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Microparticle-Mediated Assembly of Cell-Derived Vascular Tissue Rings

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Authorship

The sections of this paper were written in equal parts by all team members. The paper was edited by all team members.

Abstract

Tissue engineered blood vessels have the potential to address the clinical need for blood vessel replacements. The Rolle lab at Worcester Polytechnic Institute (WPI) has developed a method for growing rings from smooth muscle cells (SMCs) and then fusing these rings together to form tubular vessel replacements. The goal of this project was to exert control over the aggregation process of SMCs in the initial tissue ring. An adhesion system that consists of gelatin microparticles was designed to improve aggregation. Initial testing showed the gelatin microparticles can be consistently created and incorporated into these tissue rings. The microparticles do not harm the SMCs when cultured together, and the addition of the microparticles does not prevent ring formation. Though the microparticles do not hinder the aggregation speed and strength of the rings, using these microparticles does not increase these properties as expected.

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1: Introduction

The damage and subsequent loss of blood vessels in patients that suffer from cardiovascular disease is a significant health concern in the United States. Replacement vessels have been developed, but they still face significant challenges especially when small diameter vessels are needed. Current solutions include autografts, biomaterials scaffolds for cell growth, synthetic vessels, and cell- derived constructs. Cell derived constructs provide several advantages because they can provide mechanically stable blood vessel replacements of small diameters that are not prone to occlusion (Roger, et al. 2011).

The Rolle lab at Worcester Polytechnic Institute (WPI) has developed a ring culture system to create tissue engineered blood vessels made of smooth muscle cells (SMCs) (Gwyther, et al. 2011). SMCs alone are seeded into wells, and over the course of time and with the force of gravity these cells settle and aggregate to form tissue rings. These rings are then cultured on a tube to allow them to fuse into a contiguous, functional vessel. However, cell aggregation and cell viability in the center of the ring are characteristics of the rings that still require improvement.

The goal of this project works to address these concerns and exert more control on the arrangement of the cells by adding an adhesion system to the ring culture system. Minimal biomaterials added to the present culture system will interact with the cells to develop a more highly controlled cellular structure. This could improve the rate and strength of cell aggregation. Figure 1 illustrates the design approach utilized by the team. Microparticles were fabricated and then tested with cells and in the ring well system used by the Rolle lab.

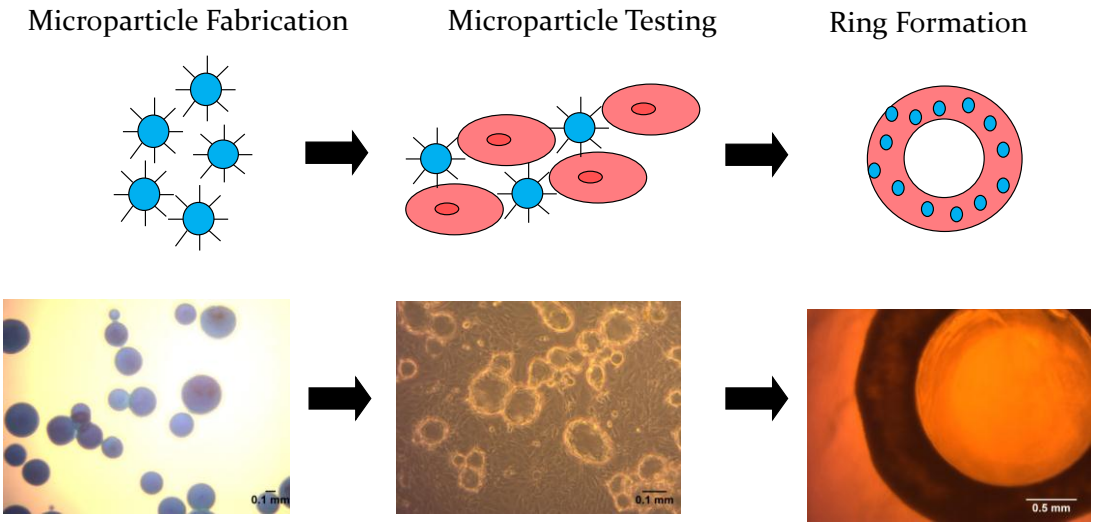

Figure 1: Final Design Approach

The adhesion system consists of gelatin microparticles that are integrated into the current ring production system by addition to the ring wells before the addition of the cells to the ring wells. These gelatin microparticles are produced using a water and oil emulsion and are then washed in acetone, isopropyl alcohol (IPA), and Dulbecco's Modified Eagle Media (DMEM). Microparticle size was assessed using light microscopy and ImageJ software. The biocompatibility of these microparticles was tested by addition of these microparticles to rat aortic smooth muscle cells (raSMCs) in tissue culture plates. This testing demonstrated that microparticles can be produced at a consistent size and that the microparticles do not contaminate cells in culture.

After biocompatibility testing, the microparticle adhesion system was then incorporated into the current ring formation method. The microparticles seeded with cells at various ratios to study the impact of the microparticles on ring formation. The external characteristics were observed visually, the internal structure was studied using various histology stains, and the mechanical properties were analyzed with tensile testing.

The microparticle adhesion system was able to form rings that are consistent with rings produced using the current fabrication method. This leaves many possibilities for future use of these particles, as well as further study into the properties of these microparticles.

2: Literature Review

2.1: Clinical Need

Cardiovascular disease causes the death of more than 2,200 Americans each day. Many of these patients suffer from atherosclerosis, which is a buildup of plaque in the vasculature. This plaque hardens the vessels and prohibits blood flow (Roger, et al. 2011). The loss of blood flow then leads to heart attack, ischemic stroke, heart failure, arrhythmias, and heart valve issues. There are several different methods of treatment for this disease. Medications like anticoagulants can be used to prevent clot buildup. Surgeries can be performed to remove the clot or insert a stent to allow blood flow through the vessel. Also, the blocked vasculature can be removed and a replacement can be positioned into the patient (Roger, et al. 2011). The replacement vessels that are currently available will be discussed in detail below.

2.2: Current Solutions

There are some existing solutions that help treat occluded or damaged blood vessels. These treatments vary in their use as well as their effectiveness. Even though these treatments do exist, there is need for improved solutions due to the limitations of each these options.

Blood thinners such as anticoagulants and antiplatelet drugs are pharmaceuticals that are used to reduce the chance of clot formation in a blood vessel (Venables 2000). These are used when a patient has an occluded but functional vessel. This is a temporary treatment that does not fix the occlusion. If treatment is discontinuous the occlusion could get worse and cause additional vessel damage. Hemorrhaging, which increases the risk of patient fatality, can also occur with the use of blood thinners (Roger, et al. 2011).

Allografts are another form of treatment for damaged blood vessels. These are vessel grafts that are taken from an individual that is the same species as the patient who is being treated. Allografts are used as a treatment option because they are relatively resistant to infection and immune responses and they do not have thrombogenic tendencies. The main issues with the use of these grafts are their limited availability and their tendency to calcify and rupture (Teebken and Haverich 2002).

Several types of vascular replacements that are manufactured *in vitro* are currently under investigation. Scaffolds are a common method of vascular replacement. These are typically made of biodegradable polymers or natural extracellular matrix (ECM) materials (Chlupac, Filova and Bacakova 2009). Synthetic vessels made of Dacron and PTFE are also used (Zacharias, Kirkman and Clowes 1987). Tissue engineered vessels made entirely of self-assembled cells are also being investigated for implant use. Each of these methods for tissue replacement has benefits and limitations (Gwyther, et al. 2011).

PLLA, PGA, or PLGA are biodegradable polymers that are commonly used as scaffold material because of their biocompatibility and controllable degradation properties. These materials, as well as other polymers, are easy to fabricate into many desired shapes, making them ideal for applications that require customization (Nikolovski and Mooney 2000). Also, the mechanical and physical properties of these polymer materials can be precisely controlled through processing techniques as well as the addition of other polymers or proteins. For example, PGA is often used to promote cellular attachment to a scaffold, and the addition of polyhydroxyalkanoate (PHA) increases the material's mechanical strength and degradation period. Of the biodegradable polymers available, PGA is the most commonly used because it can be easily handled, it is biocompatible, and it has a high porosity that promotes neovascularization

and nutrient diffusion. Additionally, the use of an acellular biocompatible material as a scaffold allows the body to supply native cells for the regrowth of tissue (Kakisis, et al. 2005). There are many characteristics that make biodegradable polymers suitable for synthetic vessel replacement, but limitations also exist. These materials are rapidly bioabsorbed and are unable to withstand the systemic pressures experienced within the vascular system (Kakisis, et al. 2005). Both of these limitations would affect the functionality of the scaffold, and could therefore lead to implant failure.

Collagen is the most common natural matrix material used in the production of vascular scaffolds. Collagen is found throughout the body as a naturally occurring ECM protein functioning in both structure and strength of tissue (Kakisis, et al. 2005). These properties make it a candidate for vascular scaffold production. The natural occurrence of collagen in the body reduces the inflammatory effects of the scaffold and provides a biomimetic environment for cellular growth. However, collagen scaffolds have demonstrated a lack of elastin when cultured *in vitro* and an unnatural orientation of the seeded smooth muscle cells. This leads to alterations in the function of the scaffold (Kakisis, et al. 2005). Cells do not respond to collagen scaffolds *in vitro* in the same way as they would respond to collagen in the body, which presents problems for scaffold success. Studies have shown that both the application of pulsatile flow or magnetic fields can alter the orientation of the cells on the scaffold (Kakisis, et al. 2005), but until the scaffold-cell interactions can be improved this solution is not ideal.

In order to find alternative ways to replace damaged vessels, synthetic blood vessels have been developed. Dacron and PTFE are the two materials that have been frequently used in the creation of these engineered blood vessels. These materials have poor elastic and compliant behavior compared to the natural tissue (Hoenig, et al. 2005). Other problems with these

synthetic grafts include inflammatory responses in the surrounding tissue and thrombosis (O'Donnell and al. 1985) (Atala, et al. 2008). These problems usually cause the vessel to fail. From the issues presented in the current treatments, it can be concluded that there is a need for a material or method that mimics the tissue in order to reduce or prevent these problems.

Tissue engineered vessels have become the targeted goal for vessel replacement. Thus far, tissue engineered vessels have been produced using cultured 2D sheets wrapped around a mandrel to produce a tube (L'Heureux 1998). During one study, 2D cell sheets were cultured for 30 days, rolled around a support, and cultured for another 8 weeks before removal. After this period of time, the tissue that formed appeared homogenous and well defined. The cells had begun to produce their own ECM and had functional thrombin receptors. However, the cell density of these tubes was lower than desired (L'Heureux 1998). These vessels demonstrate the ability to derive a graft solely from biological material, without the use of a scaffold. Limitations in the functionality of the design still exist and must be improved upon, but this method is one of the first successful developments of a tissue engineered vessel.

The tissue engineered sheet rolling method can be improved upon through the redesign of vessel formation, as seen in the compilation of SMC tissue rings. In this method, individual tissue rings are cultured, and then these rings are assembled together to form a vessel. These rings are formed through the aggregation of smooth muscle cells that have been cultured in a circular well with a rounded bottom and a post through the center. After only 24 hours the ring is visible, and each ring is cultured for at least 8-14 days. At this point, the rings are suitable for mechanical testing (Gwyther, et al. 2011). This method is significantly faster than the previous tissue engineered vessel, it creates rings of viable cells that produce their own ECM, and it contains no added or synthetic scaffold material. Though necrosis in the interior of these rings

and cellular aggregation are still issues, significant improvements to the development of tissue engineered vessels are made through this design.

2.3 Current Methods of Cellular Self-Aggregation and Adhesion

Although replication of human anatomy architecture is difficult, many researchers are moving away from the initial approaches to regenerative medicine. These initial approaches used scaffolds to engineer particular tissue and cause tissue regeneration. However, due to recent advancements in molecular engineering there is a new more complex, but beneficial approach to regenerative medicine. Instead of starting with a scaffold structural base, researchers are beginning just with cells and molecules to direct cell assembly and aggregation. This makes the tissue constructs more similar to human anatomy so they function more like the tissue rather than the scaffold, thus making them easier to integrate into the human body (Elbert 2011). There are varieties of methods, as discussed below, to cause this cellular assembly.

