

4.3.2 Alternative Designs

The team generated several alternative designs to improve adherent cell culture. The first design incorporated the continuous pumping mechanism with the combination of a scaffold to replace the usage of microcarriers. The second design also incorporated the continuous pumping mechanism, but utilized the microcarriers in a packed bed design. The third design involved maintaining the current impeller system in the stirred tank bioreactors, and instead focused on a method to connect microcarriers as they were being mixed to achieve bead to bead migration.

Stackable Scaffold Bioreactor

This design consisted of a stackable scaffold which would be placed inside the bioreactor vessel. The stackability of the scaffold offers a great advantage by being inherently compatible with scalability, granting different scalability options as more or less scaffolding could be used depending on the desired size of the bioreactor. This is the reason why the utilization of a scaffold was considered as a potential replacement for the microcarrier design, as the process of scaling up or down in bioreactor sizes will be significantly streamlined. The impeller system which is typically found in stirred tank bioreactors is replaced with a pumping mechanism that would pump the culture media from the bottom of the bioreactor to the top, thereby passing media, nutrients, and oxygen through the scaffolds for the attached cells. This pumping mechanism is important as it also enables unattached cells to be recycled within the system with the potential for them to attach onto the scaffold as they continuously pass through the bioreactor. This design can be seen below in Figure 4:
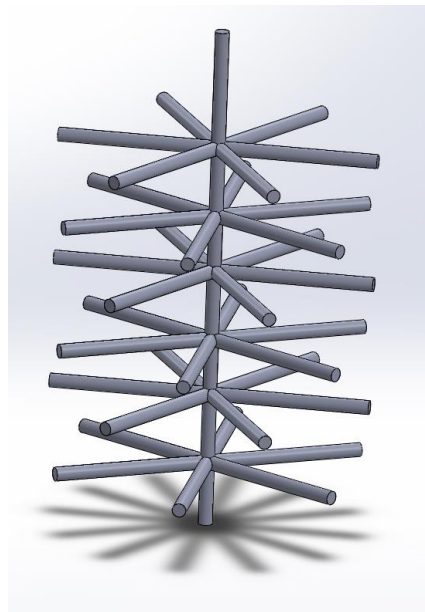
Figure 4. Scaffold Bioreactor Design

Packed Bed Bioreactor

This design focuses on addressing cellular adherence in addition to volumetric efficiency with the utilization of a packed bed of microcarriers. The typical impeller system in the current Cytodex system is replaced with a continuous pumping mechanism, where the media continuously travels through this packed bed of microcarriers, so that cells that are unable to attach are looped back into the beginning of the system in an attempt to seed them onto the beads. The replacement of the impeller system helps to reduce shear forces since the intermittent stirring method is no longer required. This continuous pumping mechanism also provides a method for nutrients and oxygen to be constantly delivered to cells seeded within the packed bed of microcarriers. The packed bed itself enables an increase in available surface area for cellular adherence since many microcarriers can be packed together within a smaller volume in comparison to the current Cytodex system, where the microcarriers are homogeneously mixed throughout the volume. In addition, cellular bead to bead migration is possible due to the dense packing of the microcarriers, further enabling confluency and cellular proliferation to achieve higher amounts of cell densities. This design can be seen below.

Figure 5. Packed Bed Design

Interlocking Microcarriers

This design involved maintaining the current stirred tank bioreactor system that is utilized in the Cytodex system, while focusing on increasing the potential surface area for cellular adherence and proliferation through interlocking microcarriers. One main issue with the current Cytodex system is that there is a lac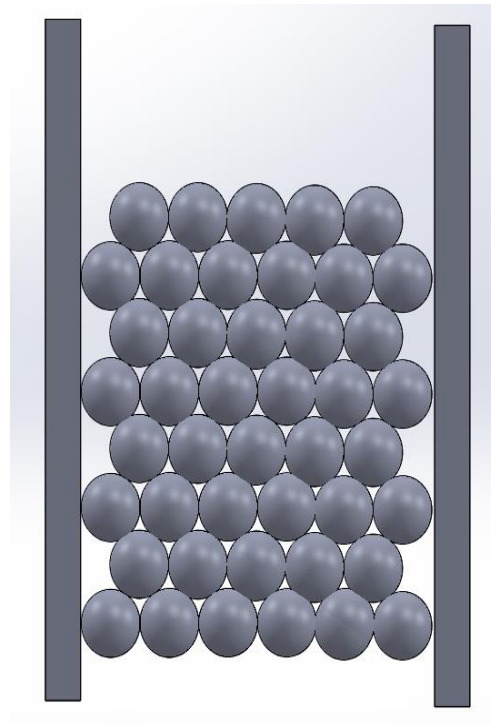k of bead to bead cellular migration, and as such microcarriers which were not seeded with cells initially will likely never be able to contain any cells since the attached cells are confined only to the bead they initially attached to. This severely limits the available surface area for cells. As such, this design incorporates the utilization of polarized glass beads, half of which are positively charged and the other half which are negatively charged. While the initial intermittent seeding method will still be utilized, as the microcarriers are mixed within the bioreactor, the polarized charges will enable the positively charged beads to become attracted to the negatively charged beads. This will increase the available surface area for the initial seeding phase, while also enabling cells to migrate between microcarriers, which will also increase the available surface area for cellular growth and confluency.

4.3.3 Final Design Selection

With mentioning the alternative designs the team had contemplated in addition to the final design, it is important to detail the process we utilized in the selection of our final design. The Pugh Selection method was utilized in conjunction with the total weights generated in the Pairwise Comparison in Section 4.1 Needs Analysis. The Pugh Selection method compares the scaffold bioreactor design, the packed bed bioreactor design, and the interlocking microcarriers design with the current baseline Cytodex system in order to generate scores that determine which design is more capable of fulfilling the design objectives. A rating of 1 indicates that the alternative design improves on a specific aspect of the baseline Cytodex system, while a rating of -1 indicates that the alternative design is actually worse in that aspect than the baseline system. A rating of 0 indicates that the alternative design and the baseline design are equivalent to each other for that specific aspect. This Pugh Selection method is detailed below in Table 8.

Table 8. Pugh Selection Method

Evaluation Criteria	Weight Factor	Baseline (Cytodex Bioreactor w/ Intermittwent Stirring)	Scaffold Bioreactor (w/ continuous pumping)		Packed Bed Bioreactor (w/ continuous pumpin)		Interlocking Microcarriers Design	
			Rating	Weighted	Rating	Weighted	Rating	Weighted
Non-disruptive to cells	4.5	0	1	4.5	1	4.5	0	0
Sterilizable	4	0	0	0	0	0	0	0
Ease of Culture	2	0	1	2	1	2	0	0
Ease of Manufacturing	0.5	0	-1	-0.5	1	0.5	1	0.5
Promotion of Maximum Confluency	3.5	0	1	3.5	1	3.5	1	3.5
Volumetric Efficiency	4.5	0	0	0	1	4.5	0	0
Scalability	0.5	0	1	0.5	1	0.5	0	0
Score		0		9.5		15.5		4

As shown, the packed bed bioreactor demonstrated the most functional benefits as it had a higher score in comparison to the scaffold bioreactor and interlocking microcarriers design. The most significant difference between the designs was their capabilities of volumetric efficiency. The scaffold bioreactor is volumetrically inefficient, due to the large amounts of dead space in between each portion of the scaffold. Although recycling of unattached cells is achieved in this design with the utilization of a pumping mechanism, the surface area to volume ratio would be very low in comparison to the packed bed design. The interlocking microcarriers design promotes the maximum possible confluency within the system, however the utilization of microcarriers in a stirred tank reactor is still volumetrically inefficient due to the large amounts of volume required. Additionally, this system is still disruptive to cells as the intermittent stirring system would be required with the impellers in place. In contrast, the packed bed design enables high amounts of packing, which would promote cellular migration between beads and increase the total surface area available for cells to spread and grow. This bead-to-bead migration is the most important aspect of the packed bed reactor, as it is volumetrically efficient and allows for cells to spread onto microcarriers which may not have previously contained any. As a result, the team decided to utilize the packed bed design as our final novel design.

4.3.4 Feasibility Studies

As the design undergoes several iterations up until the final design, the team conducted several feasibility studies to determine the practicality and functionality of the design and where potential improvements can be made. A simple preliminary cell attachment was performed in order to compare the usage of Cytodex beads with other potential substrates, such as glass beads, in order to determine if the final design should utilize the current microcarrier technology on the market or attempt to utilize another material with the hopes of improving cell attachment and bead-to-bead cellular growth. This was done by seeding cells into wells which contain a small layer of the material of interest, and waiting for an extended period of time before staining and utilizing a fluorescent microscope to observe cell behavior. Additionally, calculations were performed to ensure the feasibility behind the bioreactor system in terms of oxygenation and shear stresses. It should be verified that the bioreactor will not generate forces capable of detaching cells during the culturing process, and that enough oxygen is provided for the culture. These can eventually be fully verified once the bioreactor system is translated into adherent cell culture with the examination of the cells and their health within the packed bed. The results of these studies are detailed in Chapter 5: Final Design Verification and discussed in Chapter 7: Discussion.

A final test will be conducted comparing the current microcarrier technologies of utilizing Cytodex beads in a bioreactor with the current intermittent stirring protocol in comparison to the continuous flow bioreactor containing a packed bed of carriers. This experiment will have to be controlled, wherein the same cell density is seeded into both systems, and waiting for a period of time before the cultures are stopped at the same time. Afterwards, both systems will be examined utilizing fluorescent staining to compare cell growth, and both systems will be compared in terms of final cell count and viability. The purpose of this is to determine whether or not the new, experimental design is capable of increasing attachment efficiency as well as final cell yield. In the end, the experimental bioreactor should display equal or improved results compared to the control method of utilizing Cytodex microcarriers with intermittent stirring. The expected results are detailed in Chapter 5: Final Design Verification and discussed in Chapter 7: Discussion.

4.4 Relevant Design Calculations and Parameters

As stated previously, the feasibility of the bioreactor relies on how it affects and interacts with cells. In order to test this feasibility, we plan to examine multiple parameters pertaining to cells that are in our bioreactor as well as a standard control bioreactor to compare the two. This will allow us to determine the effectiveness of our bioreactor in terms of cell biocompatibility and cell health. Human dermal fibroblast cells will be utilized with our bioreactor, and will be maintained in accordance to WPI Professor Ambady's subculturing protocol, which can be found in Appendix D: Protocols.

The first parameter that must be examined is determining the number of live and dead cells from the bioreactors. In order to do this, we used a cell counting hemocytometer. These are able to give us the amount of total cells, but in order to determine which cells are dead or alive, we must stain the cells with Trypan Blue, where those that are alive will not be stained and those that are dead will be stained blue. Once the amount of live and dead cells was determined, we could calculate different parameters based on these numbers. One parameter we will be examining is doubling time, which is the amount of time it takes for the cells in the bioreactor to double in population size. The doubling time is determined by the following equation,

$$\text{DT} = \text{T}\frac{\ln(2)}{\ln(X_e/X_b)} \tag{1}$$

where DT is the calculated doubling time, T is the incubation time or the period of time chosen for the cells, X_b is the number of cells at the beginning of the incubation time, and X_e is the amount of cells at the end of the time indicated [28]. Another parameter needs to be examined is the cell viability, which details the amount of healthy cells in comparison to the total number of cells both living and dead. Thus, cell viability is simply the amount of live cells counted divided by the total amount of cells. This will result in a viability percentage [29].

4.5 Physical and Mathematical Models

4.5.1 Physical Modeling

When designing our bioreactor, we utilized the SolidWorks program in order to create a 3D model of our final product. SolidWorks also allowed us to see the physical properties of the model like center of mass, weight, and strength based on the material used. SolidWorks models of our bioreactor and each of its components can be found in Section 6.1 Final Design Overview.

4.5.2 Mathematical Models

Since we are creating a novel bioreactor, we understand that we will need to characterize the fluid flow within the packed bed design and the shear stresses generated. Additionally, the amount of oxygen consumed and delivered will need to be characterized to ensure the oxygenation requirements of the cells are met. These mathematical models are listed below.

Ergun Equation:

Since the final design will incorporate a packed bed bioreactor design, the characterization of the fluid flow is significant. The Ergun equation enables fluid characterization of packed bed designs by relating the pressure drop along the length of the packed bed given a constant flow rate as fluid passes through the packed bed [30]. This constant velocity is related to the constant volumetric flow rate imparted by the provided peristaltic pump. The formula for the Ergun equation is given below,

$$\Delta P = \left(\frac{150\mu(1-\varepsilon)^2 u_0}{\varepsilon^3 d_p^2} + \frac{1.75(1-\varepsilon)\rho u_0^2}{\varepsilon^3 d_p}\right) * L \tag{2}$$

where ΔP is the calculated pressure drop, L is the height/length of the packed bed, μ is the fluid viscosity, ε is the void space within the packed bed, u_0 is the fluid superficial velocity, d_p is the diameter of the particles in the packed bed, and ρ is the density of the fluid.

Shear Stress Calculation

The associated pressure drops across the length of the packed bed can be related to a corresponding shear stress which calculates the amount of shear stress each bead within the packed bed would experience [31]. This is given below,

$$\tau = \frac{\Delta P * A}{SA}$$

(3)

where τ is the calculated shear stress, ΔP is the calculated pressure drop from Equation (2), A is the cross sectional area of the fluid flow, and SA is the surface area of the packed bed.

Oxygenation

A significant parameter that must be considered is whether or not the bioreactor will properly supply the required amount of oxygen to the cells within the bioreactor. As such, the following equation details how to calculate the amount of oxygen consumed every second by the packed bed of microcarriers,

$$O_{2,C} = C_{cells} * O_{2,cell}$$

(4)

where $O_{2,C}$ is the maximum amount of oxygen consumed, C_{cells} is the maximum amount of cells in the packed bed, and $O_{2,cell}$ is the theoretical oxygen consumption rate per cell. The following equation details how to calculate the amount of oxygen supplied by the pump every second,

$$O_{2,D} = DO_2 * Q$$

(5)

where $O_{2,D}$ is the calculated rate of oxygen delivery, DO_2 is the theoretical dissolved oxygen in media, and Q is the volumetric flow rate of the pump into the bioreactor vessel.

Chapter 5: Final Design Verification

Several preliminary experiments were performed to optimize the final design and verify its design before being translated into adherent cell culture. The first experiment involved comparing the volumetric efficiencies of the novel bioreactor and the current Cytodex system by determining the maximum possible cells per unit volume in each system. The next experiment was based on determining an optimal microcarrier material for the new design, comparing the current Cytodex 1 microcarriers with larger, surface modified glass beads. The third test involved theoretical calculations aimed at examining the pressure drop across the packed bed and associated shear stresses, and whether or not enough oxygen would theoretically be delivered to cells in the packed bed. The last experiment was a fluid test in order to verify the design and ensure that there were not any leaks, and that fluid properly flowed through the bioreactor system.

5.1 Volumetric Efficiency Comparison

When comparing our novel bioreactor to the current Cytodex system, it is important to understand how utilizing a packed bed of microcarriers is more volumetrically efficient than simply having microcarriers in suspension. This volumetric efficiency can be defined as the total number of cells supported per unit volume of the reactor. The calculations can be found in Appendix E: Relevant Design Calculations.

First, a simple confluency experiment was run where dermal fibroblast cells were allowed to reach 100% confluency in a Corning T-25 flask. This flask was used since it is known that its surface area is 25 cm^2. Once 100% confluency was reached, the cells were subcultured and counted to determine the total number of cells. It was found that a surface area of 25 cm^2 contained 1.14 x 10^6 cells, indicating that the number of cells per unit surface area is 4.55 x 10^{-4} cells / µm^2. Afterwards, the surface area to volume ratio for the Cytodex system and our novel system was calculated. This ratio could be calculated for the Cytodex system as the GE Cytodex 1 manual indicated the amount of surface area per gram of Cytodex, which was 4,400 cm^2 per gram [32]. The ratio for the novel system was calculated knowing the volume of the bioreactor and the maximum total number of beads possible. Once these ratios were calculated, they were multiplied by the previously calculated number of cells per unit surface area, thereby giving the

total number of cells supported per volume of the bioreactor. This is summarized in Table 9 below.

Table 9. Volumetric Efficiency Comparison

Bioreactor System	Surface Area per Unit Volume (um^2 / L x 10^{12})	Cells per mL (x 10^5)
Cytodex System	1.32	6.01
Novel System	8.66	39.3

Once the volumetric efficiencies are calculated, they can simply be divided by one another in order to determine the increase in efficiency. As such, the novel system is 6.5-times more volumetrically efficient, as it can support 6.5-times the number of cells per unit volume than the Cytodex system.

5.2 Microcarrier Optimization

An initial experiment was run in order to observe how the cells adhered to the microcarriers including the Cytodex I microcarriers from GE Healthcare and the glass beads with various surface modifications. The glass beads utilized were Megalux Uncoated Glass Beads 30/40 Mesh, manufactured by Swarco with a diameter range of 425 - 600 µm. The surface modifications that were done to the glass beads included separate gelatin coating treatments and oxygen plasma treatments. The protocol for this experiment can be found in Appendix D: Protocols under the Microcarrier Attachment Experiment Protocol. After the beads were each seeded in triplicate in a 12 well plate and incubated for three days they were stained in Hoechst and imaged. The control microcarriers, Cytodex I, are shown in Figure 6.
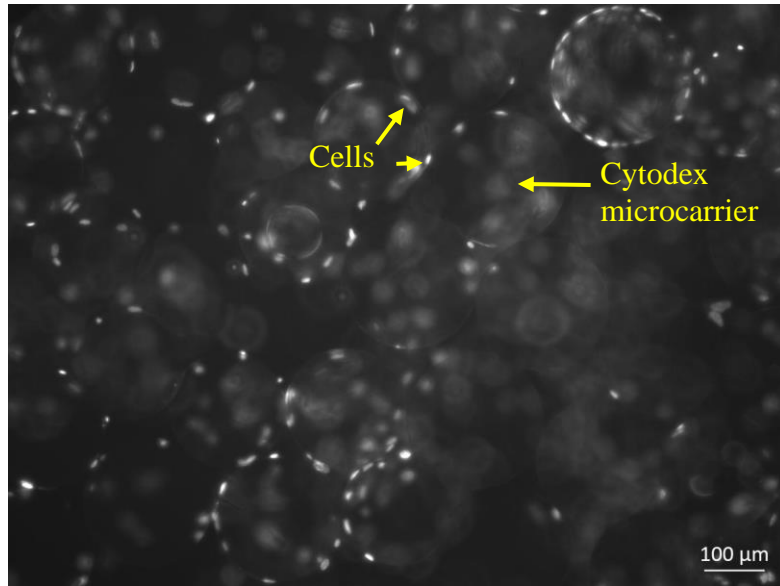
Figure 6. Cells on Cytodex 1 Microcarriers

The glass beads that were unmodified are shown in Figure 7. As can be seen there are some cells on the surface of the beads, but a majority lie in the spaces between beads.
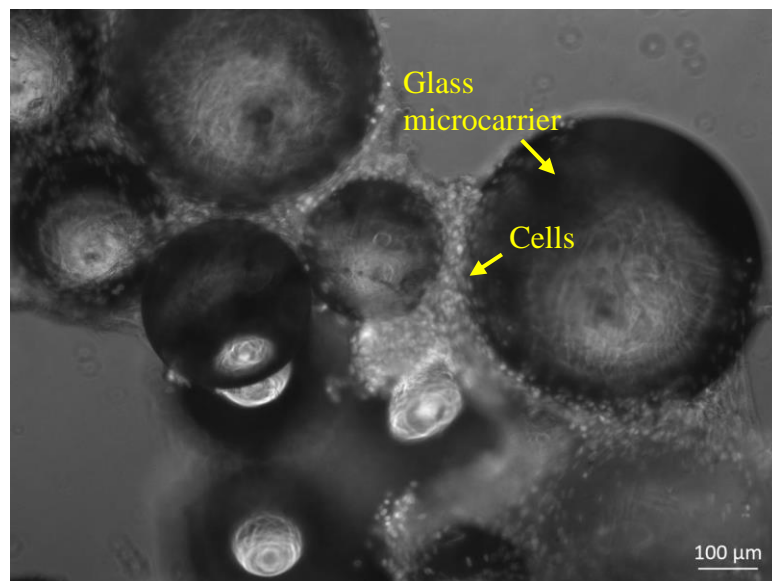
Figure 7. Cells on Unmodified Glass Beads

The glass beads that were modified using a gelatin coating show a dispersion of cells over the surface. There is some more congregation of cells in certain areas compared to others. The images of these beads can be seen in Figure 8.
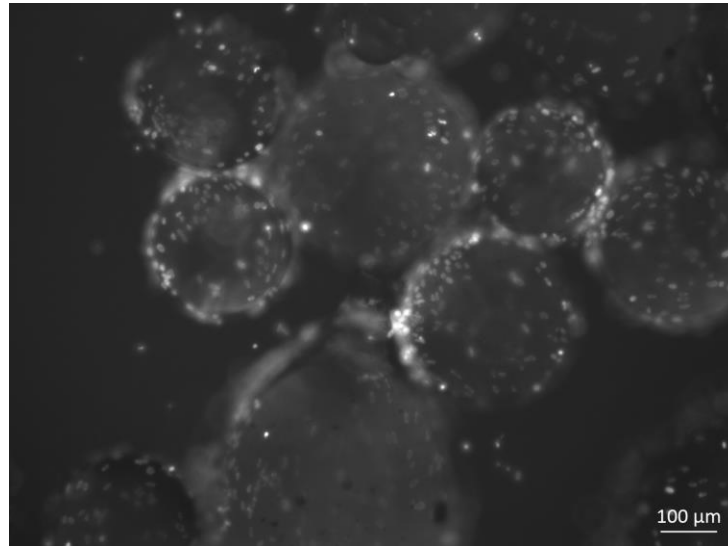
Figure 8. Cells on Gelatin Coated Glass Beads

The glass beads that were treated with oxygen plasma are shown in Figure 9. There are cells that reside on the outer surfaces of the beads throughout all sections of the bead, as well as observed migration and growth between the beads.