2.3.1: Culture System Aggregation

Many of the current systems utilized to produce oriented cellular aggregation involve the use of bioreactors and alterations to current scaffold systems. Growing tissue in an *in vitro* system is difficult, but bioreactors and scaffold based culture systems provide a finely regulated environment to promote cell growth and tissue structure (Duray, Hatfill and Pellis 1997). It is these new designs that provide insight into the alteration of this current system to improve cellular aggregation that mimics tissues from the body. Advances in the field have even brought new bioreactor and scaffold systems that cause improved cellular aggregation for normal tissue growth.

The National Aeronautics and Space Administration (NASA) recently developed a rotating wall bioreactor that creates a microgravity environment allowing cells to form into tissue constructs (Duray, Hatfill and Pellis 1997). This arranges the cells and produces an ECM similar to the native tissue. NASA has created several different types of tissue constructs using this device. It has been used in connection with microcarrier beads, as explained below. The success with this system provides important information about the effects of gravity on cellular aggregation and tissue formation. NASA developed a system to work without gravity to improve aggregation, while others have developed scaffold based culture systems that work with gravity and the cells to create tightly aggregated three-dimensional constructs (Duray, Hatfill and Pellis 1997).

The design of the culture systems has great influence on the conformational shape that a cellular aggregate takes. Current ring fabrication demonstrates that vascular tissue rings can form with a culture system that places cells in wells with a post in the middle. Youssef, et al., quantitatively characterized the force driving this type of assembly by seeding cells in micro-molded wells with varied right circular cones in the center of each well (Youssef, et al. 2011). Cells seeded in these wells formed toroidal microtissues through cellular interactions that often drove the structure up the cone. It was determined that the influence of gravity on the tissue assembly can be controlled by changing the slope of the cone and varying loading conditions. Furthermore, surface conditions and interactions of the cells were determined to be factors that influence the assembly of the cells into their three-dimensional structures (Youssef, et al. 2011). While the culture system plays a role in the assembly of microtissue structures, the components at the cellular level also have a prominent role in this assembly and are under investigation to induce cell aggregation into different three-dimensional structures.

2.3.2: Aggregation Involving Surface Molecule Attachment

A variety of methods under investigation examines and induces cellular assembly in an effort to direct cellular formation into particular constructs. One area of this research is the alteration of the surface chemistry of a cell. These alterations force the cells to react and move closer to one another in an effort to cause faster and oriented aggregation. Researchers have begun examination into surface energy alteration and the attachment of molecules such as sugars, proteins, and DNA.

Surface energy (SE) results from the bond disruption that leads to the breakdown of materials into smaller components (Ratner, et al. 2004). This type of energy plays a key role in adhesion of materials. Materials that have low SEs have a tendency to stick to materials that have relatively high SEs. Multiple properties effect whether a material has a low or high surface energy. These properties include surface area, bond type, and surface tension. The change in the surface energy of a material can be induced through the alteration of these properties, which will create a more adhesive material (Ratner, et al. 2004). Currently, little research has been performed on this topic in relation to cellular aggregation, but it has been noted that surface energy and surface tension have an effect on cellular adhesion properties (Youssef, et al. 2011). The information concerning surface energy is mainly related to materials research, but given its influence in these aspects, it has the potential to influence aggregation techniques.

Other surface alterations consist of the attachment of molecules to the surface of cells in an effort to increase the aggregation of cells into a desired structure. Sugars are one type of molecule that has been evaluated in an attempt to make cells selectively sticky. Scientists at University of California, Berkeley attached oligosaccharides to protein receptors on the surface of cells in an effort to create a molecular binding pattern for drug treatment for cancer cell

research (Service 1996). The molecular binding patterns of these sugars have alterable properties that might make it possible to improve cellular aggregation properties. These modifications were made by selectively altering the cell oligosaccharide and sialic acid groups through the attachment of a ketone. This approach to sugar modifications has not yet been applied in the body, but has shown potential in test tubes. This method has had problems with cellular apoptosis, but shows promise in the attachment of cells to scaffold material (Service 1996). This may be applicable in the current *in vitro* culture systems for blood vessel creation.

DNA attachment has also been established as a mechanism of cellular aggregation (Elbert 2011). Cell surfaces that have azide sugars react with single strand oligonucleotides. This complementary nucleotide binding mechanism allows DNA to be a viable option for cell aggregation. These nucleotides are molecularly assembled to create strands that protrude outward from the cell surface to increase aggregation. The shape of this aggregate depends on the concentration of the cells with one strand versus the number of cells with the complementary strand. When the cells are combined in a 1:1 ratio large aggregates are formed. When the cells are combined in a ratio of 1:50 microtissues are formed. The cells labeled with the less common strands are surrounded by multiple cells labeled with the complementary strand in a microtissue complex called a rosette. These microtissues are capable of generating a signaling network and can be generated by co-culturing pre-aggregated cells (Elbert 2011). It is possible to create aggregates of other shapes using DNA as the cell aggregation mechanism. This includes the formation of spheroids, honeycomb-like structures, and linear arrangements capable of contraction (Elbert 2011). The formation of these different aggregates using complimentary pairing of DNA strands could have application in the formation of blood vessels.

While the attachment of other molecules to the cells surface to increase aggregation present promising results, the use of native cell surface proteins also has possible applications for the increase in cellular aggregation. All cells have surface receptors that allow for the binding of different molecules and are involved in cellular adhesion. These molecules include integrins, the dystrophin-glycoprotein complex, the proteoglycan family, the cell adhesion molecules, and cadherins. Integrins are a category of receptors that link the actin cytoskeleton to the ECM of the tissue. They consist of alpha and beta subunits, and each combination has a unique binding specificity. Integrin binding can be controlled to regulate certain functions such as cellular adhesion, migration, and proliferation. Additionally, the dystrophin-glycoprotein complex (DGPC) is critical for proper smooth muscle function. This complex serves as the mechanical stability for the cell membrane during contraction and prevents the surface from overstretching. The proteoglycan family of syndecans is a group of adhesion transmembrane receptors with covalently attached glycosaminoglycan chains on the extracellular part of the receptor (Moiseeva 2001). However, the direct significance of their role in adhesion has not been fully studied or understood. Furthermore, the cell adhesion molecules (CAMs) are cell-cell adhesion molecules in the immunoglobulin family with transmembrane receptors that bind to and interact with particular cell surface molecules to cause adhesion. Lastly, cadherins are transmembrane molecules that are dependent on calcium ions. These are heterophilic cell-cell adhesion receptors that form a “zipper-like” structure in intercellular contact zones on cell surfaces (Moiseeva 2001). Through the study of these molecules, it is possible to identify characteristics that could help control cellular aggregation and organization to form complex structures such as blood vessels.

2.3.3: Aggregation Involving Microparticle Attachment

Certain researchers have been examining the cell directly to solve cellular aggregation problems for regenerative medicine. In regenerative medicine common practice has dictated that growing tissue is performed on scaffolds. However, with advances in these practices it has become possible to use different aggregation systems such as microparticles.

Microparticles have become an important research topic, especially in terms of drug delivery (Kohane 2007). The principles applied for these new drug delivery systems are also used in the creation of different tissues. Research has been conducted on the use of various types of microparticles to induce tissue formation.

In one study, gelatin was used to create microspheres to enhance the biological function of mesenchymal stem cell (MSC) bodies. The gelatin microspheres were created by a water in oil emulsion to chemically crosslink the gelatin in water (Hayashi and Tabata 2011). The results of this study showed that cell aggregates only formed in the curved bottom wells, and that the gelatin microspheres and MSCs had a homogenous distribution in the aggregates. It was also observed that the cell aggregates containing the largest diameter microspheres had the greatest number of living cells. The cells with the gelatin microspheres had better metabolic activity, a pertinent finding since complex cellular constructs often exhibit cell death due to a lack of nutrients. The formation of the aggregates depends on both the number and size of the microspheres. If there are too many microspheres or the microspheres are too large then there are no cell-to-cell interactions and an aggregate cannot form. The presence of the microspheres is advantageous in the creation of the cell aggregates (Hayashi and Tabata 2011).

The use of polymer microspheres in tissue engineering applications involves polymers coated in a substance to induce cell attachment and proliferation for aggregation studies. There

are studies using only the polymer microspheres, but attachment of various molecules has proven beneficial when they interact with cell surface adhesion molecules. In one study researchers created PLLA microspheres using oil in water emulsion solvent evaporation method and then attached chitosan to the surface of the microparticles (Lao, et al. 2008). When assessed *in vitro* with cells (specifically rabbit auricular chondrocytes) the microspheres exhibited significant ability to promote both the attachment of cells and cell proliferation. The chitosan is mostly responsible for this reaction, but the use of microparticles as efficient carriers of biological molecules represents their possible use in cellular aggregation (Lao, et al. 2008). If these microparticles can be coated with adhesion molecules they could induce cells to aggregate quickly, tightly, and in a more oriented fashion.

In other studies, researchers are not only creating polymer-microencapsulated microspheres, but also surface modified protein microspheres. Microparticles created from proteins are a particular interest because of their biodegradability, biocompatibility, and stability. The multiple methods to create these microparticles include crystallization, freeze-drying, calcium carbonite templating, chemical cross-linking, and sono-chemical polymerization (Chau, Agashi and Shakesheff 2008). Sono-chemically producing microparticles caused polymerization of the molecule and formed microparticle core-shell structures. Then the microparticles were conjugated on the shell to allow proteins to adhere to them. This induces the formation of hollow microspheres that can be used to encapsulate other molecules for delivery to the tissues (Richman, et al. 2011). Overall, the use and creation of microparticles varies widely and could be applicable in tissue engineered blood vessels.

In conjunction with microparticle research, the use of magnetic fields and magnetic microparticles is also an emerging concept in tissue engineering. Researchers in Japan have

developed and adapted the use of these microparticles to create blood vessels (Shimizu, et al. 2007). The researchers were able to seed a porcine vessel scaffold using these magnetic microparticles to create a 3-D vascular tissue graft. The experiment consisted of labeling three types of cells with original magnetic cationic liposomes (MLCs): NIH 3T3 cells, dermal fibroblasts, and smooth muscle cells. The average size of these microparticles was 150 nm. A cylindrical magnet was covered in a Teflon tube and was then placed in the middle of a decellularized porcine graft. Then the graft was rotated in suspension. This process shows promise for the creation of a human cell seeded vessel (Shimizu, et al. 2007). These results show that it might be possible to apply magnetic microparticles to the creation of a 3-D vessel.

The studies mentioned above exhibit different examples of microparticles used for cell aggregation. It is also possible to create microspheres with natural materials for use in cellular aggregation. Additionally, it is possible to create microspheres from polymers and attach proteins to the surface to encourage aggregation. Magnetic microparticles also present an option. Multiple methods exist to potentially achieve enhanced cellular aggregation.

2.3.4: Design Opportunities

There are currently multiple approaches to inducing and improving cellular aggregation. The main approaches are culture systems, surface modification, and microparticle research. Each option has advantages and disadvantages in their use for cellular aggregation. The options exhibiting the most promise are the culture systems built to induce aggregation into particular three-dimensional structures. The device used to create vascular tissue rings is a promising method that produces viable and well-constructed vascular tissue rings for blood vessel formation. The current device however still faces problems with tight aggregation of the cells.

This MQP team seeks to solve this problem through the development of an adhesion system that will produce viable and tightly aggregated vascular tissue rings that can be formed into blood vessels.