Figure 9. Cells on Plasma Treated Glass Beads

5.3 Ergun Equation and Oxygenation Calculations

Before running the bioreactor with adherent cells, it was determined whether or not the provided pump is capable of handling the pressure drop across the packed bed, as well as whether or not enough oxygen will be provided to the cells. These are done utilizing Equations (2), (3), (4), and (5). The physical calculations and associated assumptions can be seen in Appendix E: Relevant Design Calculations. Equations 3 and 4 were solved as a function of the flow rate, calculating what the theoretical pressu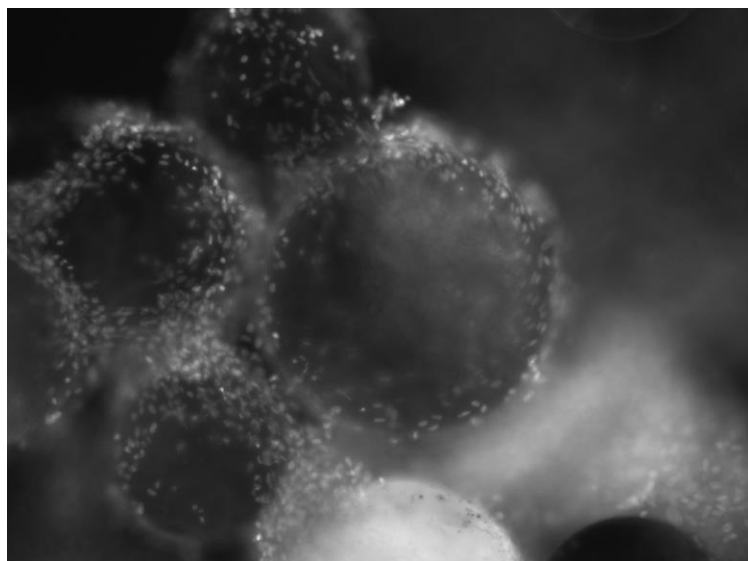re drop and associated shear stress on each microcarrier would be for a variety of flow rates. These calculations were performed under the assumption of monodispersity, where every microcarrier was the same diameter, while also assuming the densest possible packing (74%) and that media properties are similar to that of water [33]. These results are shown in Table 10 below:

Table 10. Range of Pressure Drops and Shear Stresses

Flow Rate (mL/min)	Pressure Drop (Pa)	Shear Stress (dynes/cm^2)
30	870	2.63
35	1020	3.07
40	1170	3.52
45	1320	3.96
50	1460	4.41
55	1610	4.85
60	1760	5.30
65	1910	5.75
70	2060	6.20
75	2210	6.66

As shown, these pressure drops are very small, almost negligible, especially in comparison to the back pressure of 172 kPa that the pump is capable of supporting. It is calculated that once the flow rate of the system reaches approximately 75 mL/min, the upper

limit of 6.5 dynes/cm^2 is reached, which is below the limit detailed in Section 3.2.5: Design Requirements and Specifications. Oxygenation rates were also calculated as a function of flow rate, as shown in Table 11 below:

Table 11. Oxygen Delivery Rates

Flow Rate (mL/min)	Total O2 Delivered (mg/sec x 10^{-4})
5	3.33
10	6.67
15	10.0
20	13.3
25	16.7
30	20.0
35	23.3
40	26.7
45	30.0
50	33.3

These calculations were performed under the assumption that every microcarrier within the packed bed would be 100% confluent with cells. The calculations and assumptions for determining the number of beads and cells for a specific packed bed length can be found in Appendix E: Relevant Design Calculations. For example, it was assumed that the dissolved oxygen concentration in the media is similar to that within water. Additionally, these delivery consumptions were calculated taking into account a safety factor for the amount of dissolved oxygen in water. It was determined that the maximum dissolved oxygen concentration in water

at 37 °C is 6.81 mg/L, and so a safety factor was used so that the concentration used in the calculations was 4.00 mg/L [34]. It was also assumed that the oxygen consumption rates of each cell was 1.19×10^{-7} mol O_2 per second [35] These calculated ranges of oxygen consumption can be compared to the theoretical total oxygen consumed by the system, which is 1.07×10^{-4} mg/sec. The calculations for determining the theoretical total oxygen consumption can be found in Appendix E: Relevant Design Calculations. If the total oxygen consumption rate is lower than the total delivered oxygen rate, then this would indicate that the bioreactor system is capable of supporting the packed bed in terms of supplying enough oxygen for the cells.

5.4 Bioreactor Fluid Test

After the novel bioreactor was initially fabricated, a bioreactor fluid test was performed to validate that continuous flow was achieved within the system, while ensuring that no visible external leaks were occurring. The protocol for this experiment can be found in Appendix D: Protocols under the Fluid Validation Test Protocol. After setting up the full bioreactor system, water was run throughout the system, as shown in Figure 10 below:

Figure 10. Initial Fluid Test with Water

As shown, the bioreactor was able to successfully be primed so that water was continuously flowing through it. Upon inspection at certain interfaces in the design, no visible leaks were found. Afterwards, green dye was injected in order to better visualize the liquid flow, as shown in Figure 11 below:
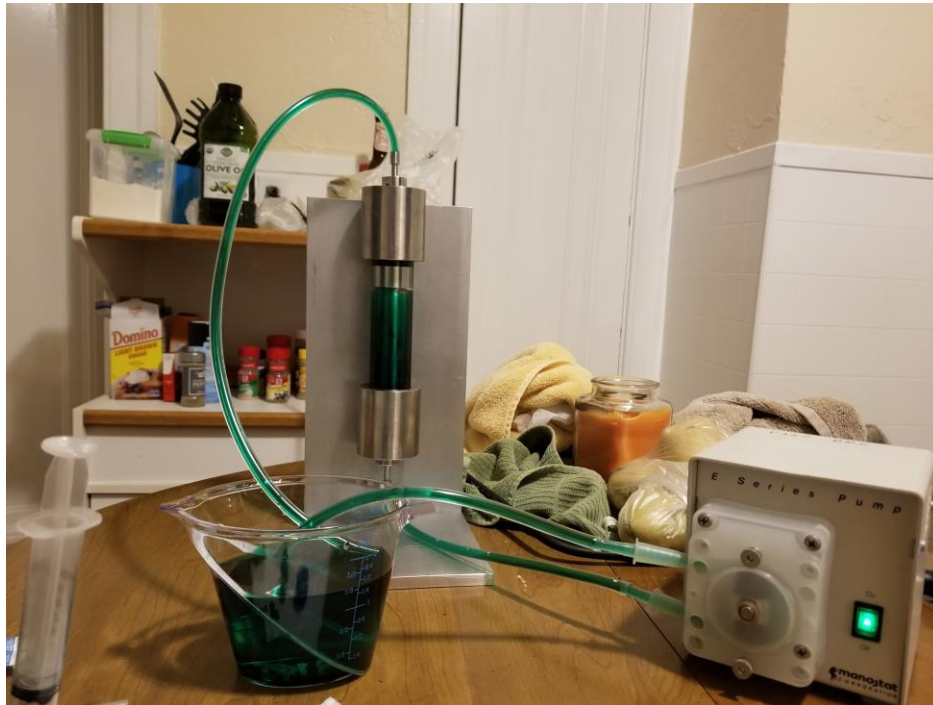
Figure 11. Initial Fluid Test with Green Dye

The green dye helps to indicate the presence of fluid within the system, showing that fluid is properly flowing into the bioreactor through the inlet hose nipple and out of the bioreactor throughout the outlet hose nipple. It should also be noted the presence of green dye within the glass tubing surrounding the middle insert. Finally, after draining the bioreactor from the water, the bioreactor was taken apart and the middle insert was upon up, showing the presence of beads as shown below:
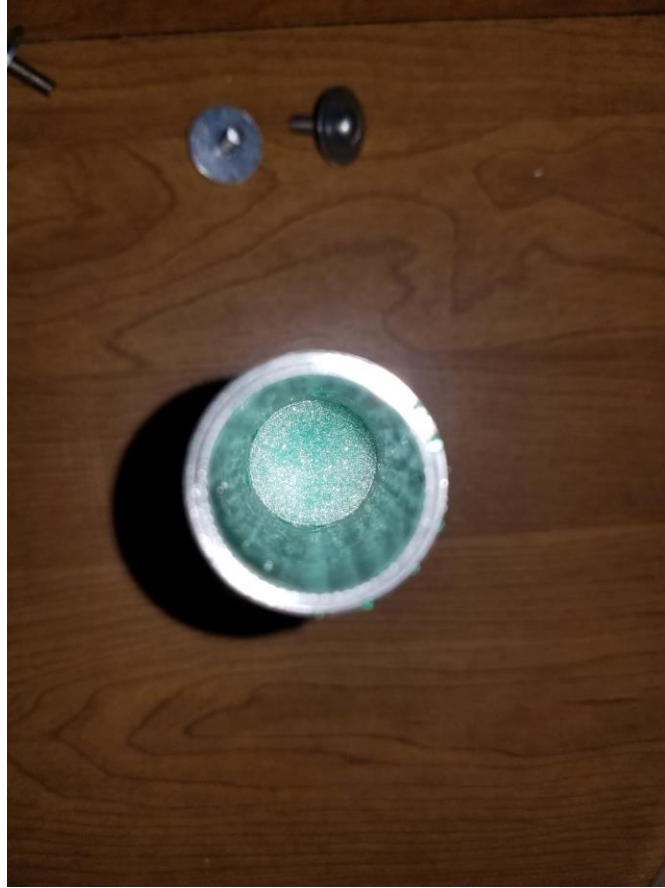
Figure 12. Middle Insert with Dyed Glass Beads

As shown, the glass beads are still present within the middle insert, indicating that they were not forcibly pushed out of the middle insert by the continuous flow. This also validates that the metal mesh screen was capable of allowing fluid through, while being small enough to contain the beads. Additionally, the green tint on the glass beads indicates that liquid properly flowed through the middle insert, since the green dye was able to pass through the beads and dye them green. In the end, this experiment validates that continuous flow was achieved in the bioreactor system and that fluid is capable of passing through the middle insert properly without disrupting the beads.

5.5 Novel Bioreactor Comparison Study

After running these initial preliminary tests, the novel bioreactor will be compared to the current adherent system with Cytodex 1 microcarriers in terms of cellular viability and density.

Both systems will be seeded at the same initial densities, and the nutrient profiles would be generated, finally resulting at the end of the study a cell density comparison. Unfortunately, due to circumstances surrounding COVID-19, the team was unable to conduct this comparison study. As a result, the next sections will detail the nutrient tests that would have occurred during the experiment, as well as the final density test that would have been conducted at the end of the experiment to compare cell densities between the two systems.

5.5.1 Nutrient Tests

Throughout the course of the experiment, nutrient and metabolite data would have been generated in both bioreactor systems for comparison, testing for specific nutrients including Glucose, Glutamax, Glutamate, Lactate, Ammonia, and Lactate Dehydrogenase (LDH) levels in the media. Conducting a comparison between the two would help to demonstrate whether or not the bioreactors are comparable in terms of nutrient depletion, especially since Cytodex adherent cultures have already been characterized in these types of studies. The protocol for conducting these nutrient tests can be found in Appendix D under the Nutrient Testing Protocol. Nutrient testing would have been conducted every day for the entirety of the comparison study in order to create nutrient curves. A sample nutrient curve is shown below [36]:
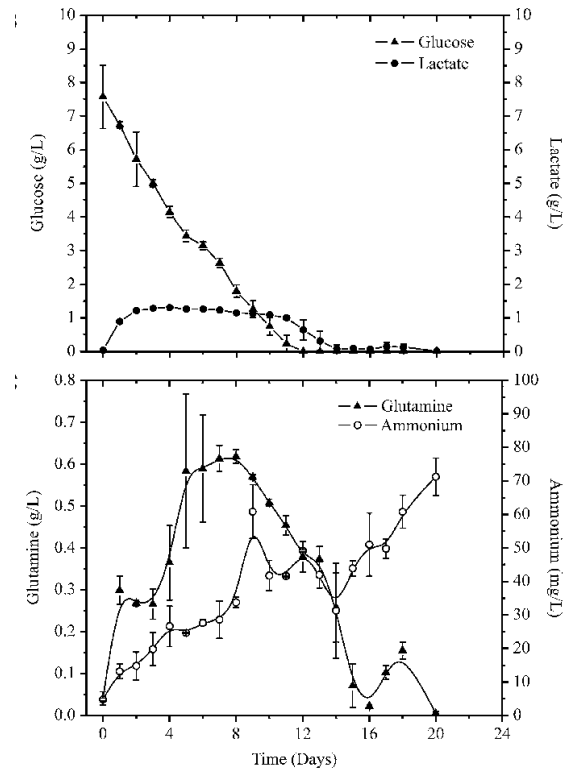
Figure 13. Sample nutrient curve

As shown, it would be expected for glucose, glutamax, and glutamate levels to decrease since these are nutrients for cell consumption, whereas lactate and ammonia levels would increase since they are cellular byproducts. LDH is a specific cytosolic enzyme which is released from the cell into the media upon cell death, and as such its levels can be utilized as an indicator for cellular viability by running LDH assays in addition to the nutrient tests. It would be expected that at the very least, both the control and novel bioreactor would generate similar trends in their respective nutrient curves. However, differences between in the novel bioreactor curves would have been expected, as this would be indicative of differences in the cell culture and amounts of cells.

A control experiment would have initially been run as well which examines nutrient growth curves for the human dermal fibroblast cells when in normal adherent cell culture in a T-flask. This generates a baseline nutrient data curve for which the bioreactor curves could be compared to as well. Additionally, utilizing this baseline nutrient curve would help to correspond nutrient depletion with confluency and cell growth. This is due to the fact that with a normal T-flask, confluency can be examined under a normal phase contrast microscope, and so doublings

in confluency, indicative of doublings in the cell population, can be correlated to specific trends in the nutrient curve. For example, a specific percentage decrease in glucose levels can be correlated to a doubling of the cell population. As such, this correlation can then be applied to the bioreactor nutrient curves to extrapolate data in terms of cell doubling.

5.5.2 Final Density Tests

At the end of the system run of both bioreactors, the final densities of cells on the two types of microcarrier technology would have been calculated and compared. The two systems would have been seeded with the same amount of cells in order to accurately compare the final cell densities at the end of two weeks. The final cell densities would have been compared for both cell attachment and cell growth. The protocol for conducting the final cell density tests can be found in Appendix D under the Final Cell Density Protocol. The differences in cell densities would have been used to highlight the impact of the novel bioreactor on adherent cell culture.

Chapter 6: Final Design and Validation

The final bioreactor system is composed of many parts, all of which are required to meet the safety and industry standards. Additionally, there are other considerations that need to be considered such as the economic impact of the device, the ethics of the device, and the manufacturability of the device.

6.1 Final Design Overview

The final design can be broken into four major sections: the main bioreactor vessel itself, the pump, the media bottle, and the tubings which act as conduits to connect everything together. A bill of materials containing all the specific materials utilized by the team can be found in Appendix B: Bill of Materials, while a simplified checklist of each section and their components can be found in Appendix C: Bioreactor Components, which will be useful when assembling the full bioreactor system.

Main Bioreactor Vessel

The main bioreactor vessel can be broken down into four components, the two end pieces, the middle glass tube, and the middle insert in the glass tube. Additionally, there is a base mount onto which the main bioreactor vessel can be screwed onto. The packed bed of glass microcarriers are held in place inside the middle insert. The full vessel setup is shown below with labels of each component:
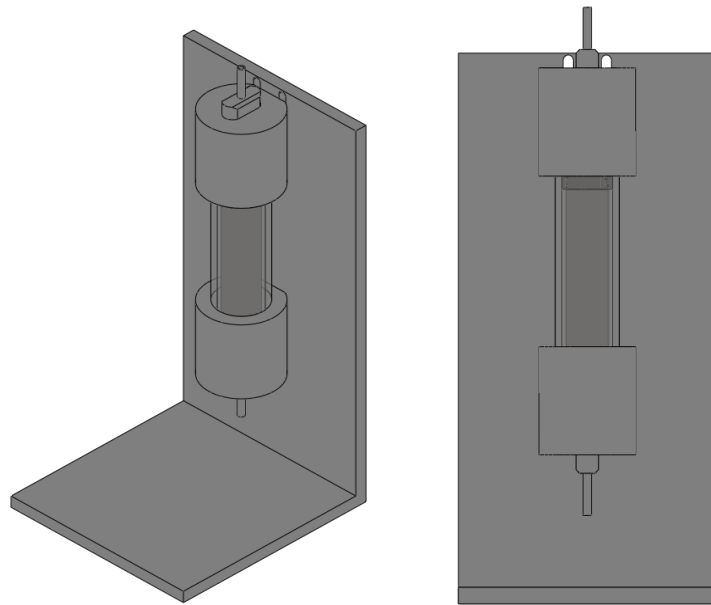
Figure 14. Solidworks models of main bioreactor vessel

ITEM NO.	PART NUMBER	Material	QTY.
1	Base	316 Stainless Steel	1
2	End Cap	316 Stainless Steel	2
3	Middle insert	316 Stainless Steel	1
4	Glass Tube	Borosilicate Glass	1
5	End Piece	316 Stainless Steel	2
6	Steel Mesh	316 Stainless Steel	2
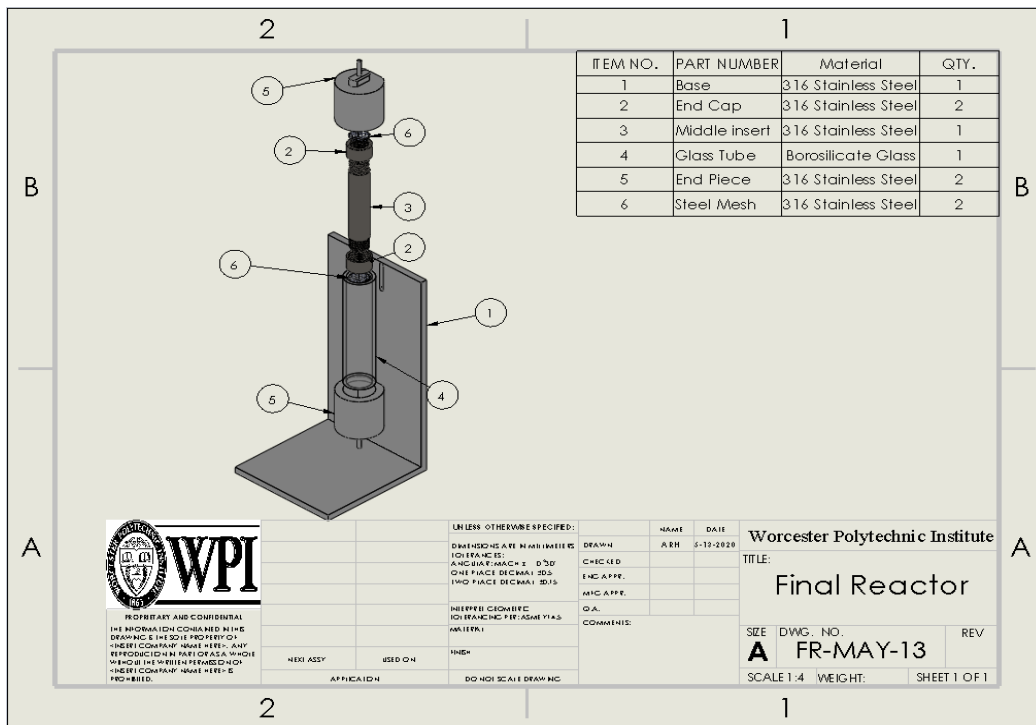
Figure 15. Solidworks drawing of main bioreactor vessel
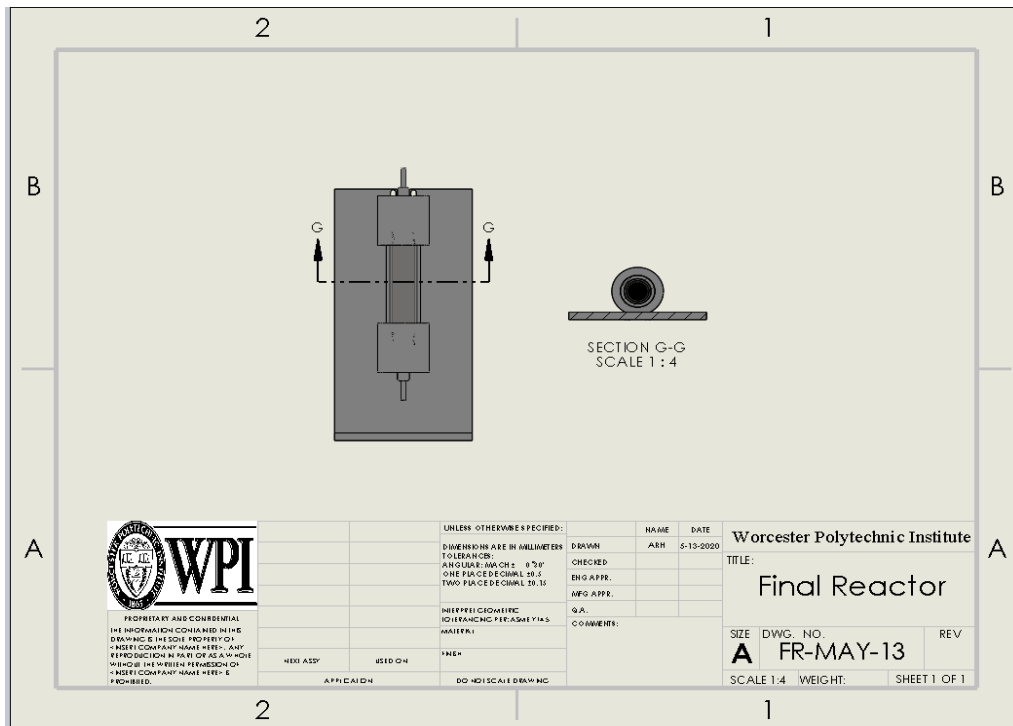
Figure 16. Solidworks drawing of bioreactor cross section

The first component to mention is the base mount. A full picture of the mount and its dimensions are shown below:
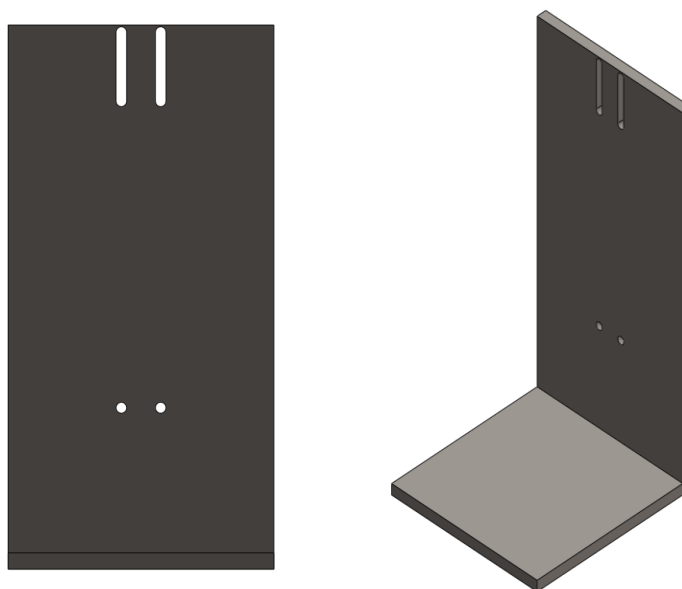
Figure 17. Solidworks models of base
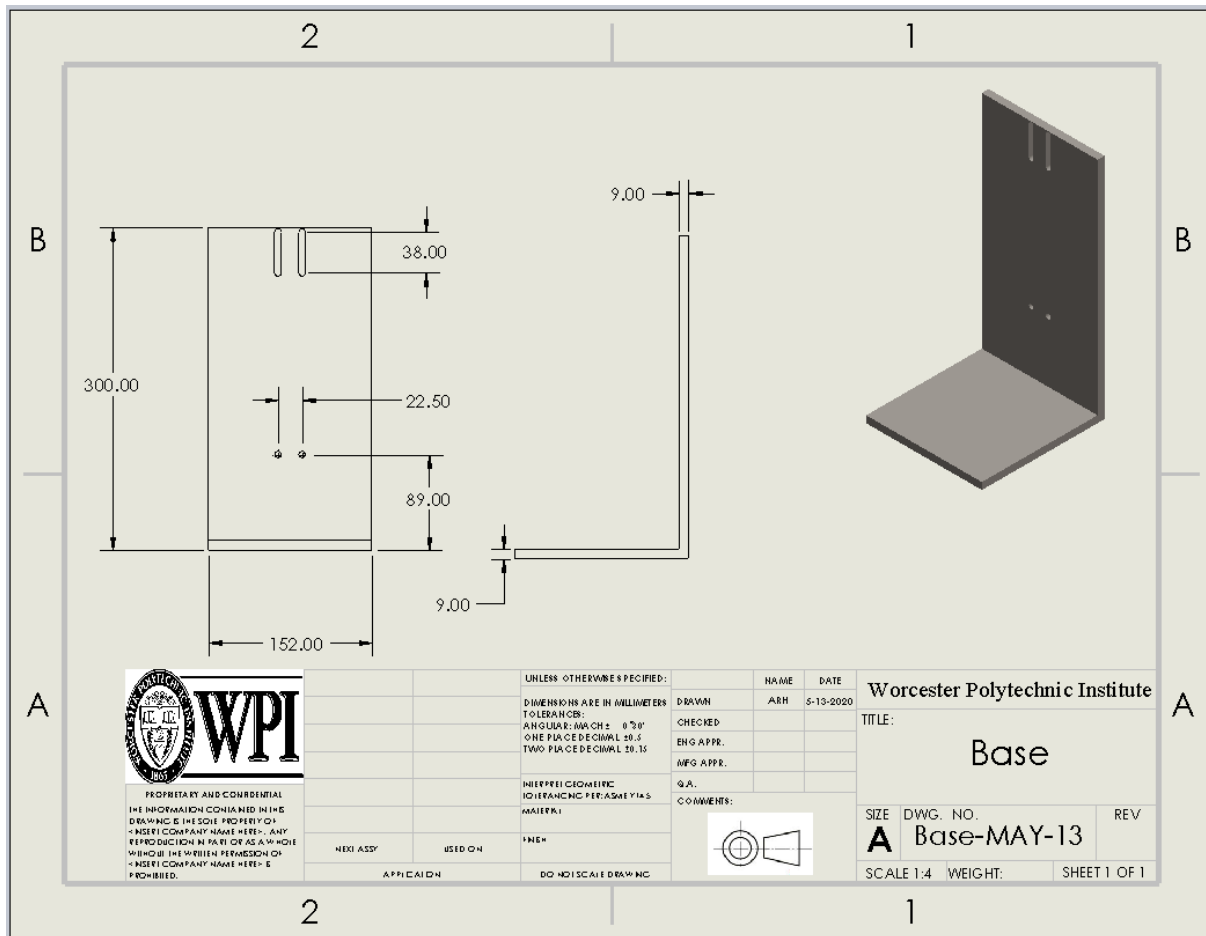
Figure 18. Solidworks drawing of base

This component is made of 316 Stainless Steel, allowing it to be autoclavable, and contains screw holes which line up with screw holes on each of the end pieces. This allows for the bioreactor vessel to be screwed onto the mount, suspending it so that it can be run vertically. This is important as this will allow the bioreactor vessel to be easily stored within an incubator, as the bioreactor can be mounted on the base, and then simply placed on a shelf.

The next components are the two end pieces. Picture of the front and back are shown below:
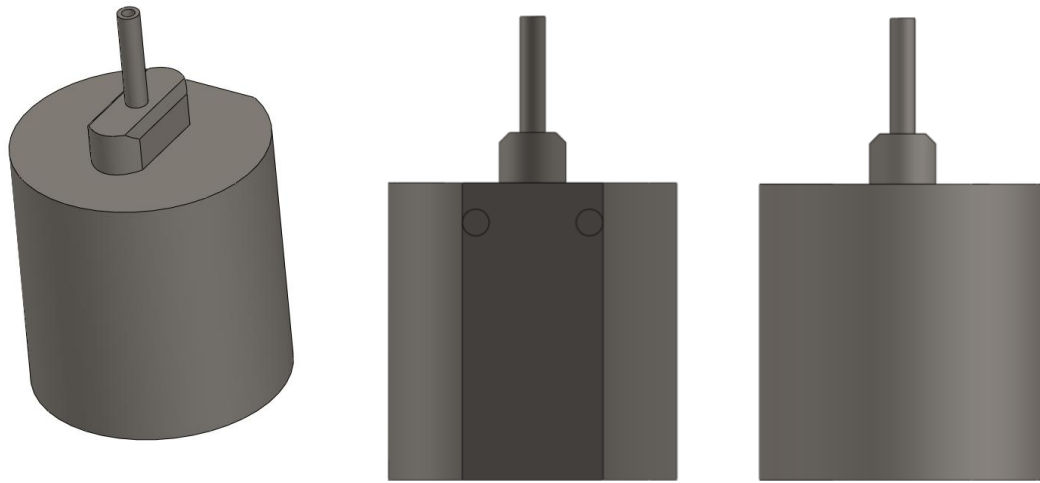
Figure 19. Solidworks models of end piece
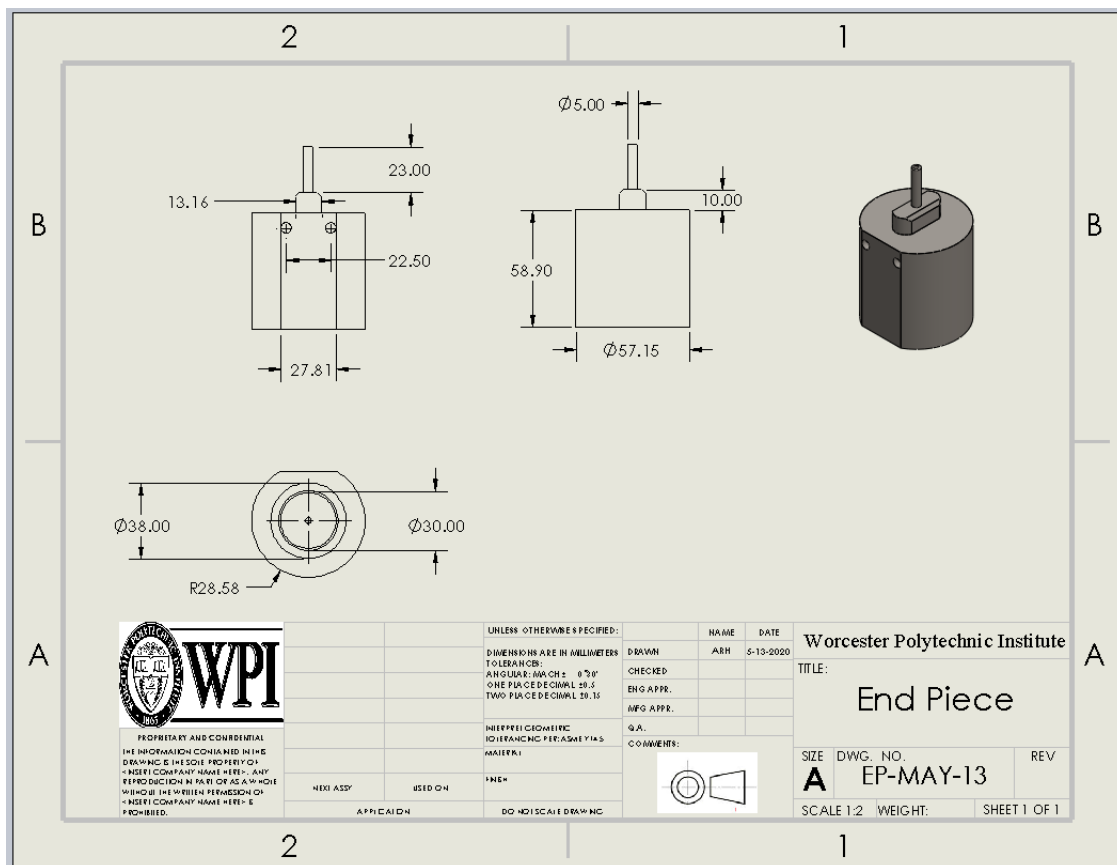
Figure 20. Solidworks drawings of end piece

Both of these pieces are made from 316 Stainless Steel as well, allowing them to be autoclavable. Both of these end pieces are attached to the top and bottom of the bioreactor vessel, serving as the inlet and outlet for the system. To accomplish this, both end pieces contain a hose nipple, allowing tubing to be connected to each one, and the end pieces themselves are hollow. Through this, cell culture media and any unattached cells will be flown into and out of the bioreactor in a continuous loop. The backs of each end piece also contain two screw holes, thereby allowing the bioreactor to be mounted onto the base mount. The inside of the end pieces is shown below:

Figure 21. Solidworks model of end piece cross-section

As shown, the hollowed opening from the hose nipple connector tapers out until the final inner diameter is reached. One important aspect of the end pieces is the placement of O-rings within each of them. A groove exists within the end piece, onto which the O-ring can be placed onto. This O-ring interacts with the glass tube, and since it is the same diameter as the glass tube, will create a water-tight seal, generating a leak-free interface so fluid will move smoothly between the glass tubes and the end pieces.

The next component is the borosilicate glass tube. This glass tube connects to the end pieces and acts as a housing unit to contain the middle insert. A picture of the glass tube is shown below:
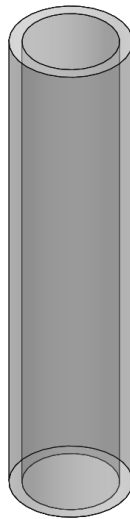
Figure 22. Solidworks model of glass tube
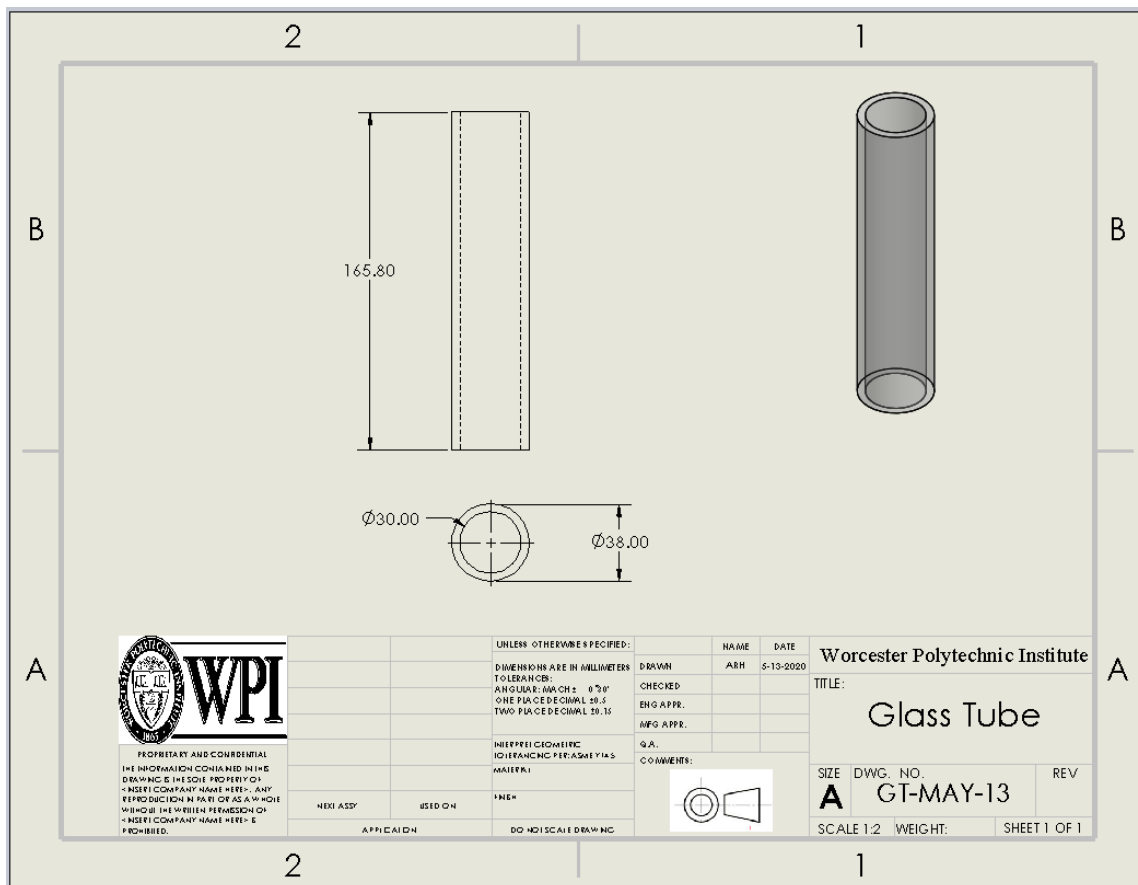
Figure 23. Solidworks drawing of glass tube

The glass tube is inserted into each of the end pieces through a twisting, pulling motion. The outer diameter of the glass tube is the same as the inner diameter for each of the end pieces, and so the interfaces created with the O-rings in each of the end pieces creates a water-tight seal. This glass tube functions as a container for the middle insert. Additionally, borosilicate glass is capable of withstanding autoclaving temperatures, thereby making it easily autoclavable with the rest of the system.