3: Project Strategy

3.1: Initial Client Statement

Below is the initial client statement provided to the team by the advisor and client Dr. Marsha Rolle:

> "We have developed a technique that allows cellular self-assembly into 3D tissue rings for vascular tissue engineering. However, some of the cells we are working with do not readily aggregate in our system. While the focus of our research is to create cell-derived tissues (without exogenous biomaterial scaffolds), a small quantity of cell adhesive microparticles may enhance our system by providing a "glue" to enable more cohesive tissue ring formation than passive aggregation alone. This may also allow us to use fewer cells for each ring construct, thus making our system more efficient. Finally, these microparticles could potentially be used as drug delivery vehicles to overcome the limitations of nutrient diffusion in our thick vascular tissue rings."

This initial client statement was then developed further by identifying objectives and constraints, and finally by revising the client statement.

3.2: Objectives

Throughout the course of the problem definition, it became clear that this project needed to meet the needs of tissue ring culture as well as define the attributes of an adhesion system. As a result, the team developed two objective trees to address these two facets of the project.

3.2.1: Adhesion System

A list of objectives was developed based on research found in published literature to examine the initial client statement. The team developed the first list of objectives for the adhesion system, as seen below in Figure 2:

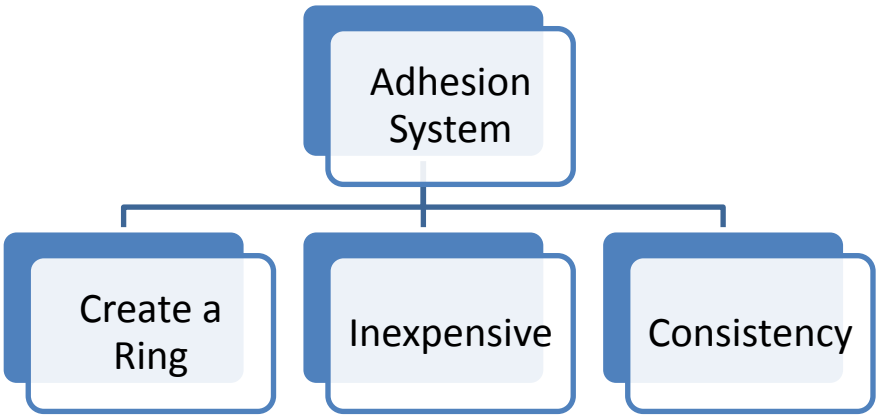

Figure 2: Adhesion System Objectives Tree

- *Create a ring*: This objective maintains that the group will create an adhesion system that causes the cells to adhere into a particular 3-D construct. The adhesion system must produce a uniform circular 3-D ring. The adhesion system should not disrupt the currently produced structure of the ring, but should enhance aggregation in this same shape.
- *Inexpensive*: The adhesion system created must allow the group to create a ring using fewer resources than currently required. This can include the use of fewer materials such as cells, media, and growth factors, or could allow for ring production in less time. This adhesion system is more useful if it can produce a ring that is testable for mechanical properties using fewer resources.
- *Consistent:* An adhesion system that produces a ring in 100% of attempts is important because the system should not reduce the efficiency of the currently used methods. Since

the client can seed cells in the scaffold system and produce rings 100% of the time, this new adhesion system must also produce rings in 100% of attempts.

3.2.2: Tissue Ring

Next, a set of objectives was developed for the tissue ring created by the system, as seen in Figure 3 below:

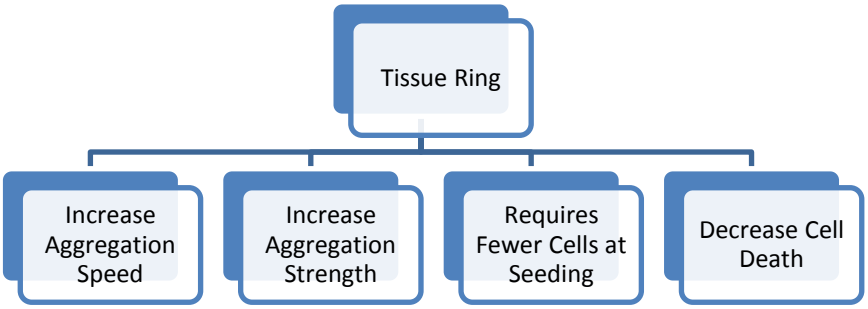

Figure 3: Tissue Ring Objectives Tree

- *Increased Aggregation Speed*: It was determined that a complete ring should form in less than 24 hours, which is the current formation period. The increased aggregation speed should also make it possible for the ring to be ready for mechanical testing in less than seven days, which is the current time mechanical testing is performed on the tissue rings. An increase in aggregation speed is important for the overall improvement of ring formation.
- *Increased Aggregation Strength*: Currently, there are still cells that do not aggregate to form tight circular rings. Increasing the aggregation strength of the ring should result in a uniform ring for testing and application in the formation of full blood vessels.

- *Requires Fewer Cells at Seeding*: The current method requires 500,000 cells in 100 µl of media to effectively create a well-formed tissue ring. The amount of cells that each ring requires is a greater expense to the client. Due to this concern, it was determined that reducing the amount of cells added is important to decrease the expense.
- *Decrease Cell Death*: In addition, many of these tissue rings are exhibiting cell death at their centers because of nutrient diffusion problems. The cells are packing together so tightly that the cells in the center are not obtaining adequate nutrients. Decreasing cell death will make each ring viable for further uses in testing and formation into blood vessels.

3.2.3: Comparison of Objectives

In order to evaluate the objectives for the adhesion system and the tissue ring, two separate Pairwise Comparison Charts were created. These charts were used to rank the objectives in order of importance so that the design can properly account for the objective with greatest priority.

The three objectives for the adhesion system were evaluated for order of importance using a Pairwise Comparison Chart. This table allowed for the determination of the order of importance between the three objectives to make sure that the design of the adhesion system exemplified each of the objectives. From this chart, it was determined that the objective "Create a Ring" is the most important because if the adhesion system cannot perform this task then it is of no use. The main priority is to help form better rings, not impede the current process. Then, "Consistency" was considered the next most important objective because the system should produce a ring with every attempt, since that is the current production efficiency. The cost of the ring creation was determined to be the least important when compared to the other two, because

it is the most flexible of the objectives. If the benefits of the changes in the new adhesion system prove worth an additional cost, that cost can be evaluated.

After determining the level of importance of each objective, the values were utilized to weigh each objective for use in evaluating conceptual design. Each score was first normalized to make it possible to create a score for the objectives. These values were normalized by adding one to the total score given to each objective because it is not possible to weight the objectives when there is a zero value. Then the normalized scores were added to obtain a total value and this value was divided into each normalized score for a weighted score, as see in Table 1.

Table 1: Adhesion System Pairwise Comparison Chart

Adhesion System Pairwise Comparison Chart						
	Create a Ring	**Inexpensive**	**Consistency**	**Total**	**Normalized Score**	**Weighted Score**
Create a Ring	X	1	1	2	3	0.50
Inexpensive	0	X	0	0	1	0.17
Consistency	0	1	X	1	2	0.33

The four objectives established for the tissue ring were evaluated for importance in the Pairwise Comparison Chart seen in Table 2. Discussion of the objectives and their rank through this chart allowed the group to determine their order of importance for the design. From the discussion of these objectives, it was determined that an increase in the aggregation speed is the most important objective. The rings should form more rapidly so that they are more readily testable. This also cuts cost because less time has to be spent culturing each ring, and fewer supplies such as media are required. After increased aggregation time, increased aggregation strength was determined to be the next most important. The rings produced will eventually need to be able to resist the pressure of blood flow, making their strength an important aspect if they are to be used as either a vessel model or clinically. Therefore, their success is partially

determined by their strength. “Reduce cell death” was deemed less important than the first two because, without a formed ring structure, the necrosis in the tissues is irrelevant. Finally, reducing the amount of cells used to create the ring was determined to be the least important objective because if the system improves overall ring creation, but still uses the same amount of cells, then the system is still useful and therefore successful. Reducing the amount of cells is a lower priority than enhancing the aggregation of cells in ring formation. Then as performed previously above, the total scores were normalized and a weighted score was determined for each of the objective listed in the chart.

Table 2: Tissue Ring Pairwise Comparison Chart

Tissue Ring Pairwise Comparison Chart							
	Increased Aggregation Speed	**Increased Aggregation Strength**	**Reduce Cell Death**	**Requires Fewer Cells at Seeding**	**Total**	**Normalized Score**	**Weighted Score**
Increased Aggregation Speed	X	1	1	1	3	4	0.40
Increased Aggregation Strength	0	X	1	1	2	3	0.30
Reduced Cell Death	0	0	X	1	1	2	0.20
Requires Fewer Cells at Seeding	0	0	0	X	0	1	0.10

3.2.4: Metrics for Objectives

After evaluating the objectives through the Pairwise Comparison Charts, a set of metrics was developed for each of the listed objectives. These metrics provide a method to measure the achievement of each objective. The objectives and metrics are listed below in Table 3:

Table 3: Objectives and Metrics

Objective and Metrics	
Objective	**Metric**
Create a Ring	Number of rings created in each attempt
Inexpensive	Estimated Dollar Amount
Consistency	Number of usable/testable rings
Increased Aggregation Speed	Amount of change in aggregation time
Increased Aggregation Strength	Amount change in strength and cells that do not aggregate
Reduced Cell Death	Number of dead cells
Requires Fewer Cells at Seeding	Initial number of cells seeded to form a tissue ring

Once the metrics were chosen a point system was created to determine if the objective meets the final design criteria. Each metric is scaled from 0 to 100 points and is based on how well the objective was met. In this case, 0 represents failing to meet the objective at all and 100 represents completely meeting the objective. The metric for each objective is listed below with descriptions of how points would be assigned in each case:

Metrics for Objectives (Adhesion System):

Objective: Create a Ring

Units: Ratings of the ring creation from 0 (worst) to 100 (best)

Metric: Assign points according to the following scale

Always produces a ring (100%)	100pts
Produces a ring 75% of attempts	75pts
Produces a ring 50% of attempts	50pts
Produces a ring 25% of attempts	25pts
Never produces a ring	0pts

Objective: Inexpensive

Units: Ratings of the expensive of entire ring creation process (adhesion system and tissue ring) from 0 (worst) to 100 (best)

Metric: Assign points according to the following scale

Under budget	100pts
On budget	50pts
Over Budget	0pts

Objective: Consistency

Units: Ratings of the consistency of the adhesion to create a ring from 0(worst) to 100(best)

Always produces correct type of ring	100pts
Sometimes produces correct type of ring	50pts
Never produces correct ring	0pts

Metrics for Objectives (Tissue Ring):

Objective: Increase Aggregation Speed

Units: Ratings of the increase in aggregation speed from 0 (worst) to 100 (best)

Metric: Assign points according to the following scale

Increase in aggregation speed	100pts
No change in aggregation speed	50pts
Decrease in aggregation speed	0pts

Objective: Increase Aggregation Strength

Units: Ratings of the increase in aggregation strength from 0 (worst) to 100 (best)

Metric: Assign points according to the following scale

Increase in aggregation strength	100pts
No change in aggregation strength	50pts
Decrease in aggregations strength	0pts

Objective: Reduced Cell Death

Units: Ratings of reduced cell death from 0 (worst) to 100 (best)

Metric: Assign points according to the following scale

No cell death	100pts
Less cell death	50pts
Same amount of cell death	0pts

Objective: Requires Fewer Cells at Seeding

Units: Ratings of the cell amount from 0 (worst) to 100 (best)

Metric: Assign points according to the following scale

Drastic decrease in amount of seeded cells (50%)	100pts
Large decrease in amount of seeded cells (62.5%)	75pts
Noticeable decrease in amount of seeded cells (75%)	50pts
Slight decrease in amount of seeded cells (87.5%)	25 pts
Same amount of seeded cells (100%)	0pts

3.3: Constraints

A list of constraints for the project was also determined to further define the limitations that control the success of the project. Without satisfying each of these constraints, the system fails. Each of the constraints required for the system to be successful are listed with descriptions below:

- *Biocompatible / Non-toxic:* The adhesion system used to aggregate the cells into a ring needs to biocompatible and non-toxic. If not, it will kill the cells and the tissue rings will no longer be viable for use.
- *No negative impact on current ring structure:* The adhesion system cannot negatively affect the current ring system and cause any deformation. If the adhesion system does not form a ring then the design has failed.
- *User Safety:* The adhesion system and the tissue rings need to be safe for the user to handle. If it is not safe to handle, the rings cannot be used in either a vascular model or clinical application.
- *Sterilizable:* The system as a whole needs to be sterilizable or it cannot be used in cell culture because of the risk of contaminating the cells. Additionally, future use of the produced rings in blood vessels for eventual implantation would not be possible without the ability to sterilize the product.
- *Cost:* The cost of the adhesion systems and the tissue ring cannot exceed $524.00, the budget of the group.
- *Time:* The design project is constrained to a 28-week period.