The final component for the vessel is the middle insert. The middle insert is responsible for holding the packed bed of microcarriers and ensuring that it is stable. Pictures of the middle insert and the screw caps as shown below:
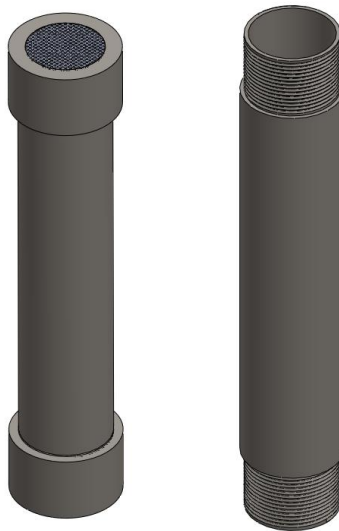
Figure 24. Solidworks model for middle insert
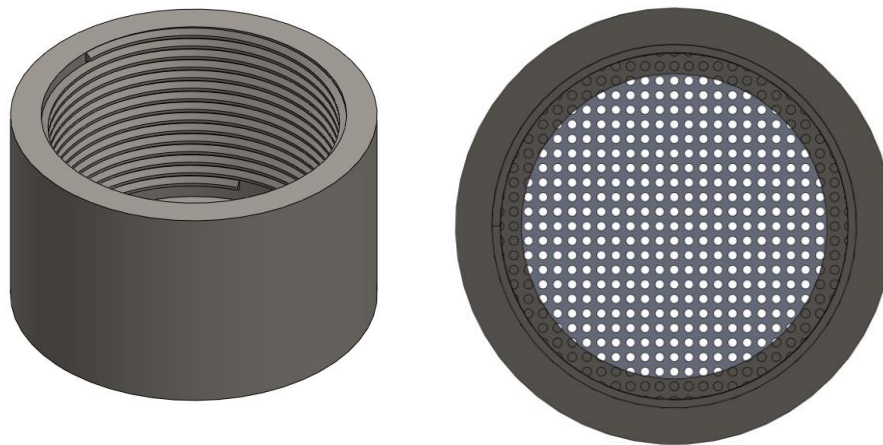
Figure 25. Solidworks model for middle insert end caps
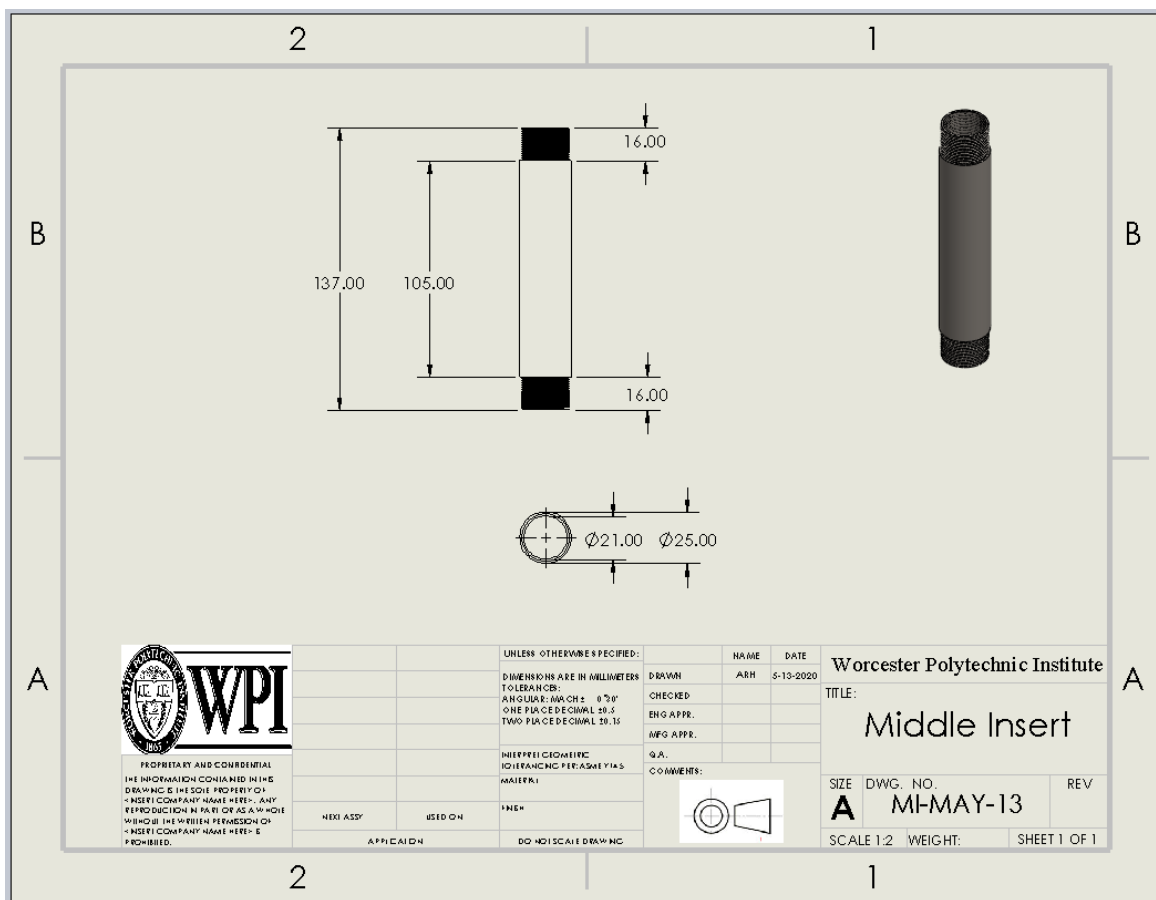
Figure 26. Solidworks drawing for middle insert
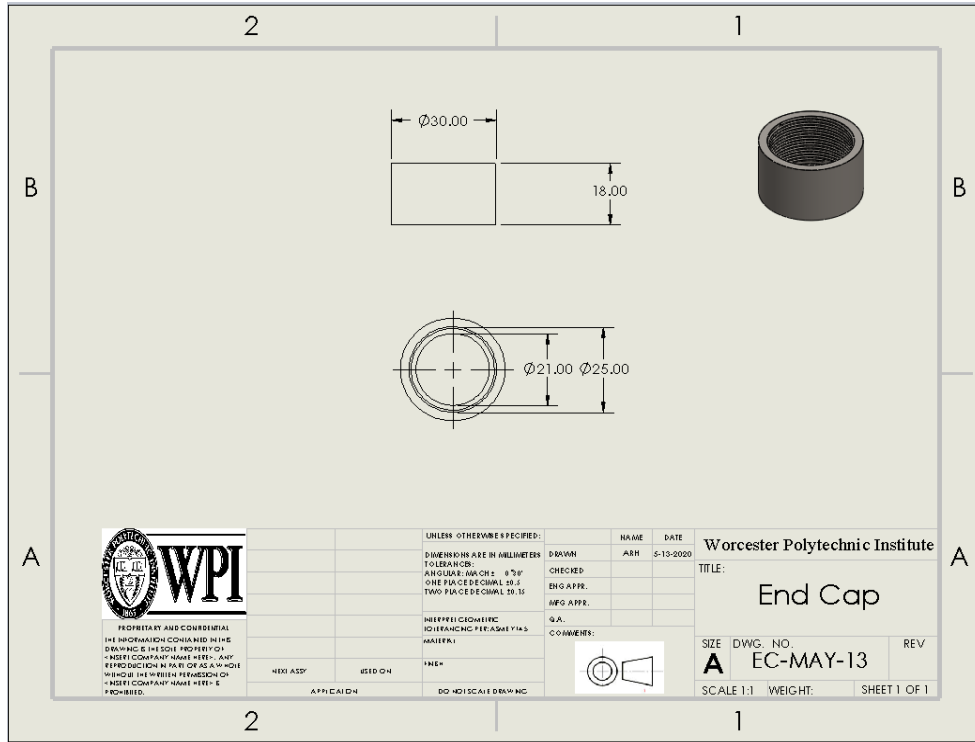
Figure 27. Solidworks drawing for middle insert end caps
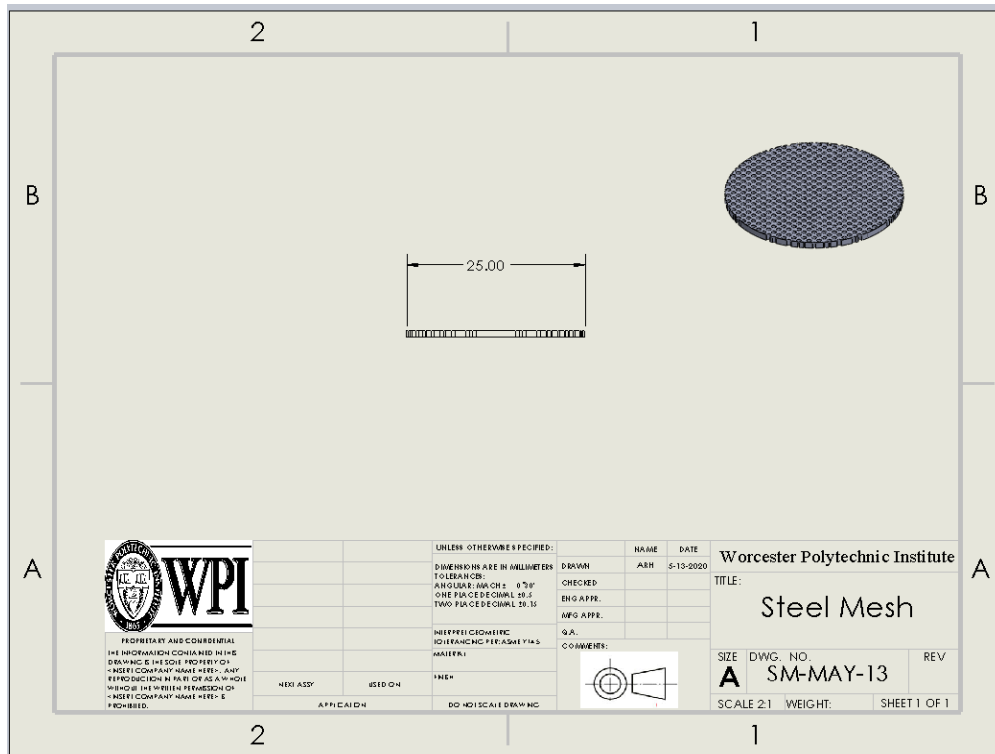
Figure 28. Solidworks drawing for steel mesh

As shown, the middle insert consists of a main vessel with two screw caps on each end. Both the main vessel and the screw caps contain threads, allowing the caps to easily be screwed onto the main vessel. Each of the screw caps contains a 316 Stainless Steel mesh screen, as well as an O-ring. The O-ring helps to create a water-tight interface with the main middle insert vessel, while the mesh screen is utilized to allow fluid flow while being small enough to hold the glass beads in place, thereby creating a packed bed of microcarriers capable of supporting bead to bead migration.

Peristaltic Pump

The peristaltic pump is an essential component to the bioreactor system, as it interfaces with the tubings in order to create continuous flow within the system. The utilization of the pump is shown below:

Figure 29. Bioreactor Interaction with Pump

The pump that it utilized is a Manostat E-Series Peristaltic Pump provided to the team by Professor Page. This pump runs at a volumetric flow rate of 56.8 mL/min, while being capable of supporting back pressures up to 172 kPa (or 25 psi) when dealing with continuous flows. As shown, the top port of the pump interacts with tubing connected to the media bottle. This is the

inlet port of the pump, while the outlet port of the pump interacts with tubing connected to the inlet hose nipple of the bioreactor vessel. As such, fluid will flow from the media bottle through the pump and into the bioreactor, eventually flowing out of the bioreactor through the outlet hose nipple and back into the media bottle. In the depiction above, the container acts as the media bottle of the system, which is described further in detail below. This establishes the continuous flow which would allow for media to be run through the packed bed of microcarriers containing cells, while any unattached cells will simply continue flowing through the system to provide more opportunities for these cells to interact with the packed bed and potentially adhere to a microcarrier.

Media Bottle

A media bottle is used in order to create a basin for cell media to continuously be pumped into and out of the bioreactor system. Unfortunately, the media bottle was unable to be set up due to circumstances with COVID-19. However, a 1L media bottle would have been utilized containing approximately 400 mL of cell media, which would have been enough volume to completely fill the bioreactor system while also having enough extra in the media bottle. The media bottle cap would have contained ports, onto which tubings can be connected to. A representative image is shown below [37]:

Figure 30. Media Bottle Setup

The representative image highlights a media bottle with two ports. For the purposes of the novel bioreactor system, three ports would have been utilized instead, with two of those ports containing dip tubes inside of the media bottle. These two ports would act as the "inlet" and "outlet" ports for the media bottle. The "inlet" port would connect to tubing that connects to the inlet port for the peristaltic pump, and through this port media and any cells in the media would be supplied into the inlet of the bioreactor. The "outlet" port of the media bottle would be connected with tubing connected to the outlet hose nipple of the bioreactor vessel. Therefore, media and any unattached cells will flow through the bioreactor outlet and back into the media bottle. Additionally, a third port will be utilized which contains a 0.22 µm filter. This filter will be utilized to help filter the air inside the incubator so that oxygen is capable of traveling into the media bottle and oxygenating the media, thereby providing an oxygen source capable of supporting the oxygen consumption of the cells in the bioreactor.

Tubings

The tubings utilized are simply polyurethane tubings found in the GH 207 lab with an inner diameter of 5 mm. It should be noted that generally polyurethane is not an autoclavable material, and as such for translation into cell culture these tubings should be replaced with silicone tubings. Silicone tubings are typically found in biomanufacturing processes due to their ability to be autoclaved with the full system.

The Packed Bed Design

As mentioned previously, the packed bed of microcarriers is contained within the middle insert. A simple depiction of this packed bed is visualized in Section 4.3.2 Alternative Designs in Figure 5. The packed bed utilizes glass microcarriers, specifically Megalux Uncoated Glass Beads 40/30 Mesh, with a diameter range of 425 - 600 µm. This packed bed is the core of the bioreactor system, as the dense packing helps to provide a volumetrically efficient system that maximizes surface area within a small bioreactor volume. The packed bed enables cellular migration in between each microcarrier, enabling a mechanism which allows for cells to migrate onto microcarriers which may not have initially contained any cells. As such, microcarriers will be maximally utilized within adherent cell culture.

The Full System

Each of these components are then combined to create the full closed-loop bioreactor system, as depicted below in Figure 31.

Figure 31. Full Bioreactor System

Through this closed-loop system, culture media containing nutrients, dissolved oxygen, and any unattached cells will constantly flow through the bioreactor vessel, promoting cellular growth and proliferation in the system while also enabling the recycling of unattached cells. This recycling is an important aspect as it will aim to maximize cellular adherence within the packed bed. The protocol to set up and break down the bioreactor system can be found in Appendix D: Protocols.

6.2 Mechanical System for Improved Insert Access

Insert Removal Process

1. Remove bioreactor from fluid system

2. Lift top end piece, insert pops up

3. Rotate glass tube forward and remove insert
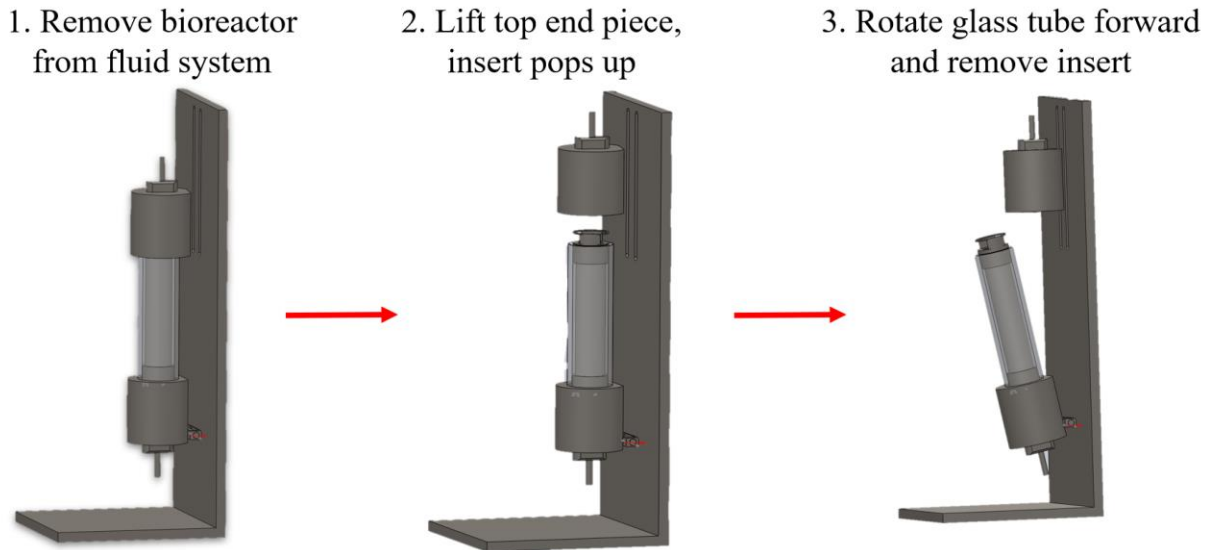
Figure 32. Insert removal process

Upon completion of the final reactor design, it was determined that the middle insert was too difficult to remove without disassembling the entire reactor, which raised both ease of access and contamination concerns. In order to address these concerns, it was determined that a mechanical system should be developed with the express goal of increasing ease of access to the insert as well as reducing human contact with the cells in the middle insert. In order to accomplish these goals, the main bioreactor vessel was outfitted with a series of modifications to its middle insert as well as the inclusion of a hinge attachment being incorporated into the bottom attachment point between the reactor shell and the base.

Constraints

Before designing the mechanical system, a few constraints needed to be recognized in order to ensure the success of the final design. Foremost, the entire mechanism was required to fit within the space of the reactor chamber. The final design of the main bioreactor vessel included only twenty-eight millimeters of clearance between the middle insert and the top of the

chamber. Therefore, the mechanism would need to be compact enough to be contained within this space while still meeting its goals. Additionally, the surface area provided within the reactor upon which to build supports for the mechanism was limited to that which was provided by the surface of the insert end caps. This was essential to maintain the fluid flow integrity of the system, and avoid disrupting or altering the flow of media through the reactor chamber. Furthermore, the mechanism was required to bear all mechanical stresses imparted upon it during both the removal process, as well as while the reactor is operating. Finally, the mechanism needed to be sterilizable through the same methods used to sterilize the middle insert itself, and would therefore be machined out of 316L stainless steel as a part of the existing designs.

Design Solution

In order to meet the design objectives, it was decided that the middle insert would be modified with a spring loaded mechanism and carrying handle that would allow for the middle insert to compress within the space of the reactor chamber and then extend to provide easy access to a technician trying to remove it.

Figure 33. Solidworks model of modified, spring loaded middle insert

In addition to the modified insert, it was decided that the reactor shell be given the ability to rotate forward and away from the reacto base in order to provide clearance to remove the

insert while not having to detach the upper end piece of the reactor. This would allow the user to slide the upper end piece up the reactor after loosening the bolts that attached it to the slots in the base, and then rotate the vessel forward in order to extract the insert. In order to accomplish this, a hinge was designed and modeled to replace the bottom base to end piece attachment point.

Figure 34. Solidworks model of hinge component

Mechanical Stress Requirements

Before designing the modifications that were to be made to the middle insert, an analysis of the expected maximum stresses that each component would need to endure was performed. This would ensure that each component would be given enough load bearing surface area in order to support the forces imparted upon it by the springs in compression as well as the removal process.

The first step undertaken to perform this analysis was to choose a spring that would be capable of supporting the weight of the insert, but not incorporate any excessive pressure that might damage the insert or the reactor chamber. For this reason, it was decided that a set of four small springs would be used. The springs used were determined to maintain a resting equilibrium length of twenty millimeters and have the ability to lift the insert ten millimeters above the glass tube upon removal of the top end piece. The weight of the backed middle insert was measured

and determined to be 6.67 Newtons, or about one pound. With these metrics determined, the following spring force equation was applied to determine the spring constant (k).

$$F/4 = kx \tag{6}$$

By substituting F and x for the weight of the insert and 0.01 meters respectively and solving for the spring constant, it was determined that each spring would hold a spring constant of 164 N/m. This value was then returned to the same equation evaluated at full compression of the spring ($x = 0.02$) in order to determine the force each spring would exert on the system when under full compression within the bioreactor. This force was multiplied by the number of springs (four) in order to acquire the total compressive force exerted by the springs on each component of the modified insert. The resulting force was calculated to be 13.37 Newtons. With the compressive force determined, the minimum surface area of the load bearing components could be determined.

Both ends of the modified insert would need to be capable of bearing the calculated compressive load once the mechanism entered compression in the sealed bioreactor, multiplied by a safety factor of 50 to account for any unforeseen additional compressive forces that may be applied. In order to calculate the minimum surface area required to withstand the compressive forces, the following equation was applied,

$$A_{sc} = 50F_c/Y_c \tag{7}$$

where A_{sc} is the minimum surface area, F_c is the compressive force exerted by the springs, and Y_c is the compressive yield strength of 316L stainless steel (170MPa) [38]. As a result, the minimum surface area required to withstand the compressive forces exerted by the springs was calculated to be $3.97 \times 10^{-7}\, m^2$.

In addition to compressive stresses, any handle designed to lift the middle insert out of the reactor chamber would need to support a tensile force equal to the weight of the weight of the insert multiplied by a safety factor of 50 which was determined to cover any unforeseen additional forces that may be imparted when lifting the insert. In order to calculate the minimum surface area, the following equation was applied:

$$A_{st} = 50F_w/Y_s \tag{8}$$

Where A_{st} is the minimum surface area, F_s is the force imparted by the weight of the insert, and Y_s is the tensile yield strength of 316L stainless steel (205MPa) [39]. As a result, the minimum surface area required to bear the tensile stresses at a safety factor of 50 was determined to be $1.3 \times 10^{-8} \, m^2$.

Design Solution

Once the mechanical stress requirements were determined, the physical modifications could be designed. Keeping the constraints and minimum surface area requirements in mind, the handle and spring loaded modifications to the middle insert could be designed. The first component of the modifications was the spring loaded mechanism. This mechanism is composed of three components (listed in appearances from left to right in the figure below): the platform, the four springs, and the housing.
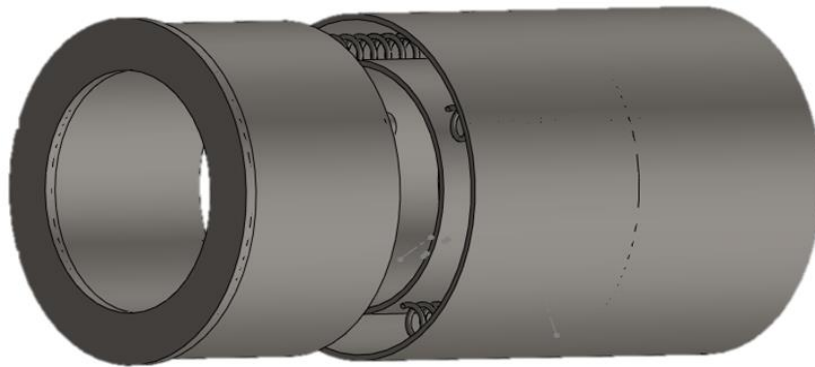
Figure 35. Spring loaded mechanism

The mechanism housing consists of a hollow cylinder designed to fit on top of the bottom cap of the middle insert. It contains a set of four pegs to hold the springs in place, and is capable of encapsulating both the springs and the platform.

Figure 36. Solidworks model of housing (no springs)

Figure 37. Solidworks model of housing (with springs)

The platform component is to fit snugly within the housing when the mechanism is under full compression, but is then used to provide a base off of which the springs can drive the middle insert upwards upon opening the bioreactor. It consists of a hollow cylinder with pegs similar to the housing in order to ensure the stability of the springs. Additionally, given that this component will be the main stress bearing feature of the spring mechanism, the stresses imparted onto the platform were calculated.

Given that the spring mechanism will not be used to lift or manipulate the insert, it only bears the compressive force applied by the springs when the insert is under compression. In order to calculate the compressive stress that the platform will undergo, the load bearing surface area was calculated using the *Solidworks* measuring tool and determined to be 259.15mm^2. With this

information, alongside the force calculated for the compressive force exerted by the springs, the compressive stress in this part can be found using the following equation:

$$\sigma_c = F_s/A_s \tag{9}$$

Where σ_c is the compressive stress, F_s is the force exerted by all four springs, and A_s is the load bearing cross sectional surface area of the platform. The compressive stress without the safety factor was calculated to be 52.054 kPa, and 2.934 MPa with the safety factor taken into consideration. Both values are comfortably below the 170 MPa yield stress of 316L stainless steel.

The second modification made to the insert was the inclusion of a handle on the top cap. This handle would be the piece that protrudes above the glass cylinder once the reactor is opened, allowing for the insert to be removed easily. The component was designed fit the curvature of the glass tube in order to limit its effect on the fluid flow of the system, as well as provide a structure that could be easily lifted with either forceps or a glass rod. The design consists of a ring handle mounted on two curves supports, as shown below:
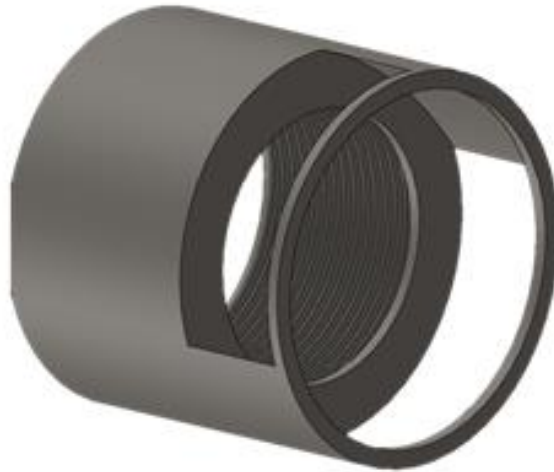
Figure 38. Modified insert cap with handle

This handle would need to withstand both tensile and compressive stresses, therefore both types of stress were calculated for each support using equation (9), substituting for the tensile force when applicable. The cross sectional load bearing surface area of each support was

calculated once again through *SolidWorks* and determined to be 16.11mm^2. The results are presented in the table below:

Table 12. Handle stress calculations

	Compressive Stress (MPa)	Tensile Stress (MPa)
Without x50 safety factor	829.9kPa	276.1kPa
With x50 safety factor	20.934MPa	6.905MPa

Finally, a hinge was added in order to attach the bottom end piece to the bioreactor base while also allowing it to swing forward in order to remove the insert. The hinge was designed using two nested brackets and a pin, as shown below:
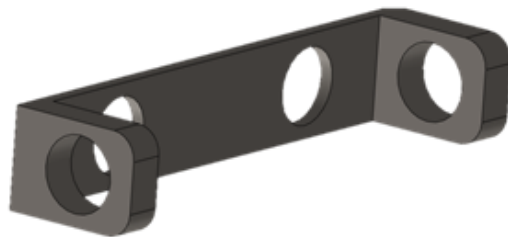
Figure 39. Outer hinge bracket

Figure 40. Inner hinge bracket

Figure 41. Hinge Pin

The hinge would be assembled and used to attach the bottom of the bioreactor to the base, as shown in Figure 34.

Maintaining Fluid Flow Integrity

      In order to maintain the integrity of media flow through the perfusion system, all surfaces of contact should be coated in a bio-safe rubber film that will allow for a complete watertight seal when the insert is under compression. These surfaces include the top and bottom ends of the modified insert, as well as the exposed lips between the spring housing and platform. The surfaces mentioned are depicted in the image below:

Figure 42. Rubberized insert surfaces

Overall Process

The overall process of removing the middle insert with the addition of both the modified insert caps as well as the hinge joint is depicted in Figure 32 above. It begins by disconnecting the bioreactor from the fluid system and placing it under a fume hood. This is followed by the loosening of the rear bolts that secure the top end piece, and sliding the end piece upwards in order to release the spring mechanism. The top end piece is then secured, and the reactor is leaned forward enough to remove the middle insert. It is then recommended that a sterilized tool, such as forceps or a glass rod slipped under the handle ring should be used to lift the insert out of the chamber. This will eliminate the need for human contact with the insert, reducing the risk of contamination. Additionally, the rubberized surfaces of both the handle and the spring loaded mechanism should create a watertight seal between both the chamber and the insert, as well as within the spring housing. This should maintain fluid flow, as well as secure the insert in a fixed position within the chamber.

6.3 Industry Standards

When designing the bioreactor system, it was important to consider the following standards/regulations when it comes to working with bioreactors and living cells. These standards were essential in determining the possible ways to achieve our goal and also in determining the success of the final design. A summary of the standards considered with the fabrication of the novel bioreactor are shown in Table 13 below:

Table 13. Industry Standards Considered in Design

ISO 11737	Sterilization of Medical Devices: Microbiological Methods, Part 1 — Determination of a Population of Microorganisms on Products
ASTM F1980:2007	Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices
ISO 13845:2016	Medical devices - Quality management systems - requirements for regulatory purposes
ISO 10993-5:2009	Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity
FDA 21CFR866:2018	Title 21, Subchapter H, Part 866 - Cell and Tissue Products

The first standard that was considered was ISO 11737 (Sterilization of Medical Devices: Microbiological Methods, Part 1 — Determination of a Population of Microorganisms on Products) [22]. Sterility is extremely fundamental for the operation of a bioreactor as contamination must be avoided before the bioreactor is inoculated with cells. As such, the sterility of our novel bioreactor will be in accordance with this standard [22]. The team designed the bioreactor system so that it was reusable, and so it must be sterilized in between uses. This standard can be used to determine the sterility of the bioreactor from use to use. The standard establishes the requirements for validation and control of sterilization, such as how to determine whether or not the reactor is sterile. If the bioreactor contains the same amount of microorganisms on it as before sterilization it is considered not sterile and must be re-sterilized and checked again.

The second standard that was considered was ASTM F1980:2007 (Standard Guide for Accelerated Aging of Sterile Barrier Systems for Medical Devices). This standard was used in terms of the sterility of the barrier system in the bioreactor [23]. It is essential that sterility is maintained over a prolonged period of time, as bioreactor operations can range from days to weeks. This standard addresses the accelerated aging theory, in which properties may change over time, affecting the proper function of a sterile barrier system. As stated previously, it is essential to ensure that the inside environment of the bioreactor is sterile when in use and no contamination will occur.

The third standard that was considered was ISO 13845:2016 (Medical devices - Quality management systems - requirements for regulatory purposes). The design of the novel bioreactor needed to be in accordance with this standard [24]. This standard provided guidelines throughout the entire design process, from ideation to prototyping and final design fabrication, in order to ensure that the product's function and quality sufficiently met the client's requirements. The product must function the way it is intended and accomplish all parts of the revised client statement. As such, this standard was relevant to the design process.

The fourth standard that was considered was ISO 10993-5:2009 (Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity) [25]. This standard is useful in determining the toxicity of a material, as it specifies various types of tests that can be performed in order to determine whether or not a material is toxic to cells when in use. This standard is important because the bioreactor could not introduce toxicity to the cells, as this would

compromise the function of the cells and the operation of the bioreactor.

The fifth standard that was considered was FDA 21CFR866:2018 (Title 21, Subchapter H, Part 866 - Cell and Tissue Products) [26]. These standards/regulations can be used when it comes to testing the bioreactor with live cells. These regulations help to define and specify many parts of the cell culture process such as culture medium, quality control kits, and microbial monitoring, which will be important in the operation and testing of the bioreactor system.

6.4 Economic Impact

The economic impact of the device lies in the microcarrier technology that is being used in the system. The novel bioreactor used glass beads as the microcarrier technology compared to the Cytodex bead technology that the control bioreactor uses. The glass beads are much more widely available and a fraction of the cost. One economic increase would be the cartridge that hosts the microcarriers. Although the system is reusable by simply re-sterilizing it, the cartridge that holds the microcarriers would be exchanged between uses and would therefore be an added ongoing expense after the initial purchase of the entire system. The entire device is made of affordable materials including stainless steel and glass and is able to be assembled in approximately two weeks, which would allow the user the ability to begin use in order to begin production quickly. An indirect economic impact would be the cost of labor for a person operating the bioreactor. The current method for adherent cell culture in a bioreactor requires a person to operate the system in the beginning stage to continuously start and stop the system in order to allow the cells to settle and attach to the microcarriers. This is no longer necessary with the novel bioreactor system, therefore eliminating additional manpower.

6.5 Societal and Political Impact

The utilization of bioreactors within the greater context of the medical field may yield societal benefits as larger scale biomanufacturing may occur with the production of biologics to treat diseases or concerns. The optimization of adherent bioreactor technology would promote scalability, thereby allowing adherent bioreactors to eventually be utilized in these large scale productions. An example of this is the usage of bioreactors within tissue engineering applications, where the bioreactor acts to reproduce physiological conditions in order to promote

and maintain cell culture with tissue engineering applications. Bioreactors are extremely useful for tissue engineering with the creation of a 3D environment that is more physiologically relevant than a simple 2D adherent culture. One specific application for tissue regeneration is the utilization of bioreactors in research aimed at producing pancreatic progenitor cells for the treatment of patients with diabetes [40]. The growth of tissue engineering will have a massive impact on the medical field, and on society in general, with the great potential to augment, maintain, or recreate functions within the body which may have been lost or negatively impacted due to disease, trauma, or medical conditions.

The potential impact on politics can be regarded with two different perspectives: in the context of politics within companies and in the context of consumerism and market regulations. Typically within companies, medical pursuits involve politics as economic profit and gain are always considerable factors with the development of technologies. Although a medical device or product may be extremely beneficial, politics can dictate whether or not the technology is developed or pursued if it is too costly. The utilization of an economically viable adherent bioreactor such as our design may help to alleviate these types of cost-benefit analysis if the price of large scale biomanufacturing can be mitigated. Additionally, political impact in terms of market regulations should be considered as well. One specific application of bioreactors is the creation of lab cultured meat, which is particularly useful in replacing current sources for meat while also mitigating environmental impact in its production. The politics regarding the production and marketing of lab cultured meat is important as large regulatory bodies such as the FDA will have to be heavily involved in ensuring proper safety and efficacy [41]. This is applicable to any medical product created utilizing bioreactors, as the FDA or other regulatory entities will have to be involved to ensure the product is able to be marketed.

6.6 Environmental Impact

The two main materials utilized in the fabrication of the bioreactor is 316 stainless steel and borosilicate glass. These materials should have minimal impact on the environment, thereby mitigating the potential environmental impact our device may have throughout its fabrication process and afterwards if it is disposed of. Stainless steel is considered an environmentally friendly product as it is 100% recyclable, while it also typically lacks coatings that could potentially be environmentally toxic if it leeches. During the production phase, stainless steel is

typically made from raw material, with up to 70% of the material being produced from recycled material [42]. Borosilicate glass should not have any notable environmental impact as well, however one aspect to note is its potential human impact during the production phase. Borosilicate glass is made from crystalline silica, and during its production crystalline silica is considered a health and disease risk if large amounts are inhaled. However, typically manufacturing facilities have proper policies and protective equipment in place to help mitigate these risks [43].

6.7 Ethical Concerns

Given that the product of our design is aimed towards adherent cell culture, this can eventually be translated into stem cell research and stem cell therapy, and therefore it is important to mention the ethical concerns pertaining to our project. Stem cell research can be controversial because it can involve the utilization and harvesting of human embryos. Since our device has no part in the development of stem cells, we do not foresee any ethical issues with the creation and use of our design.

6.8 Manufacturability

Utilizing the given schematics and SolidWorks drawings, the bioreactor system can be reproduced as long as the correct materials are utilized, which can be found in Appendix B: Bill of Materials, with the specific suppliers utilized for any specific components. Everything was purchased or manufactured in a reasonable time frame, while staying significantly under the $1,000 MQP budget.

Chapter 7: Discussion

This project was designed to create a novel bioreactor system capable of enhancing cellular attachment and confluency in comparison to current adherent bioreactor systems. Achieving this goal would help to generate higher final cell densities, thereby supporting larger-scale adherent cell operations. Several preliminary experiments were run, which highlighted that the objectives of optimizing cell attachment and creating a continuous flow were achieved.

7.1 Volumetric Efficiency Comparison

As shown in Section 5.1 Volumetric Efficiency Comparison, our novel system is found to be 6.5-times more volumetrically efficient assuming 100% confluency in both systems. With the assumption of 100% confluency, this increase in volumetric efficiency represents the upper possible limit. It is likely that the cells within both systems will not fully reach 100% confluency, and so it will be likely that the increase in volumetric efficiency is actually smaller than 6.5-times, however if both systems were utilized until they could no longer support any more cell proliferation, the number of cells in the novel system will be significantly higher if this system has the same volume as the Cytodex system. Therefore, this demonstrates the feasibility of higher final cell densities in the packed bed bioreactor when both systems are used at the same volume, with the maximum possible increase being 6.5-times.