- *Materials must be commercially available:* The materials must be commercially available so that the project team can create the adhesion system and for easy replication if the design is applied in the lab.
- *Must fit in 2mm diameter culture wells or culture system in general:* The adhesion system and all necessary materials need to fit in the standard culture system. If they do not fit in the culture system, then the tissue rings cannot be produced.

3.4: Revised Client Statement

The client statement below has been revised by the team to reflect the work done in the problem definition:

> Create an adhesion system that will enhance cell-cell, cell-ECM, and/or ECM-ECM aggregation between smooth muscle cells in the creation of tissue rings. This adhesion system should decrease the number of cells necessary for the creation of vascular tissue rings, improve the current nutrient diffusion limitations, and maintain or reduce current costs.

4: Alternative Designs

4.1: Functions and Means

The group developed a defined list of functions and means using brainstorming and the creation of a morphological chart. After a list was created a structured method was used to evaluate each mean and develop a list of conceptual designs.

4.1.1: Establishing Functions and Means

To develop the functions of this adhesion system, the team held a brainstorming session to determine the functions required to meet each of the objectives. At this session the team developed two functions of the adhesion system: make cells stick together and create space between the cells. These functions are simple, but creating designs with these functions should allow for the achievement of the objectives. The function of making cells stick together should cause the cells to aggregate faster and create a stronger ring every time a ring is seeded in the molds. The function of creating space between the cells should allow for diffusion throughout the ring and decrease cell death. This will allow for a decrease in the amount of cells necessary to create a functional ring.

After the development of the functions the team began to create means for achieving these functions. The team held a brainstorming session to identify particular means for each of the functions developed for the project. From the list of brainstormed means the team created a morphological chart, as shown in Table 4. This chart contains functions in the left column and all the potential means listed in rows.

Table 4: Morphological Chart

Morphological Chart							
Function	Mean 1	Mean 2	Mean 3	Mean 4	Mean 5	Mean 6	Mean 7
Stick Cells Together	Surface Protein Interaction	DNA binding	Magnetic Microparticles	Surface Energy	Electric Pulses	Ground Scaffold Material	Spheroids put into culture wells
Create Space	Organic Molecule Micelle	Solid Microparticle	Hollow Microparticle	Microgravity cell/media suspension	Ground/Gel Scaffold Material		

4.1.2: Elimination of Means

After the creation of the morphological chart the team used various methods to eliminate means. First, the team evaluated the means and removed any ideas that were repetitive. Ground scaffold material was removed because this mean would add larger microparticles of the materials used for microparticles. The size of the material microparticles would inhibit cell aggregation around the post because the cells would not be able to find each other. Then the team eliminated means if the mean could not meet both functions on its own or in combination with another mean. Electric pulses were removed because they can make cells stick together, but they cannot create space between cells. After the group created a streamlined list of means the team developed conceptual designs. Then the team evaluated each design through further research, feasibility, pricing, and a numerical evaluation matrix. The conceptual designs that made it through the initial phases of evaluation are briefly detailed below.

4.2: Conceptual Design Ideas

Conceptual Design 1: Magnetic Microparticles

Magnetic microparticles are made from iron oxide, which is a biocompatible material. These microparticles are coated with a polymer and then an adhesion protein, as seen in Figure 4. Microparticles can also be coated with the adhesion protein alone seen in Figure 5. Then the microparticles are added to the current ring creation system with the cells to form the proper vascular tissue rings.

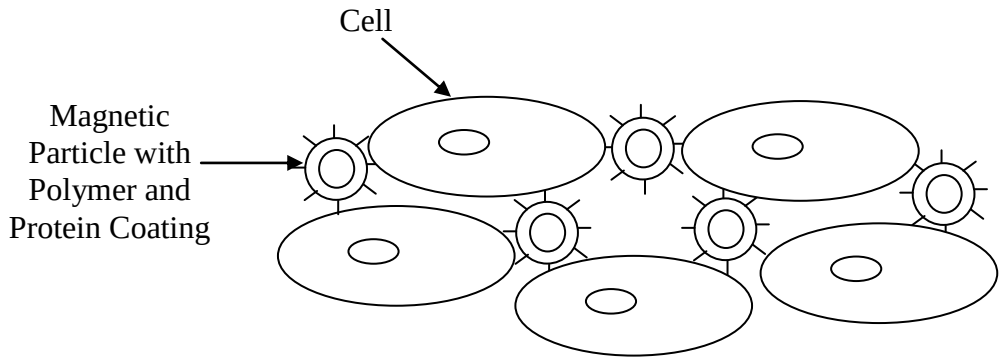

Figure 4: Diagram of Cells and Magnetic Microparticles with Polymer and Protein Coating Adhesion

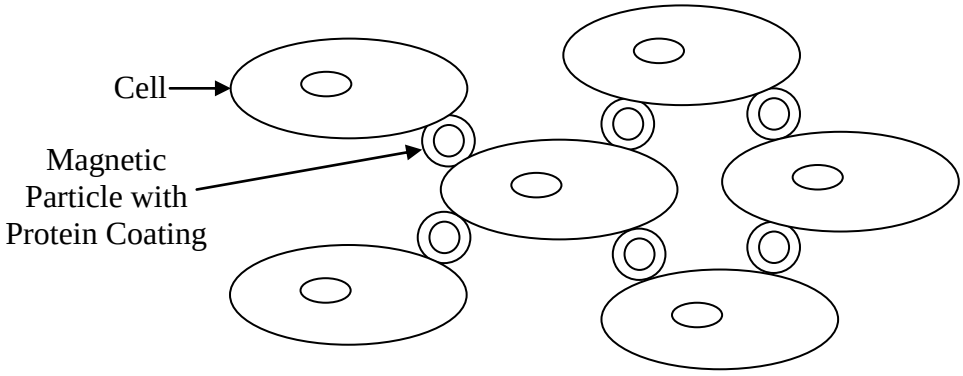

Figure 5: Diagram of Cells and Magnetic Particles with Protein Coating Adhesion

Pros	Cons
Create Space	Polar Controls
Coated with proteins/polymers	Size control difficult
Created or Purchased	Uniformity
Sticks cells together	Magnet and polymers may be cytotoxic
	Creation is more time consuming

Conceptual Design 2: Microgravity Bioreactor

The group would need to build a microgravity bioreactor, as described in the literature review, that causes the cells to form spheroids. Multiple spheroids are created and then removed from the bioreactor. The spheroids would then be placed into the current culture system to form vascular tissue rings.

Pros	Cons
Sticks cells together	No space created
Biocompatible	Create spheroid, must be seeded in ring
	No combination with other system to create space because of uneven mixing with high speed aggregation
	Expensive (Outside budget)
	Time required to make multiple spheroid for one ring is not practical
	Pack cells together to tightly causing diffusion problems

Conceptual Design 3: Microparticles

Solid Polymer Microparticles:

Solid microparticles are created using the water in oil emulsion technique with PLLA, PGA, or PLGA. After the microparticles are formed they are coated in adhesion proteins using a submersion technique. The microparticles are then placed in the current ring culture system with the smooth muscle cells to form the necessary tissue rings, as seen in Figure 6.

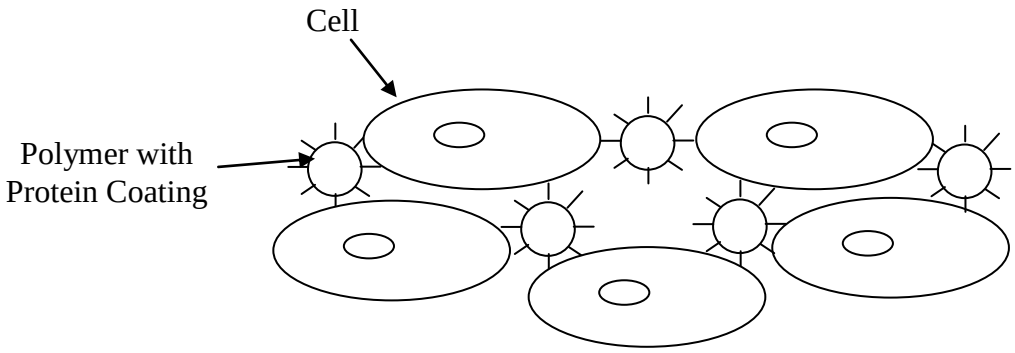

Figure 6: Diagram of Cells with Protein Coated Polymer Microspheres

Pros	Cons
Creates Space	Size control difficult
Sticks Cells Together	Process has more added time
Inexpensive	Breakdown time
Biocompatible	Proper protein attachment difficult
	Breakdown produces possible toxic substances

Hollow Polymer Microparticles:

Hollow microparticles are created sono-chemically with PLLA, PGA, or PLGA. The microparticles are then submersed in adhesion proteins to coat the microparticles. Once coated in protein, the microparticles are placed in the current ring culture system with the smooth muscle cells, as seen in Figure 7.

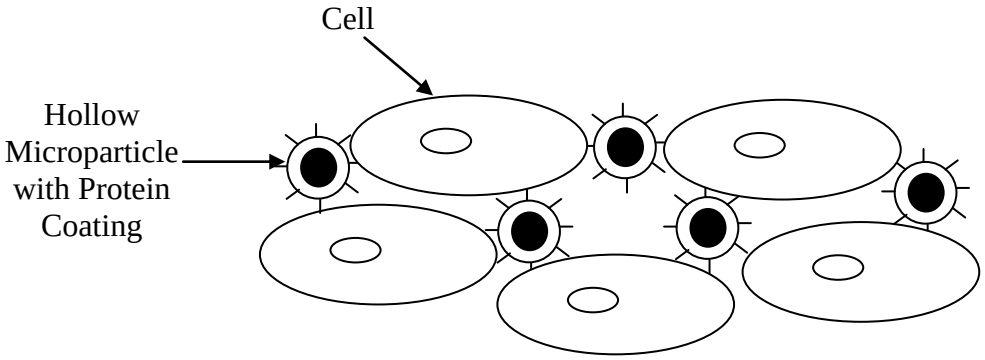

Figure 7: Diagram of Cells and Hollow Microparticle with Protein Coating Adhesion

Pros	Cons
Creates Space	Size control difficult
Sticks Cells Together	Process has more added time
Inexpensive	Breakdown time
Biocompatible	Proper protein attachment difficult
	Requires more steps than solid microspheres
	Breakdown produces possible toxic substances

Protein Microparticles:

Another design possibility is to create a microparticle completely from the protein using a water in oil emulsion. Once the microparticles are formed they can be added into the culture system with the cells to form a tissue ring. After they are added to the culture system they should adhere to the cells, as seen in Figure 8.