The main reason for this efficiency is that the packed bed will allow cells to migrate between microcarriers, something that is extremely difficult when the microcarriers are in a stirred tank bioreactor. The Cytodex system is not volumetrically efficient due to the fact that only a specific number of microcarriers can be supported per unit volume of the bioreactor, and these microcarriers are widely spread apart with dead space between each microcarrier due to the homogeneous mixing of the system. In comparison, the packed bed bioreactor minimizes the dead space between microcarriers, allowing for significantly smaller bioreactor volumes which would contain the same amount of microcarriers as the Cytodex system. As such, smaller bioreactor volumes would be needed to achieve the same number of cells as the Cytodex system since the system is more volumetrically efficient. This packing and cellular migration supports greater amounts of cells in smaller bioreactor volumes.

7.2 Microcarrier Optimization

The glass beads that were not modified resulted with cells congregating mostly in the spaces between the beads rather than on the surfaces of the glass beads. This can be seen in Figure 7. The gelatin coated glass beads did show some cells on the surfaces of the beads. However, the amount of cells on the beads was less than that of the control Cytodex microcarriers due to the difficulty of the coating process. The wide dispersity of the cell placements on the glass surface could be contributed to the fact that the gelatin coating was difficult to administer so the beads were unable to receive an equal coating throughout. The gelatin coated glass beads can be observed in Figure 8. The plasma Treated glass beads showed a much more even distribution of cells on the entire surface of the beads. This modification also displayed some bead to bead migration from cells. The oxygen plasma Treatment was able to be fully dispersed onto the entire surface of the bead allowing in even dispersion of the cells on those surfaces. The cells can be seen on the plasma treated glass beads in Figure 9. The image comparisons from the Cytodex to the plasma treated glass beads show more cells distributed throughout all layers of the bead surface as well as bead-to-bead migration of the cells on the glass beads that is not seen in on the Cytodex microcarriers.

7.3 Ergun Equation and Oxygenation Calculations

The calculations shown in Section 5.3 highlight that theoretically, the novel bioreactor system is capable of supporting adherent cell culture. This is due to the fact that for packed beds with a volumetric flow range of 30-75 mL/min, the calculated pressure drops across the packed bed were very small and almost negligible, ranging from 870-2210 Pa. When compared to the 172 kPa back pressure supported by the provided peristaltic pump, it is demonstrated that the pump would have been capable of supporting this range of flow rates since their associated pressure drops were extremely small in comparison. As such, theoretically the pump should be able to achieve continuous flow within the system, which is later confirmed with the preliminary fluid tests shown in Section 5.3. The associated shear stress with these volumetric flow rates was also calculated, ranging from 2.63 - 6.66 dynes/cm^2. The shear stress limit of 6.5-10 dynes/cm^2 is achieved when the flow rate within the system is 75 mL/min, with a calculated shear stress of 6.66 dynes/cm^2. The provided peristaltic pump runs at a flow rate of 56.8 mL/min, and therefore

should not create shear stresses that are significant enough to influence the morphology or metabolism of the cells, and should not rip off the cells from the microcarrier surfaces.

Additionally, the bioreactor system should have been able to provide enough oxygen to support the adherent cells in the packed bed. This is confirmed when comparing the theoretical total oxygen consumption rates with the theoretical total oxygen delivery rates. For a volumetric flow rate range of 5-50 mL/min, the oxygen delivery rates of 3.33 - 33.3 x 10^{-4} mg/sec were significantly higher than the theoretical oxygen consumption rate of 1.07 x 10^{-4} mg/sec, thereby confirming that the bioreactor should be able to support the oxygen requirements of the cells in the packed bed since the flow rate of the provided pump is 56.8 mL/min. However, it should be noted that all these calculations are simply theoretical. Unfortunately, due to circumstances surrounding COVID-19, the team was unable to examine adherent cell cultures in the novel bioreactor system, and therefore was unable to confirm that the cells were capable of being supported.

7.4 Bioreactor Fluid Test

The fluid test with the final design confirmed that no leaks were present in the bioreactor system and that continuous flow was achieved. This continuous flow is significant because it is the main mechanism behind seeding the cells onto the microcarriers in the packed bed, while providing nutrients, oxygen, and any unattached cells so that they have the opportunity to adhere to microcarriers in the system. One important observation was the fact that when the fluid test was run, it was observed how green dye built up around the middle insert. Ideally, there should be no fluid movement around the middle insert containing the beads, since the media should be directed through the end pieces and up into the middle insert. However, the presence of green dye around the insert highlighted that this was not the case. This is due to the fact that the glass tubes purchased were not perfectly uniform in the inner diameter across their lengths. Due to this, during the manufacturing process the outer diameter of the middle insert could not have been manufactured exactly to match the inner diameter of the tubing. If this was possible, this would allow the middle insert to simply be press-fit into the tube, naturally creating a water-tight seal so that the media would only be capable of moving through the middle insert rather than around it. This was an inherent limitation with the purchased glass tube, and as such should be something that is improved upon in the future. The ability of the media to build up around the

middle insert may impact the continuous flow of unattached cells, as it is possible for cells to instead build up around the middle insert instead of flowing through the packed bed. However, besides this limitation, the fluid test confirmed that the objective of continuous flow was achieved.

7.5 Novel Bioreactor Comparison Study

Unfortunately, a comparison study was not able to be conducted due to circumstances with COVID-19. As a result, there is no concrete data on whether or not the novel bioreactor would have been capable of supporting adherent cell culture in an enhanced manner in comparison to the current bioreactor system. The following section will thereby discuss the expected results of this study.

7.5.1 Nutrient Tests

The generation of nutrient curves would have allowed comparisons between the current intermittent stirring bioreactor system and the novel bioreactor system. It would be expected that both contain similar nutrient curves, comparable in terms of trends to the sample nutrient curve given in Section 5.5.1. However, deviations from between the two curves would potentially indicate differences between the two bioreactor systems, which is the expected result. For example, if the novel bioreactor was capable of supporting enhanced attachment efficiencies, this would result in higher cell densities. Due to this, nutrient depletion and waste generation would be faster corresponding to these higher cell counts, resulting in a nutrient curve with more exaggerated trends in comparison to the adherent intermittent stirring system.

One important aspect of this test would have been the utilization of a control, which simply would have been nutrient testing of the cell culture in a regular T-flask. These baseline nutrient curves would establish how nutrient depletion or waste generation typically trends in a normal adherent culture before being translated into a bioreactor system. These trends can then be related to observed cell population doubling by observing the relative confluency of the cell culture. As such, trends in nutrient levels can be corresponded to cell doublings, establishing expected percentage increases or decreases whenever the population doubles. Ideally, these same trends would be observed within the bioreactors, however as previously stated, it is expected that

the novel bioreactor would have experienced higher cell densities. As such, it would have been possible to extrapolate information on cell doubling and cell growth within the novel system based on the generated nutrient curves and how the percentage increases or decreases would have compared to the control nutrient curves. It would have been expected for the cell doubling to still remain approximately 24 hours, however the team would have been able to observe when these doublings occurred, helping to characterize growth within the bioreactor and how cell growth compared with the Cytodex bioreactor.

7.5.2 Final Cell Densities

The final cell density test between the novel bioreactor system and the current intermittent stirring system would have compared cell attachment efficiency. From the microcarrier comparison study it is expected that the novel bioreactor system would have allowed bead to bead migration of cells, which is not currently achieved by the intermittent stirring system. This bead to bead migration would have resulted in higher cell densities for the novel bioreactor system because the beads had more surface area for cells to attach to and grow on. Also from the volumetric efficiency calculations between the two bioreactor systems it would have been expected that the novel bioreactor system would have an expected 6.5-fold increase allowing for a larger area for cells to adhere to. This could potentially result in 6.5 times more cells compared to the current state of the art system. A higher attachment efficiency of the novel system would allow for a potential increase in production of cells using a bioreactor system.

Chapter 8: Conclusions & Recommendations

With the final bioreactor system being constructed, several objectives were achieved with the end goal of improving adherent efficiency and viable cell densities for adherent cell bioreactor systems. First, optimization of microcarrier cell attachment was achieved through the examination of various microcarrier materials and demonstrating the possibility for bead to bead migration with cells in a packed bed system. The team was able to perform the glass bead attachment experiment comparing Cytodex 1 beads and glass beads with various surface modifications in static culture. The experiment proved that at the very least glass beads modified with gelatin and plasma treatment were comparable to the Cytodex 1. The plasma treated glass beads showed a more unified distribution of cells on the entire surface of the glass bead compared to that of the gelatin treated beads. This was most likely due to the difficulty of uniformly treating all areas of the glass beads with gelatin. The plasma treated glass beads showed the migration of cells from one bead to another bead in order to increase the surface area the cells were limited to grow on. The migration of cells could result in higher cell densities because they are not limited to the surface area of just one bead, as is for the current state of the art system.

Afterwards, with the bioreactor being constructed, continuous flow was demonstrated as feasible with the system. This continuous flow is important because it would theoretically help to meet the objective of increasing attachment efficiency through the recycling of unattached cells within the closed-loop system. Additionally, the various verification calculations demonstrated that the flow created by the peristaltic pump would not have generated a significantly high shear stress on the cells, and the system should have been able to provide sufficient oxygenation for the cells. Unfortunately, the team was unable to examine the performance of the bioreactor with cells, and therefore was unable to demonstrate whether or not the bioreactor was capable of enhancing cellular attachment in comparison to the current intermittent stirring adherent system. This would meet the objective of promoting maximum confluency by allowing cells to migrate onto microcarriers which previously did not contain any cells from the initial seeding process. Volumetric efficiency was demonstrated with the novel bioreactor as calculations highlighted that our packed bed bioreactor achieves a 6.5-fold increase in volumetric efficiency in

comparison to the Cytodex microcarrier system. This is significant as it would allow for higher cell densities to be achieved in lower amounts of volume.

In terms of limitations, one important limitation was discovered through the fluid test, which demonstrated how fluid is capable of flowing around the middle insert rather than solely be directed through the packed bed. This was due to the fact that the glass tube purchased for the project was not perfectly uniform in its inner diameter along its length, and therefore the middle insert could not have been designed to be the exact inner diameter of the glass tube, which would have allowed the insert to be press-fit into the tube. This should be taken into consideration in any future work, and any glass tube utilized in recreating this system should either have a constant inner diameter or should be manufactured independently in order to ensure a proper fit with the middle insert and the glass tube. Another limitation for the design was the utilization of the incubator in GH 006. There are a wide variety of incubators with varying sizes, and the incubator available for the team was on the smaller side. As a result, the incubator could not be manufactured to the size of regular benchtop bioreactors, and was restricted to the size of the small incubator. If possible, a larger incubator should be utilized for the bioreactor so that it is not limited in terms of height and size, which may allow for more optimal packed bed lengths.

In terms of future work, there are several recommendations that the team suggests. The team suggests that the comparison study be completed following the protocol in Appendix D: Protocols titled Bioreactor Comparison Test. This would allow for the novel system to be tested in order to show the possible impact it may have on adherent cell culture.

The insert in which the glass beads are packed was unable to be made see-through due to time and manufacturing restrictions. It is suggested that the manufacturing process be modified in order to allow the insert to contain a hole in which the user can observe and image the cells on the beads. This would allow for the packed bed to be imaged without taking the system apart and therefore stopping and contaminating the system.

An additional recommendation would be to test different cell lines in the system with the plasma treated glass beads. This would allow for a validation of the system in terms of how it works with other forms of adherent cells. The cells that were used in the static culture experiment were human dermal fibroblast cells and it would be assumed that other adherent cell lines would produce similar results in terms of attachment.

A final recommendation would be to optimize the bioreactor design. In terms of optimizing the design, it would be ideal to make the reactor incubator-independent like most current benchtop bioreactors. This would involve adding a thermal jacket for temperature regulation, an oxygen supply for proper culture oxygenation, and nutrient supply for the system. The reactor may also be modified to interface with process control sensors like current benchtop bioreactors so that temperature, pH, and oxygen can be directly measured. Additionally, the scalability of the bioreactor should be confirmed with the manufacturing and testing of larger-sized models to ensure that in the end, this bioreactor design will be capable of supporting larger biomanufacturing processes.

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Appendix

Appendix A: Interviews with Chris Bellerive

Throughout the course of the project, we had met with Chris Bellerive from WPI's Biomanufacturing Education and Training Center, who had served as our client to provide insight into the current biomanufacturing industry, the current state of the art, and limitations associated. The questions are provided below along with a paraphrased version of Chris's answers.

Meeting 1 - November 12, 2019

Q: What are the existing issues with the microcarrier system of GE Cytodex beads?

A: The challenging issue is trying to attach cells onto the microcarriers within the stirred tank bioreactor. Some cells are capable of attaching while others are not. So the current issue right now is how do we increase efficiency? During the intermittent stirring process, ramping up the agitator can potentially strip cells off the beads that are already attached. Ideally, the shears shouldn't rip off cells while also maintaining mixing. Additionally, there is the issue of utilizing surface area to increase the amount of cells that attach versus those that do not. Maybe there could be a way to create a system that would create more surface area? For example, what if the microcarriers are able to lock together, thereby creating more surface area to help attach cells and provide more surface for the existing attached cells to continue to grow.

Q: Why utilize the microcarriers instead of using other forms of scaffolding?

A: There really isn't anything out there for larger scale bioreactor, mainly just the microbeads. A potential idea could possibly be creating a scaffold, maybe with an adjusted impeller design, so that lower shears would avoid stripping cells, while the scaffold would offer a large surface area for cells to attach to.

Q: What are the challenges and effects of shear stresses?

A: There currently isn't too much information out on shear forces within the bioreactor system. Ideally, you cannot have high shear as this would be damaging to cells. However, there are

already optimized impeller designs such as pitched blade designs that aim at helping with issues surrounding shear force. Shear isn't much of an issue for current suspension cells, however it would be very useful to look at for adherent cells, such as stem cells or those that are more shear-sensitive.

Q: What do you use to quantify various nutrients and metabolites in the media?
A: We utilize a Cedex reader which contains optical sensors and assays which contain enzymes and reagents to measure this type of data from cell media. These systems are "off-line", meaning that they are separate from the bioreactor system and a sample from the bioreactor must be used each time. We look at cellular byproducts such as ammonia and lactate. These are main concerns that need to be looked at since high concentrations would make them toxic to the culture. You could also look at Lactate dehydrogenase as an indicator of viability. We also look at nutrients such as glutamate and glutamine.

Meeting 2 - March 6, 2020
Q: What does the BETC utilize as bottles for their flasks and benchtop bioreactors?
A: For media bottles, the BETC utilizes caps from ChemGlass, which have ports on them to attach tubing onto. There are a variety of designs with a varying number of ports. Maybe your group can start with the designs that have 3 or 4 ports.

Q: What are the specific concentrations of Cytodex that the BETC utilizes for their bioreactor operations? How do you determine what initial seeding density you will utilize?
A: For our Cytodex bioreactors, we typically utilize 3-7 grams of Cytodex per liter of volume within the bioreactor in our previous experiments. In terms of the seeding densities, the initial density will be dependent on the size of the cells, the surface area of the beads, and the doubling times of the cells.

Q: How would you approach gathering nutrient data and what kind of data can we gather from this?
A: You should look into nutrient depletion within bioreactors, as you may be able to determine cell growth and extrapolate the doubling time and growth rate from these. For example, you

could start with a control study where you determine the nutrient uptake of the cell line in a normal T flask. Then, you could look at this data and use it as a baseline for the bioreactor nutrient depletion.

Q: How would we quantify the nutrient uptake?

A: You can utilize glutamax, ammonia, and lactate assays, among the other nutrients you would want to look into. These can all be done with the Roche Cedex machines here in the Cedex.

Appendix B: Bill of Materials

Material	Cost (USD)	Manufacturer
Cytodex I beads (25g)	335.00	Sigma-Aldrich
Megalux Uncoated Glass Beads 30/40 Mesh	N/A (sample provided by manufacturer)	Swarco
Glutamax (100 mL)	37.17	Gibco
DMEM (500 mL)	26.22	Gibco
FBS (50 mL)	65.50	Sigma-Aldrich
Penicillin/Streptomycin (100 mL)	25.00	Sigma-Aldrich
Cells (HUVEC/Fibroblast) (106 cells)	279.00	
316L Stainless Steel	5.77-6.35 per kg	N/A (supplied by Tom Partington)
Manostat E-series Economy Peristaltic Pump	Discontinued	Cole-Palmer
Pyrex Reusable Media Storage Bottle - 1L	194.94 / case of 10	Fisher Scientific
ChemGlass 3-Port Assembly for 1L Media Bottle	$326.98	ChemGlass
38.1mm (1 1/2 in) O.D. Corning Pyrex Glass Tubing	$29.95 (2 pcs)	Wale Apparatus
Precision Stainless Steel Wire Cloth - 316 Stainless Steel - 50 x 50 Mesh Size	$23.27 (12"x12" sheet)	McMaster-Carr
5 mm Inner Diameter Polyurethane Tubings	N/A	N/A (supplied from GH 207 lab)

Appendix C: Bioreactor Components

This appendix will break down each of the bioreactor components found in the system and the material they are made of. The main bioreactor system consists of three main components:

1. The bioreactor vessel
2. The Manostat E-series Economy Peristaltic Pump
3. The media reservoir with bottle cap ports

The bioreactor vessel consists of five parts:

1. A base mount made of 316 Stainless Steel with screws to hold the bioreactor vessel vertically
2. A bottom end piece made of 316 Stainless Steel, acting as the inlet for the system as it is connected to the pump via tubing. This end piece also contains an O-Ring inside of it to create a water-tight interface with the borosilicate glass tube
3. A top end piece made of 316 Stainless Steel, acting as the outlet for the system as it is connected to the outlet port on the media reservoir via tubing. This end piece also contains an O-Ring inside of it to create a water-tight interface with the borosilicate glass tube
4. A middle borosilicate glass tube in between each of the end pieces, acting as a chamber to contain the middle insert
5. A middle insert made of 316 Stainless Steel that is situated inside the glass tube, acting as the chamber to hold the packed bed of microcarriers

The middle insert is also composed of three separate components:

1. A top end piece that is screwed onto the middle insert. This top piece will contain a 316 Stainless Steel mesh to prevent the flow of microcarriers from the packed bed out of the middle insert. This end piece also contains an O-Ring inside of it to create a water-tight interface with the middle insert
2. A bottom end piece that is screwed onto the middle insert. This bottom piece will contain a 316 Stainless Steel mesh small enough to contain the packed bed of microcarriers. This

end piece also contains an O-Ring inside of it to create a water-tight interface with the middle insert

3. The middle insert itself, with threaded ends on the top and bottom which will allow the end pieces to be screwed onto it

Appendix D: Protocols

Title: Adherent Cell Culturing Protocol

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1. Scope and Purpose

The purpose of this protocol is to detail how to properly subculture adherent cells with the transfer of the current cell culture into one with fresh growth media. This protocol can be utilized universally with minor changes to either the complete medium or the volumes used depending on the specific cell line. In the context of this MQP, this protocol is used to subculture the cells before being utilized in any preliminary verification study or before being transferred into the bioreactors.

2. Materials

- 5 mL DPBS (-)
- 3 mL 0.25% Trypsin-EDTA
- At least 15 mL complete growth medium
- Incubator (37°C, 5% CO2)
- 5 mL & 10 mL serological pipettes
- Pasteur pipettes
- Vacuum pump
- Microscope
- Centrifuge

3. Procedure

1. Before bringing the current culture plate with cells into the hood, check culture plate under the microscope to
   a. Check the health and degree of confluency of the cells

      b. Ensure that cells are not contaminated

2. Bring plate inside hood

3. Attach sterile Pasteur pipette to tube attached to vacuum pump, carefully aspirate medium

4. Using serological pipette, gently add **5 mL DPBS (-)** alongside of plate, rinse cells

5. Attach Pasteur pipette to tube attached to vacuum pump, carefully aspirate DPBS (-)

6. Using serological pipette, gently add **3 mL of 0.25% Trypsin-EDTA** solution along the side of the plate, Close the lid

7. Incubate the plate on slide warmer at 37C for **5-10 minutes**

8. After incubation, check the cells under the microscope to make sure that cells are detached and loose, bring the plate inside the hood

9. Using fresh serological pipette, **add 2 mL complete medium** to the plate. This neutralized the trypsin, the **total volume is now 5 mL**

10. Using same serological pipette, disperse cells by repeated pipetting, avoid air bubbles

11. Transfer cell suspension to fresh, sterile 15 mL conical tube, repeat pipetting to break up cell clumps

12. **If you plan on doing a cell count, you can use a sample of cell suspension at this point**

13. Spin the tube at **200G** for 5-10 minutes in the centrifuge, after centrifugation, look for the cell pellet at the bottom of the tube

14. Using the vacuum set up, carefully aspirate the medium using a fresh sterile Pasteur pipette leaving about 0.5 mL fluid in the tube

15. Resuspend cells in an appropriate amount of complete media, depending on ratio of subculturing (1:3, 1:4, 1:5, 1:10) or plate specific amount of cells as needed for experiment. A good rule of thumb if you counted the cells is to resuspend the cells at 1 million cells/mL

16. Plate enough cells into a fresh plate, making the total volume of medium to 10 mL (if you are plating 1 mL cells, add 9 mL complete media)

17. Check cells under microscope

18. Transfer plate to incubator for continued incubation for desired duration

4. Definitions

Confluency - Percentage of the surface of the culture dish covered by the adherent cells

Complete Media - Growth media made previously consisting of Dulbecco's Modified Eagle Media, Penicillin/Streptomycin, Fetal Bovine Serum, and Glutamax

Title: Microcarrier Attachment Experiment Protocol

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1. Purpose

The purpose of this protocol is to detail a preliminary microcarrier attachment experiment examining cellular attachment and bead to bead migration on a variety of microcarrier substrates. This includes the Cytodex 1 microcarriers from General Electric, as well as glass beads subjected to various surface modifications. This experiment will help to determine the best microcarrier substrate available to the group before translation into the bioreactor system. The protocol will go through the various preliminary steps of sterilization and applying surface modifications to the glass beads before culturing with cells.

2. Materials

- Cytodex 1 microcarriers from GE
- DPBS
- Forceps
- Scooper
- 70% Isopropyl alcohol
- Plastic mesh
- Megalux glass beads 30/40 mesh
- 0.1% Gelatin Solution (ATCC® PCS-999-027™)
- Plasma Cleaner in SL 219
- Complete Medium
- Incubator (at 37°C and 5% CO2)
- Phase contrast microscope

3. Procedure

Sterilization

1. First must sterilize forceps, scooper
2. Add into tubes of isopropyl alcohol for 1 minute
3. Rinse by moving items into tubes of DPBS

4. Repeat several times for several rounds of sterilization

5. Must also sterilize plastic mesh to be used to move glass beads to be coated in gelatin

6. Repeat steps, however soak mesh in petri dish of isopropyl alcohol instead of a tube

7. Place glass beads in autoclave bag and send to lab manager for beads to be autoclaved

Gelatin Coating

1. Decontaminate the external surface of the 0.1% Gelatin Solution bottle by spraying with 70% ethanol.

2. Using aseptic technique and working in a laminar flow hood or biosafety cabinet, add 1.0 mL of 0.1% Gelatin Solution per 10 cm2 of culture surface area (e.g., 2.5 mL if using a T-25 flask).

3. Using forceps, place sterilized mesh which contains autoclaved beads into dish

4. Rock culture flask to coat the surface; place in a 37°C incubator (with or without 5% CO2) for at least 30 minutes, and up to overnight.

5. Using forceps, remove sterilized mesh and the coated beads from dish into new dish

6. Add 5.0 mL of complete growth medium per 25 cm2 of culture surface area (e.g., 5.0 mL if using a T-25 flask).

7. Place the gelatin-coated beads in a 37°C, 5% CO2 incubator for at least one hour to equilibrate before inoculating with the cell suspension.

Plasma Treatment

1. Place autoclaved glass beads into a 60 mm plate and place the plate inside the plasma cleaner in SL 219

2. Turn the plasma cleaner on, then the vacuum on

3. Place the cap over the entrance and turn the black dial to seal the entrance

4. Turn the dial on the plasma cleaner to high and ensure the plasma cleaner is on by checking the side for a purple flame. Duration of the plasma cleaning should be 1-2 minutes

5. Turn everything off, and slowly release the black dial to take off the cap

6. Take plate out and lightly shake in an effort to turn over the glass beads in the plate

a. Plasma treatment only affects the top exposing layers, therefore this shaking will attempt to expose any surface that may not have been exposed in the first plasma treatment

7. Repeat steps 1-5

8. Remove plate with beads, cover and bring to the hood

Glass Attachment Experiment

1. Using the 24 well plate template below, place the appropriate microcarriers into their corresponding columns

   a. The microcarriers are transferred into each well by utilizing the sterilized scooper to scoop autoclaved glass beads, modified glass beads, and Cytodex beads until the bottom layer of the well is completely covered

   b. A1, B1 and C1 will contain the control (no beads in the well, simply adherent cells seeded on the well surface)

   c. A2, B2, and C2 will contain Cytodex 1 beads

      i. Refer to Control Bioreactor Protocol steps 1-5 for preparing and swelling the Cytodex 1 microcarriers

   d. A3, B3 and C3 will contain glass beads (unmodified)

   e. A4, B4, and C4 will contain the gelatin-coated glass beads

   f. A5, B5, and C5 will contain the plasma-treated glass beads
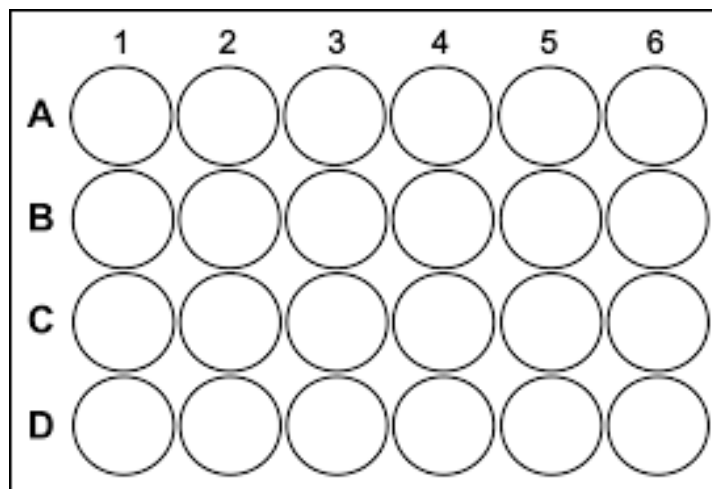
2. Seed ~4000 cells into each well (refer to the Cell Subculturing Protocol on how to plate cells from a subculture)

3. Fill each well with up to 2 mL of complete medium

4. Take initial images of each well under phase contrast microscope and note when T=0 is

5. Place 24 well plate into incubator at 37°C and 5% CO_2

6. Check and image each well at T=6 and T=16 hours

7. After T=16 hours, refer to the Hoechst Staining Protocol in order to fluorescently image each well

4. Definitions

N/A

Title: Hoechst Cell Staining Protocol

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1. Purpose

The purpose of this protocol is to take video and images of cells that are adhered to microcarriers using fluorescence microscopy in order to show cell growth and migration in static microcarrier culture. During this experiment Hoechst Dye was used in order to image cell nuclei on microcarriers.

2. Materials

- Hoechst Dye
- 24-well plate with microcarriers and cells from microcarrier experiment
- Metal Scoop
- Fluorescence microscope

3. Procedure

1. First, Hoechst dye was obtained from the lab
2. Next, 2 uL of Hoechst dye was added directly to each of the 5 wells which need to be imaged, each containing 2 mL of culture media, in order to achieve a 1ug/mL final concentration
3. Mix the dye/media solution via repeated pipetting
4. After, wells were put in an incubator at 37 degrees Celsius for 10-15 mins to allow the dye diffuse through the cells
5. After letting the wells sit, the beads in each well were carefully transferred to corresponding empty wells to ensure only the cells on the microcarriers are being imaged. This was done using a sterilized metal 'scoop'.
6. Finally the wells were put under a fluorescence microscope in order to take video and images of the cells at different cross-sections of the beads

4. Definitions

Hoechst dye - Nucleic acid stain that emits blue fluorescence when bound to double stranded
DNA

Title: Novel Bioreactor Setup and Breakdown

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1. Purpose

The purpose of this protocol is to detail the steps in setting up the bioreactor so that it is ready for the preliminary fluid test or the final comparison study. It is necessary to understand the naming of each component (refer to the Final Design section) and how they come together to comprise the final bioreactor design. Breakdown of the bioreactor will also be necessary at the end of the study for conducting final cell density comparisons

2. Materials

- Novel bioreactor components (listed in the Final Design section)
- Manostat E Series Economy peristaltic pump
- 5 mm ID polyurethane tubing
- Megalux glass beads 30/40 mesh (plasma treated modification)
- 1 L media bottle
- ChemGlass 3-Port Assembly for 1 L media bottle

3. Procedure

Bioreactor Setup*

1. Insert the glass tube into one of the stainless steel end pieces containing the appropriate O-ring, twisting and applying force so that the tube pops into place
2. Screw off the top cap of the middle insert and scoop in required amount of glass beads into the insert; screw the top cap back onto the middle insert and place the middle insert inside the glass tube
3. Repeat step 1 with the other stainless steel end piece, closing off the top of the glass tube so that both ends are now in place with each steel end piece
4. Line up the back ends of the steel end pieces so that the screw holes on each back end is facing the screw holes on the base mount

5. Place washer around a screw and insert into one of the aligned screw holes of the base mount and one of the steel end pieces; use a flat-head screwdriver to screw the screw into place, make sure not to overtighten

6. Repeat step 5 with the 3 other aligned screw holes so that the bioreactor is mounted onto the base mount

7. Connect 5 mm ID polyurethane tubings onto the hose nipples on both the top and bottom steel end pieces

8. Connect the bottom polyurethane tubing to the outlet port of the Manostat E Series Economy peristaltic pump

Media Bottle Setup

1. Fill the media bottle with 400 mL media

2. Screw on the ChemGlass 3-port cap onto the media bottle

3. Connect a 5mm ID polyurethane tubing from the inlet port of the media bottle to the inlet port on the Manostat E Series Economy peristaltic pump

4. Connect the top polyurethane tubing of the bioreactor to the outlet port of the media bottle

Bioreactor Breakdown*

1. Remove the inlet polyurethane tubing from the inlet port on the media bottle and place it into empty flask within incubator

2. Run pump to drain the rest of the fluid in the bioreactor system into the media bottle

3. Once the bioreactor is drained, remove both polyurethane tubings from the top and bottom hose nipples

4. While holding the bioreactor, unscrew one of the screws from the base mount and place screw and washer to the side

5. Repeat step 1 for the other 3 screws, making sure to hold onto the bioreactor so it will not fall over once the screws are removed from the base mount

6. Remove the bioreactor from the base mount and slowly pull and twist on the top steel end piece to remove it from the top of the glass tube

7. Remove middle insert from the glass tube and set aside in the hood

8. Repeat step 6 for the bottom steel end piece to completely disassemble the bioreactor

* Should be performed under a biosafety hood

4. Definitions

N/A

Title: Fluid Validation Test Protocol

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1. Purpose

The purpose of this protocol is to test the fluid flow throughout the experimental bioreactor before conducting any sort of biological testing with cells and media. This is to ensure that there are no leaks within the system, and that the flow will be continuous throughout the entire system. This sort of validation is an important initial step, and will allow the team to troubleshoot any aspects of the bioreactor designs should any problems be discovered.

2. Materials
- Media bottle
    - Media bottle filled with 400 mL water to completely fill bioreactor system and have extra in bottle
- 5 mm ID polyurethane tubing
- Experimental bioreactor fully set up
    - Middle insert must be filled with glass beads
- Peristaltic pump
- Dye (can be any color)
- Syringe & needle

3. Procedure
1. Set up the bioreactor, tubings, and peristaltic pump according to the described designs and images as shown in the design section
2. Fill up the media bottle with a 400 mL volume of water, then place the media cap bottle on top. This amount should be enough to fill up the bioreactor and tubing system with fluid, while having enough in excess to maintain a constant source that will never run out
3. Connect the two open ends of the tubings to two ports on the media cap bottle on the media bottle to create the closed-loop bioreactor system
4. Turn on the peristaltic pump and wait for the bioreactor system to "prime" over time

a. "Priming" simply means when the bioreactor system and the tubings become full with the fluid in the media bottle

5. Once the bioreactor system is primed, check around each area where a connection occurs throughout the system, this would include:
    a. Tubing connections to the media cap ports
    b. Tubing connections to the peristaltic pump
    c. Tubing connections to the top and bottom of the bioreactor
    d. Glass tube connections to each stainless steel end pieces

6. If any problems with leakage occur, start troubleshoot process
    a. Look into modifying the system, potentially with an outer securing layer of tubing around the connection or utilizing additional O-rings to create water-tight seals wherever needed

7. If no problems with leakage occur, proceed to the fluid dye process

8. Connect syringe and needle by simply twisting needle into Leuer lock

9. Draw 3 mL volume of dye into the syringe, and then place needle directly into the polyurethane tubing connected to the media cap port associated with the "inlet" of the bioreactor design

10. Inject 1 mL of the dye into the system and follow the flow of the dye as it moves throughout the bioreactor system from the inlet into the outlet of the bioreactor and into the media bottle
    a. Be sure to note if the dye does not fully loop through the closed-loop bioreactor system, as this would then indicate that there is an issue with the continuous flow of the bioreactor

4. Definitions

N/A

Title: Control Bioreactor System Protocol

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1. Purpose

The purpose of this protocol is to detail how a standard bioreactor system is set-up and run. The protocol will begin by explaining the preparation of the microcarriers in the system. This entire system will be used as the control in order to test the new bioreactor system against.

2. Materials

- Cytodex 1 Microcarriers
- PBS (Ca^{2+}, Mg^{2+}-free)
- Culture Medium
- GE Bioreactor system

3. Procedure

1. Weigh out the desired amount of microcarriers to use in the bioreactor system. The typical concentration that will be present in the stirred-tank bioreactor is 2-3g/L.
2. Add dry Cytodex microcarriers to a siliconized glass bottle and swell in PBS for at least 3 hours at room temperature with occasional gentle agitation. The concentration of PBS to Cytodex is 50-100 mL/g.
3. Remove the supernatant and wash the Cytodex with gentle agitation in 30-50 mL of PBS per gram of Cytodex for a few minutes.
4. Discard the PBS and add fresh PBS then sterilize by autoclaving with steam from purified water.
5. Prior to use, allow the microcarriers to settle, decant the supernatant and rinse the microcarriers in warm culture medium (20–50 mL/g Cytodex) in order to reduce dilution of the culture medium by PBS trapped between and within the microcarriers. Allow the microcarriers to settle again and remove the supernatant and resuspend the microcarriers in a small volume of culture medium and transfer to the culture vessel.
6. The media and microcarriers are added to the stirred-tank bioreactor through the top port.

7. In order to allow the anchorage cells to attach to the microcarrier, the microcarriers and the medium (⅓ of total medium volume) are intermittently stirred several times and allowed to settle in between each stirring.

8. In order to maintain a microcarrier culture, the stirring should then remain continuous in order to keep the microcarriers suspended in the culture medium.

9. The frequency and extent of medium replenishment depends on cell type, culture density, culture medium and gas tension. The usual procedure is to start with replenishing 50% of the medium volume every 3 days, but to take advantage of conditioning effects, replenishment should not take place within the first 2 days of culture. If a sample is taken, 10–20% of the medium volume can be replaced with fresh medium.

10. The cultures should remain at confluence throughout the process so in order to ensure this should be continuously tested using the probes: pH, temperature, and dissolved oxygen.

4. Definitions

Complete Media - Growth media made previously consisting of Dulbecco's Modified Eagle Media, Penicillin/Streptomycin, Fetal Bovine Serum, and Glutamax

Intermittent Stirring - Stirring within the bioreactor at a set RPM, but with periods of stirring followed by periods of non-stirring

Continuous Stirring - Stirring within the bioreactor system at a continuous set RPM without any interruptions

Title: Nutrient Testing Protocol

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1. Purpose

The purpose of this protocol is to detail the testing of nutrient levels in the media of the bioreactor system. Daily testing of the nutrient levels will help to ensure normal cell culture conditions are occurring, where the adherent cells are consuming specific nutrients, while also releasing detectable waste byproducts as they continue to grow and proliferate. In the end, the team will be able to analyze this data for any specific trends to determine whether or not the cells are able to remain healthy, and if media replacement is necessary if cellular byproducts accumulate at high levels. This analysis will be looking into Glucose, Glutamax, Glutamate, Lactate, Ammonia, and Lactate Dehydrogenase (LDH) levels in the media.

2. Materials
- Roche Cedex Bio Analyzer
- Sampling cuvettes from Roche
- Experimental bioreactor system
- Syringe

3. Procedure
1. Turn off peristaltic pump to stop the flow
2. Place syringe into the sampling polyurethane tubing connected to one of the media bottle cap ports for sampling
3. Take off hemostats and open clamp on the sample port tubing
4. Pull syringe back in order to draw sample directly from the media bottle
5. When ~3 mL reached in tube, close clamp and reapply hemostats
6. Remove syringe from tubing and place ~2 mL directly into the sampling cuvette; sterility not major issue when transferring sample but be sure to be quick and precise after removing sample to limit exposure to open environment

7. Bring cuvette to Roche Cedex Bio Analyzer and place into an open tube in the sampling area

8. On the Cedex software, input an ID for the sample and select Glucose, Glutamax, Glutamate, Lactate, Ammonia, and LDH sampling tests to be performed

9. Begin processing and wait for results to appear on the screen showing nutrient levels

10. Directly record these values into lab notebook

11. Use the last 1 mL of the sample to perform LDH assay (refer LDH Assay Protocol) to obtain cell cytotoxicity data

12. Repeat daily

4. Definitions

Roche Cedex Bio Analyzer - Bioprocessing monitor from Roche capable to running assays to detect nutrient levels

Title: Final Cell Density Protocol

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1. Purpose

The purpose of this protocol is to assess the final cell densities of both our novel bioreactor and the conventional bioreactor systems for comparison. Both reactors will have been seeded at the same initial cell density, and therefore differences in the final cell density will highlight the impact of the novel bioreactor's design on adherent cell culture.

2. Materials

- 100-1000 microliter pipet
- Associated pipet tips
- 15 mL conical tube
- Cell culture disc
- Trypsin
- Incubator
- Biosafety Hood
- Wrench
- Scoop
- Scale
- 200-micron strainer

3. Procedure

1. Refer to the Novel Bioreactor Setup and Breakdown Protocol to properly breakdown bioreactor to gain access to the glass beads in the middle insert
2. Scoop a mass of beads out of the insert (0.5 gram) and place into an empty cell culture plate.
3. Pipet 5ml of trypsin into the culture plate, cover, and gently agitate. Place the plate in incubation for 10-15 minutes.