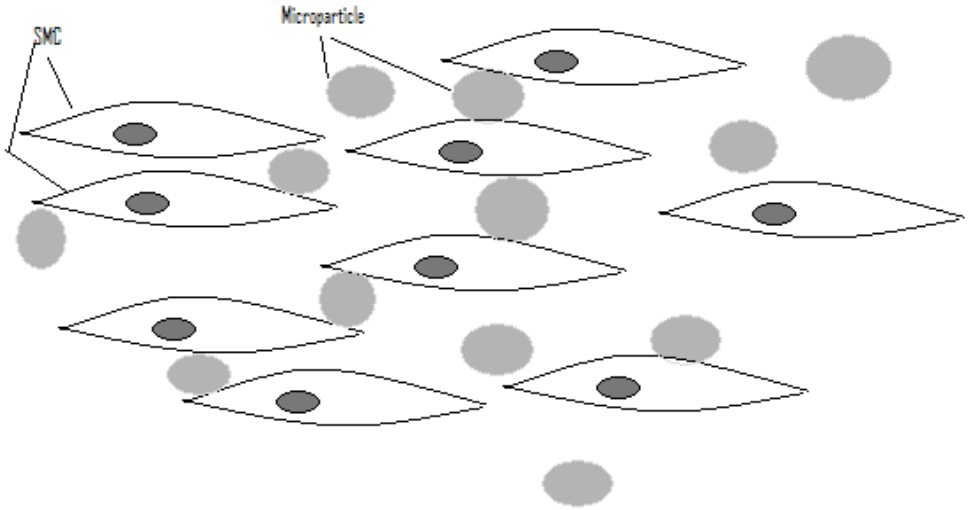

Figure 8: Diagram of Cells and Protein Microparticle Adhesion

Pros	Cons
Completely protein (natural)	Size control difficult
Create Space	Process has more added time
Inexpensive	
Biocompatible	
Stick cells together	
Non-toxic breakdown	

Conceptual Design 4: Lipid Micelles

A lipid micelle is created using phospholipids that will self-assemble when placed in solution. Then proteins are attached to the lipid micelle, either on the surface or into the bilayer. This lipid micelle is then placed into the culture system with the smooth muscle cells to form vascular tissue ring, as seen in Figure 9.

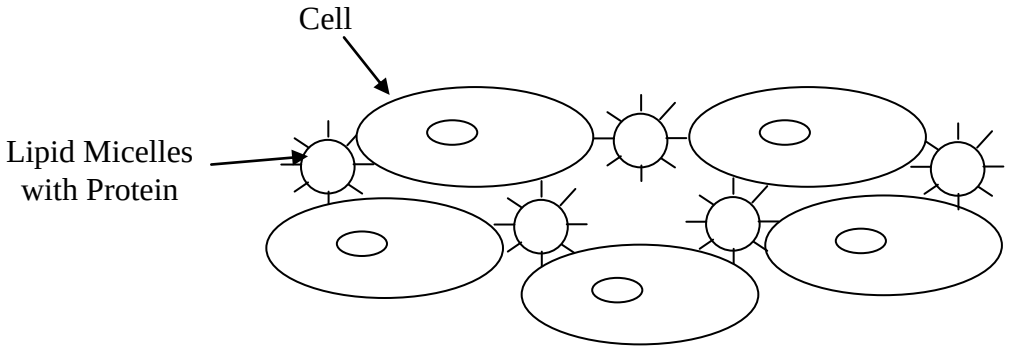

Figure 9: Diagram of Cells and Lipid Micelle with Protein Adhesion

Pros	Cons
Inexpensive	Might go into cells rather than stick to surface, then no space
Biocompatible	Might absorb media needed for cells

Conceptual Design 5: DNA Attachment

This design was conceptualized based on the work of (Elbert 2011). The adhesion of cells will be mediated by complimentary DNA. The DNA molecules will be conjugated to the surface of the cell. The complimentary pairing will occur between DNA strands on smooth muscle cell or DNA strands on the cells and strands on intermediate microparticle to take up additional space, as seen in Figure 10.

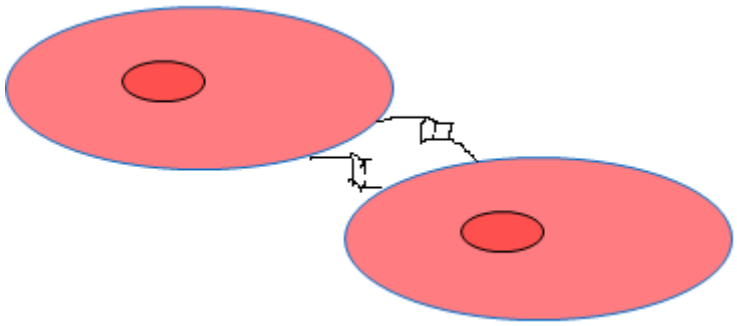

Figure 10: Diagram of Cells adhered through DNA Attachment

Pros	Cons
Simple attachment	Expensive
Can be attached to microparticles for space	Specifically built proteins
Natural (Non-toxic)	

Conceptual Design 6: Surface Energy Mediation

Another design alternative uses the concept of surface energy to allow for adhesion. The material chosen will have a lower surface energy than the smooth muscle cells. This will allow the material to adhere to the cells while inhibiting other substances to adhere to it, as demonstrated in Figure 11.

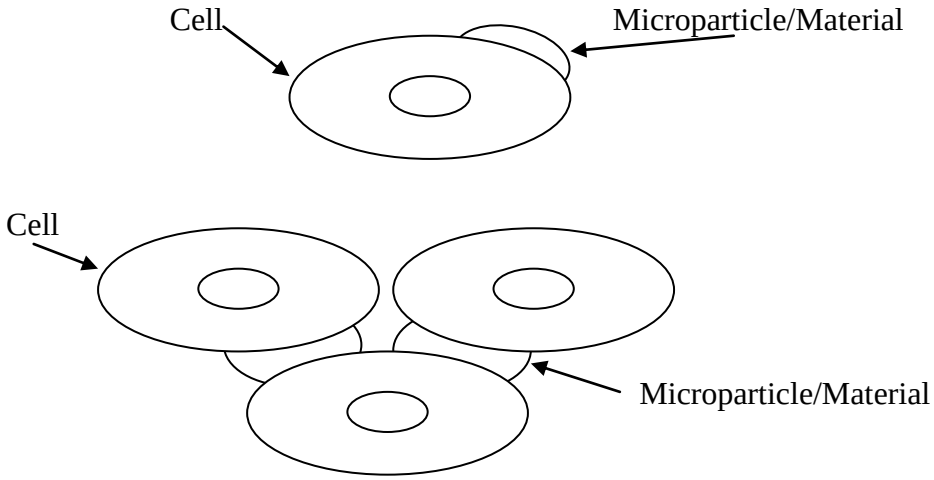

Figure 11: Diagram of Cells and Surface Energy Adhesion with Microparticles

Pros	Cons
Creates space	Have to control surface energy of every material in culture system
Sticks cells together	Longer process for microparticle creation and surface energy control
Inexpensive	

Conceptual Design 7: Spheroids of cells in ring wells

Cells will be grown into spheroids. Multiple spheroids will then be placed into the ring molds and cultured to form tissue rings, as seen in Figure 12.

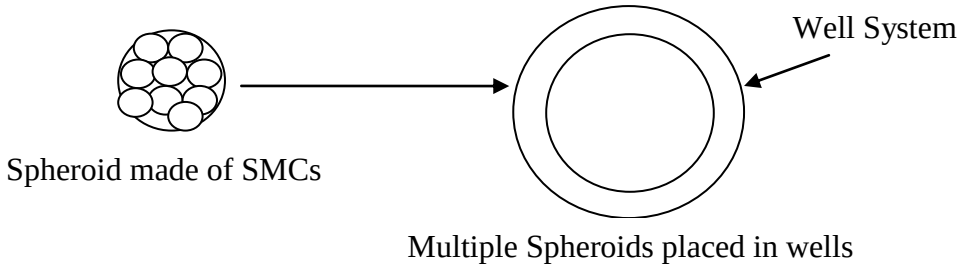

Figure 12: Diagram of Spheroid Adhesion

Pros	Cons
Increased aggregation strength	Does not necessarily decrease cell amount
	Same/More time

Conceptual Design 8: Protein Solution

As seen in Figure 13, protein solutions of a particular concentration will be added to the well system when creating rings. This protein should aid in cellular aggregation and form well-adhered tissue ring.

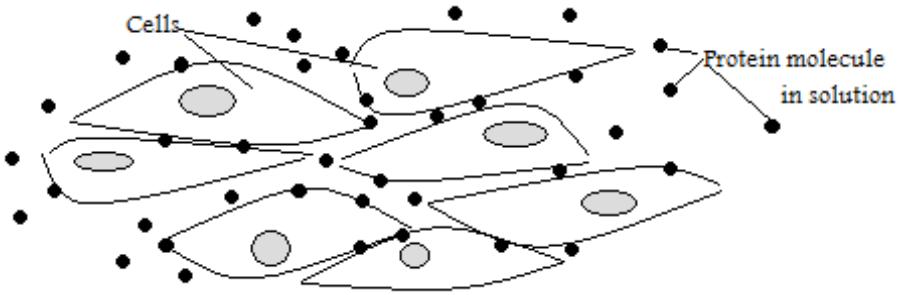

Figure 13: Diagram of Cells with Protein Solution

Pros	Cons
Viewable under a fluorescent microscope to verify incorporation	Does not decrease cell amount
Inexpensive	Will not create space to decrease cell death
No added time	
Does not complicate procedure	
Biocompatible	
Aggregate cells together	

Numerical Evaluation of Conceptual Designs

After the examination of each of these conceptual designs, the group filled out a numerical evaluation matrix, as seen in Table 5, to determine the feasibility of each designs. The team created charts that listed the constraints and objectives on the left and means across the top. Then each design was evaluated based on these parameters. If a design did not meet a constraint that box was checked off and the design was immediately eliminated. The designs that were eliminated this way are the following: DNA, magnetic microparticles, the microgravity bioreactor, and surface energy.

DNA was eliminated based on the constraint of cost. It was determined that it would cost $6,000.00 to buy 100 complimentary pairs of DNA strands. The strands have to be specifically built to stick out perpendicular to the cell and attach for cell aggregation. Magnetic microparticles were also eliminated because of cost. The microparticles of the correct size are too expensive. The microparticles must be smaller than the cells or proper three-dimensional

aggregation will not occur. The microgravity bioreactor was also eliminated due to cost. It was considered too expensive to build. Surface energy was eliminated because of time constraints. Proper control of the surface energy of each material in the design system was considered too time consuming and not easily reproducible.

Once designs were eliminated using the constraints the others that remained were compared using the objectives. Each design was given a score based on the preset metrics in Chapter 3, and how well it met each objective. Then these values were multiplied by the weights of the objectives. Each score was then tallied to determine the ranks of each conceptual design.

Table 5: Numerical Evaluation of Conceptual Designs

NUMERICAL EVALUATION MATRIX																
Means		**Protein Microspheres**	**Protein Microspheres**	**Protein Alone**	**Protein Alone**	**Polymer Microspheres with proteins**	**Polymer Microspheres with proteins**	**Lipid Micelles with protein**	**Lipid Micelles with protein**	**Cellular Spheroids**	**Cellular Spheroids**	**DNA**	**DNA on particle**	**Magnetic microspheres**	**Microgravity**	**Surface Energy**
	Objective Weights	**Normal Score**	**Weighted Score**	**Normal Score**	**Weighted Score**	**Normal Score**	**Weighted Score**	**Normal Score**	**Weighted Score**	**Normal Score**	**Weighted Score**					
Constraints:																
Biocompatible																
No impact on ring structure																
User safety																
Sterilizable																
Cost												X	X	X	X	
Time																X
Commercially available materials																
Fits into culture wells																
Adhesion System Objectives:																
Create a Ring	0.50	100	50	100	50	100	50	100	50	100	50					
Inexpensive	0.17	50	8.5	50	8.5	50	8.5	50	8.5	100	17					
Consistent	0.33	100	33	100	33	100	33	100	33	100	33					
Tissue Ring Objectives:																
Increase Speed	0.40	100	40	100	40	100	40	100	40	100	40					
Increase Strength	0.30	100	30	100	30	100	30	100	30	100	30					
Fewer Cells	0.10	100	10	50	5	100	10	100	10	50	5					
Decrease Death	0.20	100	20	50	20	100	20	100	20	100	20					
Total		650	191.5	550	176.5	650	191.5	650	191.5	650	195					

Proteins important for aggregation were evaluated through a numerical evaluation matrix, as seen in Table 6. First, the proteins were evaluated using the constraints. Vitronectin and osteopontin were eliminated because of their cost. Vitronectin costs $200.00 - $ 250.00 and osteopontin costs $300.00 - $500.00 for an insufficient amount. Each protein costs more than half of the project budget. This means that the team could not test more designs if either material was purchased. The remaining conceptual designs were then compared using the adhesion

system objectives. They were not compared using the tissue ring objectives because the proteins alone would not achieve these objectives. Each protein was given a score based on the preset objectives and that score was multiplied by the weights and then tallied for a final ranking of the proteins.

Table 6: Numerical Evaluation of Possible Proteins

Protein Numerical Evaluation Matrix											
Means		Fibronectin	Fibronectin	Gelatin	Gelatin	Laminin	Laminin	Collagen	Collagen	Vitronectin	Osteopontin
	Objective Weights	Normal Score	Weighted Score	Normal Score	Weighted Score	Normal Score	Weighted Score	Normal Score	Weighted Score		
Constraints:											
Biocompatible											
No impact on ring structure											
User safety											
Sterilizable											
Cost										X	X
Time											
Commercially available materials											
Fits into culture wells											
Adhesion System Objectives:											
Create a Ring	0.50	100	50	100	50	100	50	100	50		
Inexpensive	0.17	50	8.5	100	17	50	8.5	100	17		
Consistent	0.33	100	33	100	33	100	33	100	33		
Tissue Ring Objectives:											
Increase Speed	0.40	N/A		N/A		N/A		N/A			
Increase Strength	0.30	N/A		N/A		N/A		N/A			
Fewer Cells	0.10	N/A		N/A		N/A		N/A			
Decrease Death	0.20	N/A		N/A		N/A		N/A			
Total		250	91.5	300	100	250	91.5	300	100		

The team also utilized this tool to compare the different polymers for microparticle creation. The polymers were evaluated using the same process as described previously. From this numerical evaluation matrix, as seen in Table 7, it was determined that PGA did not meet the constraint of materials being commercially available. The other two materials were then compared using the weighted objectives.

Table 7: Numerical Evaluation of Possible Polymers

Polymer Evaluation Matrix						
Means		PLGA	PLGA	PLLA	PLLA	PGA
	Objective Weights	Normal Score	Weighted Score	Normal Score	Weighted score	
Constraints:						
Biocompatible						
No impact on ring structure						
User safety						
Sterilizable						
Cost						
Time						
Commercially available materials						X
Fits into culture wells						
Adhesion System Objectives:						
Create a Ring	0.50	100	50	100	50	
Inexpensive	0.17	50	8.5	100	17	
Consistent	0.33	100	33	100	33	
Tissue Ring Objectives:						
Increase Speed	0.40	N/A	N/A	N/A	N/A	
Increase Strength	0.30	N/A	N/A	N/A	N/A	
Fewer Cells	0.10	N/A	N/A	N/A	N/A	
Decrease Death	0.20	N/A	N/A	N/A	N/A	
Total		250	91.5	300	100	

After unfeasible designs were eliminated, the team began work on developing an experimental procedure. During the creation of this procedure it was determined that the team required an immunofluorescence kit to properly visualize the proteins in the culture rings. This kit is vital to test and evaluate the majority of the designs. The test kit, which the team purchased, cost $370.00. This price decreased the available budget significantly, which necessitated a reevaluation of the numerical evaluation matrixes, specifically a review of the cost constraint. Upon reevaluation, as seen in Table 8 and Table 9, changes were made to both the protein and polymer numerical evaluation matrices. On the protein numerical evaluation matrix both fibronectin ($120.00) and laminin ($170.00) no longer met the constraint of cost. Additionally, PLGA was eliminated on the polymer numerical evaluation matrix because the polymer alone cost $175.00.

Table 8: Reevaluation of Possible Proteins

Protein Numerical Evaluation Matrix									
Means		Collagen	Collagen	Gelatin	Gelatin	Laminin	Fibronectin	Vitronectin	Osteopontin
	Objective Weights	Normal Score	Weighted Score	Normal Score	Weighted Score				
Constraints:									
Biocompatible									
No impact on ring structure									
User safety									
Sterilizable									
Cost						X	X	X	X
Time									
Commercially available materials									
Fits into culture wells									
Adhesion System Objectives:									
Create a Ring	0.50	100	50	100	50				
Inexpensive	0.17	100	17	100	17				
Consistent	0.33	100	33	100	33				
Tissue Ring Objectives:									
Increase Speed	0.40	N/A		N/A					
Increase Strength	0.30	N/A		N/A					
Fewer Cells	0.10	N/A		N/A					
Decrease Death	0.20	N/A		N/A					
Total		300	100	300	100				

Table 9: Reevaluation of Possible Polymers

Polymer Numerical Evaluation Matrix					
Means		PLLA	PLLA	PLGA	PGA
	Objective weights	Normal Score	Weighted score		
Constraints:					
Biocompatible					
No impact on ring structure					
User safety					
Sterilizable					
Cost				X	
Time					
Commercially available materials					X
Fits into culture wells					
Adhesion System Objectives:					
Create a Ring	0.50	100	50		
Inexpensive	0.17	100	17		
Consistent	0.33	100	33		
Tissue Ring Objectives:					
Increase Speed	0.40	N/A	N/A		
Increase Strength	0.30	N/A	N/A		
Fewer Cells	0.10	N/A	N/A		
Decrease Death	0.20	N/A	N/A		
Total		300	100		

In addition, after reevaluating the matrices and reorganizing the options for the designs the group determined that removing lipid micelles was the best option. This choice was based not only on time concerns, but also cost and research. There is significant research on the use of the other designs for cellular aggregation, and not much research on the use of lipid micelles for such purposes. A majority of the literature expressed the use of lipid micelles more for drug delivery directly to cells because of its ability to enter cells through the membrane structure (Torchilin 2007). This would negatively impact the current structure of the ring.

4.3 Alternative Designs

After evaluation of the conceptual designs, four alternative designs were developed. The first is the addition of protein molecules in solution to the culture rings. The second is the addition of protein microparticles to the culture rings. The third is the addition of protein coated polymer microparticles to the culture rings. The culturing of cellular spheroids, which are then placed into the culture rings, is the fourth alternative design.

5: Design Verification

5.1: Preliminary Cell and Ring Culture

In order to start preliminary experiments using each different adhesion system, the team produced a baseline model of the SMC ring properties through the culture of tissue rings without the incorporation of an adhesion system. Observing these results allows for the understanding of how the adhesion system affects the formation and properties of the tissue rings.

The team first started culturing cells using adult rat smooth muscle cells (ARSMC). These cells were used in all preliminary testing as well as the testing for the adhesion system design alternatives. Throughout culture, a hemocytometer was used to determine the cell count. Concentration was calculated according to the following equations:

cells/mL of suspension = average # of cells per square * dilution factor * 10000

total amount of cells = cells/mL of suspension * total volume of suspension

In the original culture, as seen in Figure 14, the average number of cells per square was 69. Using the calculation above, the team determined that there were 690,000 cells/mL (total of 6,900,000 cells), and that there were 3,450,000 in each of the T75 flasks. These flasks were then placed in an incubator (37°C) to allow the cells to continue to aggregate and proliferate.

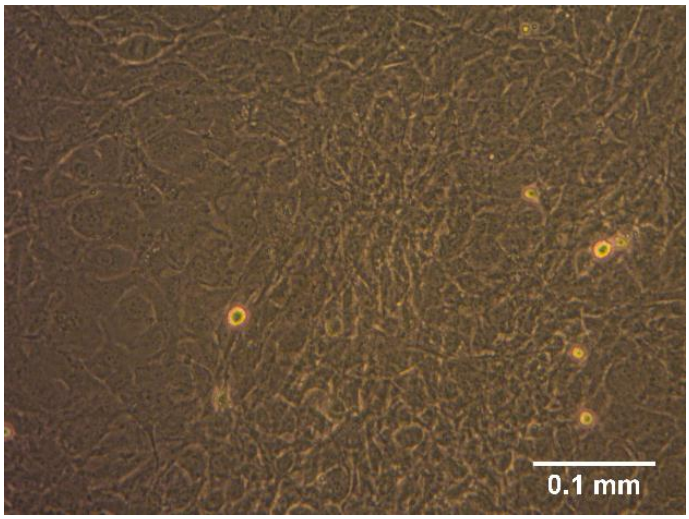

Figure 14: Adult Rat Smooth Muscle Cells 3 Days after Initial Plating (20x)

The cells were observed every day to ensure media replacement and passaging occurred when needed. The cells were passaged approximately every two days through a 1:10 dilution. During the second passage, the total amount of cells was divided into five T75 flasks and the remaining cells were properly disposed of. After that, the cells in each flask were passaged when needed (using the 1:10 dilution), however only 10% of the cells were kept so that there were always only five T75 flasks of cells. After a fourth passage, some of the cells were frozen for future use and some were used in the creation of tissue rings. One flask of cells was used to fill 10 vials each containing 500,000 cells and was frozen. Another flask containing cells was used to make 12 tissue rings. The cells were suspended and a cell count was performed. Unlike the usual cell count method where cells are counted in the corner squares of the hemocytometer grid, the cells were counted in the every other square except the corner ones. This method was used to be consistent with process that is described in the literature. The total number of cells was calculated by the formula below.

(#cells/#squares) * hemocytometer factor * dilution factor * #mL suspended

A total of 6,640,000 cells were calculated to be in the solution. This solution was diluted to a concentration of 500,000 cells in 100ul for each agarose ring well mold. Using the formula below, it was determined that the cells needed to be suspended in 1.328mL of media.

#mL needed for suspension = (total# cells * mL)/ total cells needed for 12 rings

Before the cells were added to the mold, media was added to the space between the well and the mold to allow the agarose to absorb some of the media to help deliver nutrients to the cells. Rings were created using a micropipette to place 100μL of the suspended cells into each agarose ring mold. After 24 hours more media needed to be added to the wells so that the mold was completely submerged. The cells need at least 24 hours undisturbed to produce a ring stable enough to allow for the transportation of the well and the addition of more media. Due to time restrictions, the media was added before the 24 hours and only gave the cells about 19 hours to form a ring and aggregate, which could have attributed to problems with ring formation during this initial trial experiment. Every two days the media was changed, and after seven days from the initial seeding of the rings final observations were made.

During this culture, only 8 of the 12 rings formed, one of which can be seen in Figure 15. The other four wells contained partial rings. Some of the rings were not uniform in thickness, which could potentially be due to an uneven incubator surface.

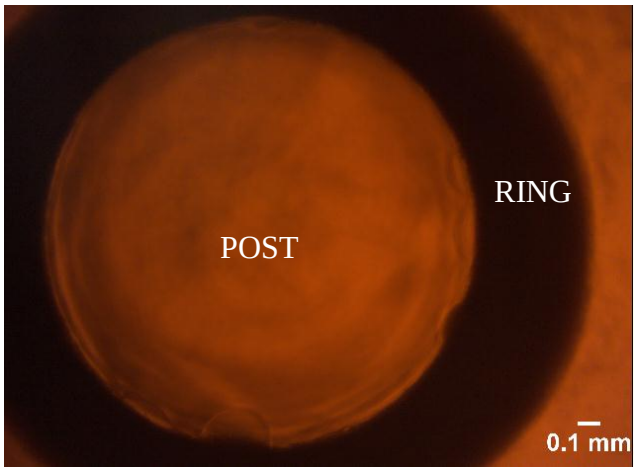

Figure 15: SMC Tissue Ring Seeded with 500,000 cells at 7 days of culture (4x)

Thickness of the tissue rings was determined by taking a picture of each individual ring under 4X magnification and using ImageJ to measure the thickness. First a ruler was placed under the same magnification and a picture was then taken. Using ImageJ, a line was drawn on the picture of the millimeter to determine how many pixels existed per millimeter. A scale of 919 pixels/mm was measured and used to determine the thickness of the rings. Measurements were taken in four different locations of the ring and were averaged to determine the thickness. The average thickness of one of the rings cultured for seven days was 0.45mm.