4. After 10-15 minutes, remove the plate from the incubator, check to confirm that the cells are lifted into suspension. Apply 5ml of media to the plate, and agitate gently.

5. Pipet the 10ml of media and beads using a serological pipet and then pass it through a 200-micron filter into the 15ml tube. Use an additional 5ml of media to rinse the beads thoroughly.

6. Place the tube in a balanced centrifuge, spin for 5 minutes and remove. Aspirate media, and resuspend cell pellet in 10mL of media and mix thoroughly.

7. Prepare a slide, pipet 7 microliters onto the glass slide, and perform a cell count.

Appendix E: Relevant Design Calculations

This section details all the relevant design calculations which were utilized in characterizing the novel packed bed bioreactor system. This includes calculations related to volumetric efficiency, pressure drops across the packed bed, shear forces experienced on each microcarrier, and the amount of oxygen delivered/consumed within the system. The first calculation, however, is determining the total number of cells supported within the packed bed, as this is a baseline calculation that the rest of the calculations utilize.

Total Number of Beads Calculation
In order to perform any calculation, the total number of beads within the bioreactor system must be known. This is performed by dividing the volume of the bioreactor vessel by the volume of beads, under the assumption that the densest possible packing efficiency if 74%, meaning that 74% of the bioreactor vessel volume contains beads [33]:

$$\text{Beads}_{\text{total}} = \frac{\eta * \text{Volume}_{\text{tube}}}{\text{Volume}_{\text{beads}}} = \frac{0.74 * \text{Volume}_{\text{tube}}}{\text{Volume}_{\text{beads}}}$$

The volume of the bioreactor glass tube can be defined as its length multiplied by its area. The volume of a bead is simply the volume of a sphere, which is defined as $4/3*\pi*$radius of the microcarrier. As a result:

$$\text{Beads}_{\text{total}} = \frac{0.74 * \text{Volume}_{\text{tube}}}{\text{Volume}_{\text{beads}}} = \frac{0.74 * \text{L}_{\text{tube}} * \text{A}_{\text{tube}}}{\text{Volume}_{\text{beads}}} = \frac{0.74 * \pi r_{\text{tube}} * \text{L}_{\text{tube}}}{\frac{4}{3} * \pi * r_{\text{bead}}}$$

The radius of the bioreactor vessel is simply the inner diameter of the glass tube divided by two. The inner diameter of the glass tube is known as 30.1 mm. The diameter of the glass bead is known as 512.5 µm or $512.5*10^{-6}$ m, as the provided glass beads are Megalux Uncoated Glass Beads with a known diameter range of 425 - 600 µm. 512.5 µm is used for the diameter under the assumption of monodispersity. The length of the packed bed to be used for this calculation and all subsequent calculations is 0.1 m, as this is nearly the entire length of the middle insert while leaving just a little bit of wiggle room inside the insert. As such, the total number of beads can be calculated as:

$$\text{Beads}_{\text{total}} = \frac{0.74 * \pi r_{\text{tube}}^2 * \text{L}_{\text{tube}}}{\frac{4}{3} * \pi * r_{\text{bead}}^3} = \frac{0.74 * \pi * (\frac{30.1 \text{ mm}}{2 * 1000 \text{ mm/m}})^2 * 0.1\text{m}}{\frac{4}{3} * \pi * (\frac{512.5 * 10^{-6}\text{m}}{2})^3}$$
$$= 7.47 \text{ x } 10^5 \text{ beads}$$

As such, with a packed bed the length of 0.1m inside the middle insert, the total number of beads is 7.47 x 10^5.

Volumetric Efficiency Calculations

This section will focus on how the volumetric efficiency is calculated for both the Cytodex and Bioreactor systems and then compared. First, the total number of cells per surface area is calculated with the 100% confluency experiment. The total number of cells was found to be 1.14 x 10^6 cells. Knowing that a T-flask has a surface area of 25 cm^2:

$$\frac{\text{Total \# cells}}{\text{Surface area}} = \frac{1.14 \text{ x } 10^6 \text{ cells}}{25 \text{ cm}^2} * \frac{1 \text{ cm}^2}{1 \text{ x } 10^8 \text{ μm}^2} = 4.55 \text{ x } 10^{-4} \text{ cells/μm}^2$$

Next, the total surface area per unit volume of the bioreactor system must be calculated. Utilizing the GE manual, it is known that for every gram of dry weight of the Cytodex microcarriers, there is a total surface area of 4400 cm^2 [32]. When interviewing with Chris Bellerive from the BETC, it was determined that typically 3-7 g of Cytodex is used per L of the bioreactor system. As such, the surface area per unit volume can be calculated:

$$\frac{\text{SA}}{\text{V}}_{\text{Cytodex}} = \frac{3 \text{ g Cytodex}}{1 \text{ L volume}} * \frac{4400 \text{ cm}^2}{1 \text{ g Cytodex}} * \frac{1 * 10^8 \text{ μm}^2}{1 \text{ cm}^2} = 1.32 \text{ x } 10^{12} \text{ μm}^2\text{/L}$$

The surface area per unit volume of the novel bioreactor system can be calculated knowing the total number of beads in the system. This is done by multiplying the surface area of one bead by the total number of beads in the system to determine the total surface area, as shown:

$$\text{SA}_{\text{bead}} = 4\pi r^2 = 4\pi * (\frac{512.5 \text{ μm}}{2})^2 = 8.25 \text{ x } 10^5 \text{ μm}^2\text{/bead}$$
$$\text{SA}_{\text{packed bed}} = \text{SA}_{\text{bead}} * \text{Beads}_{\text{total}} = 8.25 \text{ x } 10^5 \text{ μm}^2\text{/bead} * 7.47 \text{ x } 10^5 \text{ beads}$$
$$\text{SA}_{\text{packed bed}} = 6.16 \text{ x } 10^{11} \text{ μm}^2$$

Next, the volume of the packed bed system can be calculated, knowing that the length is 0.1 m and the diameter is 30.1 mm:

$$\text{V}_{\text{bed}} = \text{L}_{\text{bed}} * \text{A}_{\text{tube}} = \text{L}_{\text{bed}} * \pi r_{\text{tube}}^2 = 0.1 \text{ m} * \pi(\frac{30.1 \text{ mm}}{2 * 1000 \frac{\text{mm}}{\text{m}}})^2$$
$$= 7.12 \text{ x } 10^{-5} \text{ m}^3$$

Now, the surface area to volume ratio of the packed bed can be calculated:

$$\frac{SA}{V}_{bed} = \frac{SA_{packed\ bed}}{V_{bed}} = \frac{6.16 \times 10^{11}\ \mu m^2}{7.12 \times 10^{-5}\ m^3} * \frac{1\ m^3}{1000\ mL} = 8.66 \times 10^{12}\ \mu m^2/L$$

Now that the surface area to volume ratios are calculated for both systems, the total number of cells supported per volume can be determined by multiplying each ratio by the previous total number of cells per unit surface area calculation. The calculated cells supported per volume is the volumetric efficiency of the system. As such, volumetric efficiency can be calculated for the Cytodex system as shown, where *VE* is the volumetric efficiency:

$$VE = \frac{SA}{V} * \frac{Total\ \#\ cells}{Surface\ area}$$

$$VE_{Cytodex} = \frac{SA}{V}_{Cytodex} * \frac{Total\ \#\ cells}{Surface\ area}$$
$$= 1.32 \times 10^{12}\ \frac{\mu m^2}{L} * 4.55 \times 10^{-4}\ \frac{cells}{\mu m^2} * \frac{1\ L}{1000\ mL} =$$
$$6.01 \times 10^5\ \frac{cells}{mL}$$

The same equations are applied for the packed bed system:

$$VE_{bed} = \frac{SA}{V}_{bed} * \frac{Total\ \#\ cells}{Surface\ area}$$
$$= 8.66 \times 10^{12}\ \frac{\mu m^2}{L} * 4.55 \times 10^{-4}\ \frac{cells}{\mu m^2} * \frac{1\ L}{1000\ mL} =$$
$$39.3 \times 10^5\ \frac{cells}{mL}$$

Now that the total number of cells per volume is calculated for both systems, the volumetric efficiencies can simply be divided to determine the increase in volumetric efficiency between the packed bed system and the Cytodex system:

$$\frac{39.3 \times 10^5\ \frac{cells}{mL}}{6.01 \times 10^5\ \frac{cells}{mL}} = 6.56$$

As such, it is determined that the packed bed reactor is 6.56-times more volumetrically efficient than the Cytodex system since it can support 6.56-times more cells per unit volume.

Ergun Equation Calculations

This section will focus on how the Ergun Equation as described in Equation (2) is utilized to derive the pressure drop across the packed bed of microcarriers as a function of the flow rate provided by the peristaltic pump. Several assumptions were utilized for this equation:

- The void space between each particle is 0.26, under the assumption of the densest possible packing of spheres being 74%
- Fluid properties such as viscosity and density of the cell culture media are assumed to be that of water at 37°C, since the bioreactor will be running in the incubator
- Monodispersity is assumed for the spheres, as the sample given from the manufacturers contains a range of diameters from 415-600 µm; as such, the median diameter of 512.5 µm is assumed

First, we start off with the Ergun Equation, as described by Equation (2):

$$\Delta P = \left(\frac{150\mu(1-\varepsilon)^2 u_0}{\varepsilon^3 d_p^2} + \frac{1.75(1-\varepsilon)\rho u_0^2}{\varepsilon^3 d_p}\right) * L \tag{2}$$

where ΔP is the calculated pressure drop, L is the height/length of the packed bed, μ is the fluid viscosity, ε is the void space within the packed bed, u_0 is the fluid superficial velocity, d_p is the diameter of the particles in the packed bed, and ρ is the density of the fluid.

With the given assumptions, we know:

$\varepsilon = 0.26$
$\rho = 997$ kg/m^3
$\mu = 6.922*10^{-4}$ kg/(m*s)
$d_p = 512.5$ µm $= 512.5 * 10^{-6}$ m
$L = 0.1$ m

The fluid superficial velocity is a variable that can be calculated because with a provided volumetric flow rate, while the area of the bioreactor vessel is known as 30.1 mm:

$$u_0 = \frac{Q}{A} = \frac{Q\,[m^3/s]}{\pi\left(\frac{30.1\ mm}{2} * \frac{1\ m}{1000\ mm}\right)^2} = \frac{Q\,[m^3/s]}{7.12\ x\ 10^{-4}\ m^2}\ [m/s]$$

Now, all of these constants can be plugged into the Equation (2) to derive the pressure drop as a function of velocity:

$$\Delta P = (\frac{150 * (6.922 * 10^{-4} \frac{kg}{m*s}) * (1 - 0.26)^2 * (u_0 \frac{m}{s})}{(0.26)^3 * (512.5 \text{ x } 10^{-6}m)^2}$$

$$+ \frac{1.75 * (1 - 0.26) * (997 \frac{kg}{m^3}) * (u_0 \frac{m}{s})^2}{(0.26)^3 * (512.5 \text{ x } 10^{-6}m)}) * (0.1 \text{ m})$$

$$\Delta P = 1.23 \text{ x } 10^6 * (u_0) \frac{kg}{m*s^2} + 1.43 \text{ x } 10^7 * (u_0)^2 \frac{kg}{m*s^2}$$

A sample calculation is shown below with a flow rate of 50 mL/min:
First, the fluid velocity is calculated utilizing the volumetric flow rate:

$$Q = 50 \frac{mL}{min} * \frac{1 \text{ x } 10^{-6} \text{ m}^3}{1 \text{ mL}} * \frac{1 \text{ min}}{60 \text{ s}} = 8.33 \text{ x } 10^{-7} \text{ m}^3/s$$

$$u_0 = \frac{8.33 \text{ x } 10^{-7} \text{ [m}^3/s]}{7.12 \text{ x } 10^{-4} \text{ m}^2} = 1.17 \text{ x } 10^{-3} \text{ m/s}$$

Once the fluid velocity is calculated, it can be plugged into the simplified Ergun Equation

$$\Delta P = 1.23 \text{ x } 10^6 * (1.17 \text{ x } 10^{-3}) \frac{kg}{m*s^2} + 1.43 \text{ x } 10^7 * (1.17 \text{ x } 10^{-3})^2 \frac{kg}{m*s^2}$$

$$\Delta P = 1.46 \text{ x } 10^3 \text{ Pa}$$

Shear Stress Calculations
Now that pressure drops can be calculated from the Ergun Equation, these pressure differentials can be utilized to calculate the shear stress experienced on the surface of each microcarrier with Equation (3). This equation will be utilized to derive the shear stress as a function of the volumetric flow:

$$\tau = \frac{\Delta P * A}{SA} \quad\quad\quad (3)$$

where τ is the calculated shear stress, ΔP is the calculated pressure drop from Equation (2), A is the cross sectional area of the fluid flow, and SA is the surface area of the packed bed.

The cross sectional area of the fluid flow can be described as,

$$A = \frac{\pi D^2 \varepsilon}{4}$$

where A is the cross sectional area of the fluid flow, D is the diameter of the bioreactor vessel and ε is the packed bed void space. The diameter of the bioreactor vessel is known as 30.1 mm, while the voice space of the packed bed is known as 0.26, as described above since the densest possible packing is assumed as 74% for spheres.

Determining the surface area of the scaffold will involve knowing the surface area of beads within a specific volume. The surface area of the scaffold can be defined as the surface area of the total number of beads in a volume of the packed bed. This is simply the surface area of one bead multiplied by the number of beads previously calculated above, which will yield the total surface area. As such, the surface area of the scaffold as a function of the length of the packed bed can be derived as:

$$SA = SA_{beads} * 7.47 \text{ x } 10^5 \text{ beads}$$
$$SA = 4\pi r_{bead}^2 * 7.47 \text{ x } 10^5 \text{ beads}$$
$$SA = 4\pi(\frac{512.5 \text{ x } 10^{-6}\text{m}}{2})^2 * 7.47 \text{ x } 10^5 \text{ beads}$$
$$SA = 6.16 \text{ x } 10^{-1} \text{ m}^2$$

Now that the scaffold surface area is a function of length, the shear stress calculated from the Ergun Equation is a function of length, such that:

$$\tau = \Delta P * \frac{\pi D^2 \varepsilon}{4} * \frac{1}{6.16 \text{ x } 10^{-1} \text{ m}^2}$$

A sample calculation is shown below, utilizing the pressure drop calculated from the Ergun Equation for a volumetric flow rate of 50 mL/min:

$$\tau = 1.46 \text{ x } 10^3 \text{ Pa} * \frac{\pi(30.1 \text{ mm / } 1000 \text{ mm/m})^2 * (0.26)}{4} * \frac{1}{6.16 \text{ x } 10^{-1} \text{ m}^2}$$
$$\tau = 4.41 \text{ x } 10^{-1} \text{ Pa} = 4.41 \text{ dynes/cm}^2$$

When these calculations are performed for the volumetric flow range of 30-75 mL/min, it is found that the shear stress limit of 6.5-10 dynes/cm² is reached at around a flow range of 75 mL/min, indicating that the provided peristaltic pump with a flow rate of 56.8 mL/min will not exceed this shear stress limit.

Oxygenation Calculations

Oxygenation validation involves the calculation of the total amount of oxygen that is delivered into the bioreactor system in comparison to the total amount of oxygen that is consumed within the system. First, this will involve calculating the total amount of oxygen consumed by the system. This is done utilizing Equation (4), which is

$$O_{2,C} = C_{cells} * O_{2,cell} \tag{4}$$

where $O_{2,C}$ is the maximum amount of oxygen consumed, C_{cells} is the maximum amount of cells in the packed bed, and $O_{2,cell}$ is the theoretical oxygen consumption rate per cell.

This calculation is performed under the assumption that the packed bed is at maximum confluence, with each cell consuming oxygen at its maximum possible rate. The total amount of beads within the packed bed system has already been calculated above as 7.47×10^5 beads. This can be converted into the total number of cells under the assumption of maximum confluency on every bead. Utilizing the previous confluency calculations, it was found that when a bead is 100% confluent, it will contain 375 cells per bead. As such:

$$C_{cells} = 375 \frac{cells}{bead} * 7.47 \times 10^5 \text{ beads}$$
$$C_{cells} = 2.80 \times 10^8 \ cells$$

Next, the maximum theoretical amount of oxygen consumed by a fibroblast cell must be determined. Examining literature, it was found that 1.19×10^{-17} mol of oxygen is consumed by a fibroblast cell every second [35]. As such the total amount of oxygen consumption within a packed bed as a function of the length of the packed bed is calculated as:

$$O_{2,C} = 2.80 \times 10^8 \text{ cells} * 1.19 \times 10^{-17} \frac{\text{mol } O_2}{\text{cell} * s} = 3.33 \times 10^{-9} \frac{\text{mol } O_2}{s}$$

$$O_{2,C} = 3.33 \times 10^{-8} \frac{\text{mol } O_2}{s} * \frac{32 \text{ g } O_2}{\text{mol } O_2} * \frac{1000 \text{ mg}}{1 \text{ g}} = 1.07 \times 10^{-4} \text{mg } O_2/\text{sec}$$

This states that the total amount of oxygen consumed by a packed bed with the length of 0.1 m is 1.07×10^{-4} mg every second. This will be the baseline comparison that the total amount of oxygen delivered will be compared against.

Next, the total amount of oxygen delivered by the system must be determined as a function of flow rate. This is done utilizing Equation (5), which is:

$$O_{2,D} = DO_2 * Q \tag{5}$$

where $O_{2,D}$ is the calculated rate of oxygen delivery, DO_2 is the theoretical dissolved oxygen in media, and Q is the volumetric flow rate of the pump into the bioreactor vessel.

First, the theoretical amount of dissolved oxygen in the media must be determined. This is performed under the assumption that this amount of dissolved oxygen is equivalent to the amount of dissolved oxygen in water. Additionally, this should be calculated at 37°C since this will be operating within incubator conditions. As such, it was found that the maximum dissolved oxygen concentration in water at 37°C is 6.71 mg/L [34]. A safety factor is factored into this concentration since it is highly unlikely that this maximum concentration will be achieved within the incubator, and as such the dissolved oxygen concentration is taken as 4.00 mg/L. This can be converted into:

$$DO_2 = 4.00 \ \frac{mg}{L} * \frac{1 \ L}{1 * 10^6 mm^3} = 4 \times 10^{-6} \ mg/mm^3$$

Now, the total amount of oxygen delivered into the system can be derived as:

$$O_{2,D} = Q * 4 \times 10^{-6} \ mg/mm^3 \ [mg/s]$$

An example calculation is shown with a volumetric flow rate of 50 mL/min. The volumetric flow rate of the pump is given as a constant 50 mL/min, which can be converted into:

$$Q = 50 \ \frac{mL}{min} * \frac{1 \ min}{60 \ s} * \frac{1000 \ mm^3}{1 \ mL} = 8.33 \times 10^2 \ mm^3/s$$

Now, this can be plugged into the total amount of oxygen delivered function:

$$O_{2,D} = 8.33 \times 10^2 \ mm^3/s * 4 \times 10^{-6} \ mg/mm^3 \ [mg/s]$$
$$O_{2,D} = 33.3 \times 10^{-4} \ mg/s$$

This states that for a flow rate of 50 mL/min, the total amount of oxygen delivered to the packed bed is 33.3 x 10^{-4} mg every second. In comparison, 1.07 x 10^{-4} mg of oxygen is consumed every second, thereby verifying that the bioreactor vessel would be able to support the oxygen requirements of the packed bed of microcarriers with a length of 0.1 m.