5.2: Preliminary Alternative Design Testing

5.2.1: Protein Molecule Solutions

5.2.1.1: Collagen Solution

The team also decided not to pursue collagen solution any longer as an alternative design at the same time as deciding to stop collagen microparticles research. This decision was due to the difficulty of working with the material and problems associated with fluorescently tagging

such a viscous material. This decision was also made because there was no longer any collagen available for the team to work with, and the time constraint limited the team's abilities to pursue this particular portion of the project.

5.2.1.2: Gelatin Solution

In an attempt to assess this design option, the team tagged a 2 µg/mL concentration of gelatin solution with an Alexa Fluor 594 Protein Labeling Kit. However, the team needed to determine if the concentration of gelatin sent through the resin was decreased from the process. The volume of the fluorescently labeled protein solution was not sufficient for concentration measurements. Because the team was unable to determine these factors and due to time constraints the team was not able to test gelatin solution in ring formation.

5.2.2: Spheroids

The team attempted two small pilot tests to create cell spheroids and determine its feasibility as an alternative design. The first pilot test utilized microcentrifuge tubes to attempt to form a spheroid of cells. It was assumed that the shape of the microcentrifuge tube would allow the cells to aggregate downwards by gravity and form a spheroid in the small point of the tubes. During this initial test, the team used 0.5 mL and 1mL microcentrifuge tubes, 15 of each. A plate of cells was trypsinized and then resuspended in the proper amount of media to yield 100,000 cells in 100 µl of media. Once resuspended, but altered to calculate for 100 µl of the cell suspension with 100,000 cells. In order to make a ring 500,000 cells are necessary, so it would follow that 5 spheroids with 100,000 cells each are necessary. Then 100 µl was pipetted into each centrifuge tube. Then the tubes were capped and placed in a 37^{o}C incubator for 24

hours. After 24 hours, the tubes were examined and an attempted media change was performed. The examinations showed that one major spheroid was formed, but also multiple smaller spheroids were formed within each tube. Additionally, changing the media was very difficult because some spheroids were lost in the old media that was removed. However, picking up the spheroid and maneuvering them to another location with forceps was very easy. Once these things were noted a second pilot test was performed to determine if it was possible to yield better results.

In the second pilot test, the team decided to use only 0.5 ml microcentrifuge tubes because of their smaller shape and more defined point at the end. Additionally, the amount in each tube was changed to 100,000 cells in 50ul. The procedure for placing this amount in each tube was the same as from the initial test in every other aspect. These were allowed again to incubate for 24 hours and after that point the microparticles were observed and handling tests were performed. Again, it was observed that one spheroid was not formed: a main spheroid in each tube was formed and then multiple smaller ones were also formed in the tube, as seen in Figure 16. It was also noticed that again moving the spheroid with forceps from place to place was simple. Based on the results from these two pilot tests it was determined that this method would make it impossible to control cell count in the spheroids placed into the well culture system. This is important because decreasing cell number is a main objective of the team. Another factor that was considered was the fact that it would take five tubes to form enough spheroids to make one tissue ring, and this is a very inefficient system to form a tissue ring. It makes the current system more complex and more time consuming just to form multiple or even one ring. After consideration of these factors and the constraint of time, the team decided to eliminate spheroids as one of its alternative designs.

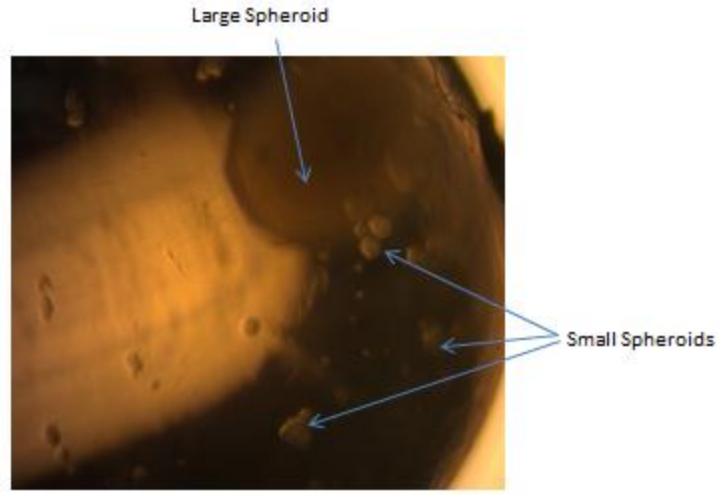

Figure 16: Spheroids in 0.5 mL Microcentrifuge Tube after 24 Hours of Culture (20x)

5.2.3: Microparticles

5.2.3.1: Protein Coated Polymer Microparticles

After further evaluation of creating protein coated polymer microparticles, the team decided to eliminate the idea as a viable design option. This decision was made based upon a cost analysis, applicability of these microparticles in the system, and time constraints. Obtaining all the materials necessary to create this particular type of microparticle was just within the remaining budget of the group. However, it was decided that the remaining funds would be better spent on more viable and currently working design options. The decision to create these microparticles was also based upon the process needed to make polymer microparticles. The particular process to make this type of microparticle is more time intensive then the other working design. It also requires more chemicals and hazardous organic solvents (ex. methylene chloride) that require extensive washes to remove properly. Due to the hazardous nature of these chemicals to both user and the cells it was determined more beneficial and safer to follow a more natural route of microparticle formation utilizing only proteins. These considerations and the

time constraints to finish the project led to the removal of protein coated polymer microparticles as a possible alternative design.

5.2.3.2: Collagen Microparticles

The team attempted to make collagen microparticles using two different methods: a water in oil emulsion and buffer solutions. The water in oil emulsion and cleaning of the microparticles was performed. In this process, 3 mL of collagen was added to150mL oil heated to 60^{o}C in a glass bottle and then spun at 300 rpm. However, during the cleaning of the microparticles it was noted that microparticles were not formed. Instead the collagen formed a gelatinous mass, as seen in Figure 17, and after centrifugation with acetone it formed a collagen sheet as the top of the tube as seen in Figure 18. The team determined that without organic solvents to crosslink the polymer it would not be possible to form microparticles through this emulsion method. The collagen was destroyed in the process because of the high temperature and harsh chemical used in this particular technique. After this attempt at making microparticles, the team was pointed in another direction by their advisor to try to form microparticles through the same method used to form collagen threads in the Pins lab at WPI (Pins, Christiansen and Silver 1997).

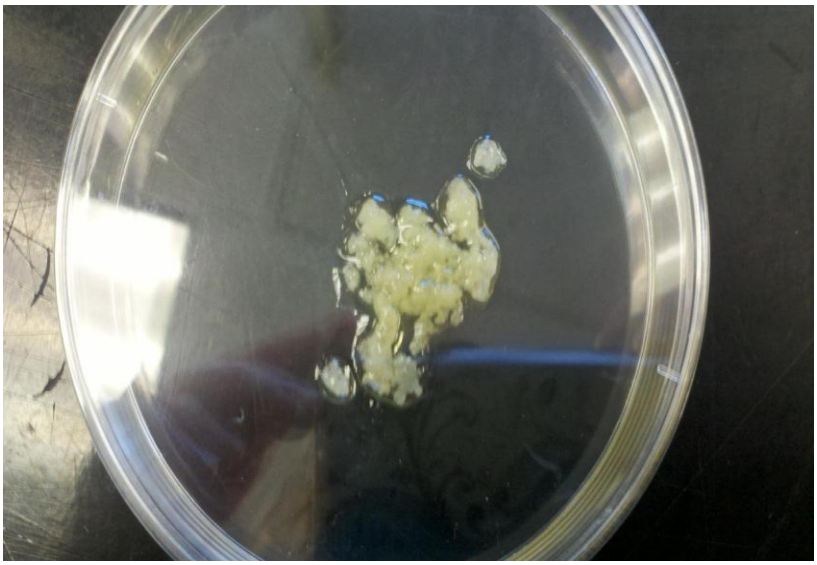

Figure 17: Collagen Mass in Petri Dish Produced from Water in Oil Emulsion

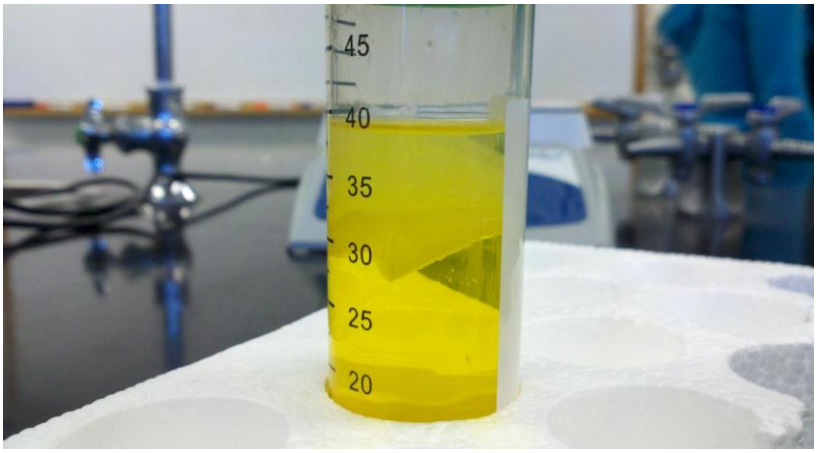

Figure 18: Collagen Sheet in Acetone and Oil after Water in Oil Emulsion and Centrifugation at 5000 rpm

Through research, the team examined this technique, which utilized the use of two buffers to form threads. Using these two buffers, a fiber formation buffer and a fiber incubation buffer, the team attempted to create microparticles by adapting the microparticle procedure and collagen fiber formation technique. In this procedure, 150 mL of the fiber formation buffer was heated to 37^{o}C and then placed on a magnetic stir plate with a magnetic stirrer. About 3mL of

collagen, which was stored on ice and protected from the light, was placed drop-wise using a 1000ul pipette into the buffer, 1.5 ml into two different glass bottles. This procedure was completed in two different glass bottles: one bottle was spun at 300 rpm and one at 500 rpm. These two speed values were chosen because they provided the most valuable results when creating gelatin microparticles. It was noted that during this process there was no microparticle formation. However, the solution was still allowed to incubate for 24 hours to see the possible results. After 24 hours it was examined that and microparticles were not formed, but instead small threads formed in this buffer, as seen in Figure 19 and Figure 20. Due to a lack in microparticle formation and an inability to adequately separate the buffer and the collagen, the next step of placing the collagen in the next buffer for 24 hours was not pursued for further testing.

Figure 19: Collagen Threads in Fiber Formation Buffer Spun at 300 rpm

Figure 20: Collagen Threads in Fiber Formation Buffer Spun at 500 rpm

Collagen microparticles were removed as an alternative design option because of the difficulty working with the material and the difficulty it added to other procedures. This particular substance required more care in working with it and other chemicals some of which were more volatile and out of budget to obtain. Furthermore, due to difficulties working with the material the group decided that buying collagen to work with would not be worth wasting the budget. The original amount of collagen was supplied to the group from the on campus labs and since they could not supply more, the team decided buying more would most likely produce not viable result. This decision was due to the previous results mentioned above and the time constraint pressed upon the team.

5.2.3.3: Gelatin Microparticles

Preliminary Attempt at Microparticle Creation

As one of the pilot studies, gelatin microparticles were created using a water in oil emulsion procedure that was found in the literature (Hayashi and Tabata 2011). Since this was a pilot study and was not to be used during future experimentation, this experiment was not done

in a sterile environment. An aqueous gelatin solution was made using 2g of gelatin with 18ml of water in a 50ml centrifuge tube. The centrifuge tube containing this solution was placed in a 60°C water bath for 15 minutes, while swirling the mixture intermittently. The tube was then cooled to 40°C. Approximately 600mL of olive oil was heated to 40°C in a beaker. Olive oil was chosen based on its prior use in literature (Hayashi and Tabata 2011), cost, and availability. The gelatin was then added to the olive oil using a micropipette so that the gelatin was dispersed into the oil. A magnetic stirrer was used to mix the solution at 200- 400 rpm for 10 minutes. The beaker was then covered with parafilm and placed into storage at 4°C to allow the microparticles to form.

Once the solution reached 4°C, the oil was centrifuged at 5000 rpm at 4°C for five minutes to allow any suspended microparticles to settle, allowing for easier collection. Once the microparticles were collected, the oil was then disposed of. Cold acetone was used to wash the microparticles 3 times to ensure that all of the oil had been removed. This solution was then centrifuged at 5000 rpm at 4°C for five minute. A new pellet of all the microparticles, as seen in Figure 21, accumulated at the bottom of the centrifuge tube and the excess oil and acetone were then disposed of.

Figure 21: Dry Gelatin Microparticle Pellet Produced from Water in Oil Emulsion

The microparticles were then placed under a microscope to visualize size and to compare them to rat smooth muscle cells. As seen in Figure 22, the microparticles were very small in comparison to the smooth muscle cells. This is possibly because the microparticles were not rehydrated before adding them to the cell culture.

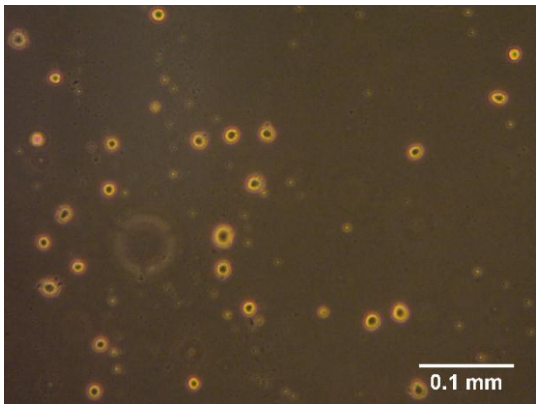

Figure 22: Microparticles and raSMCs (10x)

The microparticles that were used for further testing were created by the following standard operating procedure:

1. Make an aqueous gelatin solution
    a. Measure out 1g of gelatin on weigh paper using a scale and 9 ml of water in a centrifuge tube using a scale
    b. Combine in centrifuge tube
2. Make a 40^{o}C hot water bath
3. Heat gelatin solution in water bath at 40^{o}C for 15 minutes inverting intermittently
4. Heat 150 mL olive oil in bottle to 40^{o}C in container
5. Add gelatin solution to olive oil drop-wise using a 1000 µl micropipette in a circular motion to avoid placing droplets on top of one another.
6. Stir using a magnetic stirrer that completely covers the bottom of the container at 500 rpm for 10 minutes
7. Place in a refrigerator to decrease the temperature to 4^{o}C (about 24 hrs) which will allow the microparticles to form
8. Centrifuge top portion of olive oil (this should barely have any microparticles) at 5000 rpm for 5 minutes in centrifuge tubes
9. Pour off olive oil into a container to save for another use in making microparticles
10. Add 5-10mL of acetone to the oil and gelatin solution (Note: Work with acetone in fume hood)
11. Stir the solution at 60rpm for 30 seconds
12. Separate solution into centrifuge tubes

13. Centrifuge each tube at 5000rpm for 5 minutes
14. Discard supernatant from each tube
15. Add 40-50mL of acetone to each centrifuge tube
16. Invert each centrifuge tube vigorously, until the pellet is resuspended
17. Centrifuge each tube at 5000rpm for 5 minutes
18. Remove supernatant from each tube
19. Repeat steps 14 to 17 until the oil is removed (microparticles will be able to move freely and should be the same color as the gelatin powder used to make microparticles)
20. Combine all microparticles into a beaker
21. Allow to dry in fume hood (about 2-3 hrs)
22. Once dry move microparticles to centrifuge tube
23. Store in 4^{o}C fridge

After this procedure was completed, the microparticles were the washed and sterilized using the following procedure:

1. Measure out necessary amount of microparticles
2. Place in a 50mL centrifuge tube
3. Fill the tube to 45mL mark with room temperature IPA
4. Shake tube vigorously or vortex at highest speed
5. Place in centrifuge at 200 rcf for 5 minutes
6. Aspirate off IPA in culture hood
7. In same hood add cold DMEM to 45mL mark
8. Shake vigorously or vortex at highest speed
9. Place in centrifuge at 200 rcf for 5 minutes
10. Aspirate off DMEM in culture hood
11. In same hood add cold DMEM to 45 ml mark
12. Vortex microparticles at highest speed setting until microparticles appear dispersed.
13. Centrifuge at 200 rcf for 5 minutes
14. Aspirate off DMEM in culture hood
15. Add the necessary amount of warm full media to microparticles for desired concentration
16. Shake tube vigorously.

Note: Solution can be stored after step 12. Complete steps 13 through 16 immediately before use with cells.

Microparticle Formation and Initial Plating

In order to make cellular rings with gelatin microparticles, tests were needed to determine the feasibility of creating the microparticles as well as their interactions with the rat smooth

muscle cells. Using the existing protocol, microparticles were created by a water and oil emulsion (Hayashi and Tabata 2011). Once the microparticles were made, they were soaked in Trypan Blue to enhance visibility. The microparticles were then added to isopropyl alcohol (IPA) for sterilization. A small sample of this mixture was taken to determine if the microparticles had actually formed. As seen in Figure 23 it is possible to create microparticles from gelatin.

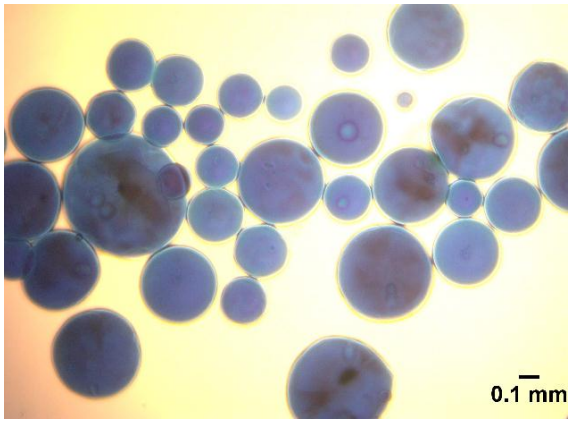

Figure 23: Trypan Blue Stained Microparticles (4x)

It was possible to create gelatin microparticles and as a result a test was needed to determine how the rat smooth muscle cells would react with the microparticles. This will confirm the feasibility of adding microparticles with cells to make cellular rings. The microparticles that were mixed with IPA were made into a pellet by centrifuging the mixture at 200 rcf. By creating a pellet the IPA could then be aspirated and replaced with sterile water to wash the microparticles. Because cells would swell in the presence of water, the microparticles needed to be rinsed thoroughly to remove water from the microparticles. This mixture was then centrifuged at the same speed and the water was aspirated and replaced by DMEM. The DMEM and microparticle mixture was centrifuged at the same speed and the DMEM was aspirated and replaced with media. This mixture was then placed in an incubator at 37° C which is the temperature at which the cells incubate. A small sample of microparticles was taken to determine

the concentration and amount of microparticles that were in the mixture. The concentration of the microparticle mixture was found by placing a known amount of liquid in a 35mm plate, taking a picture of the microparticles, counting the amount of microparticles in the picture, measuring the area in the picture using ImageJ and then using the equation below:

(x #of microparticles/ x mm^2)(x mm^2 in plate/ x ml added to plate) = x microparticles/ x ml

Once total amount of microparticles were determined, it was then possible to calculate how many cells were needed to test initial ratios of microparticles to cells for 1:1, 2:1, and 1:79. The 1:79 microparticle:cell ratio was a random sample due to the extra amount of cells and microparticles left over from the two previous ratios. After 24 hours, each sample was examined under a microscope. Both the 1:1 and 2:1 samples did not demonstrate significant cell-microparticle adhesion. This was confirmed when shaking the culture plate, as very few particles were stationary when moved. This demonstrates a lack of attachment between the cells and particles. The 1:79 plate, however, demonstrated much more adhesion between the cells and the microparticles. When this culture plate was shaken, a majority of the microparticles remained stationary, demonstrating they were able to adhere to the surrounding cells. This result was used to justify further experimentation with varying ratios of microparticles:cells, where the cell count greatly outnumbers the microparticle count. Images taken using light microscopy, as shown in Figure 24, were used to characterize the cell-microparticle interaction. In this image, microparticles and cells were plated at a ratio of 1:79, and the particles were stained with trypan blue for visualization. As can be seen, the cells remain viable in the presence of microparticles, confirming the microparticles are biocompatible and non-toxic.

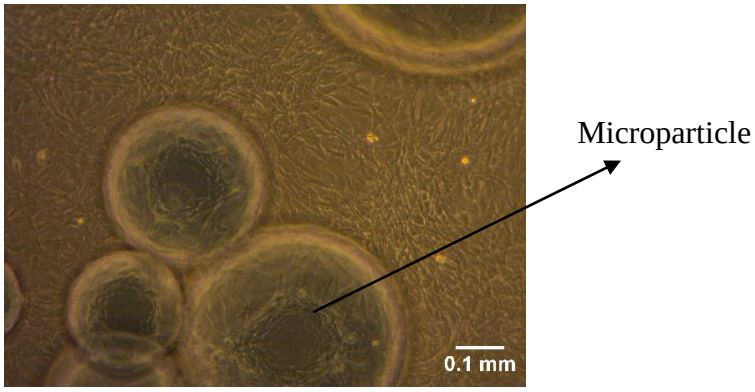

Figure 24: Cell Growth around Microparticles: Biocompatibility Test after 48 Hours of Culture (10x)

However, there are multiple alterations that need to be made to improve the efficiency of the design. Since it was visually confirmed that the microparticles were larger than the cells, it was determined that the microparticle production should be altered in such a way to reduce the size of the microparticles. This could potentially improve the cells interaction with the microparticles. It was also noted that soaking the microparticles in trypan blue was unnecessary because the microparticles are visually distinct from the cells when they are cultured together. After initial plating, a variety of tests were performed to produce the most efficient batch of microparticles for plating and tissue ring creation with the cells.

Determining Emulsion Speed

In order to move forward with testing the gelatin microparticles with rat smooth muscle cells, the method used to create the microparticles needed to be verified. The initial batch of microparticles that was added to the cells was made at 300rpm. The size of these microparticles ranged from 60-600µm, which is too large to integrate with the cells. Ideally, the microparticles must fall into a size range that allows for proper cellular interaction. Based on literature review, the microparticles should be similar in size to the cells being used (Hayashi and Tabata 2011). However, based on the fabrication method being used, the microparticles cannot be

manufactured as small as the cells. Instead, the emulsion speed must be adjusted to create microparticles small enough to still encourage cellular interactions while allowing for controllable fabrication. One test that was performed was how to make microparticles at the correct size (about 50 micrometers). Because the original batch was created at 300rpm, three new batches were made at 300, 500 and 600 rpm. Once these were created a small sample of each was taken, placed in IPA and examined under a microscope. The microparticles made at 300rpm were within the range of 60-600 μm, the 500 rpm batch was within 15-50μm and the 600rpm batch ranged from 5-50 μm. It was then determined that microparticles made at 500rpm gave the desired size range. It was also noted that microparticles could be created reproducibly using this method: following this procedure, a similar size distribution of microparticle diameters was seen with every batch produced.

Microparticle Aggregation Problems

After making the first batch of microparticles, a microparticle aggregation issue was observed. Microparticle aggregation was characterized by random attachment of microparticles to one another, where more than three particles were attached. Various quantities of attached particles were observed in solution, and these inconsistencies prevent the accurate calculation of particle number and distribution within solution. Many tests were needed to determine what variable was causing this microparticle aggregation to occur. Variables that were tested one at a time were the liquid used to wash the microparticles, the speed the microparticles were centrifuged at during the wash steps, the temperature at which the liquids were added to the microparticles, and the concentration of the microparticles in the liquid. After testing it was determined that microparticle concentration in solution was a main contributing factor of

microparticle aggregation. Further research was done to find ways to help fix or prevent these problems, and vortexing the mixture was one of the methods used to help fix this issue.

Centrifuge Speed

The team initially thought that the problems with microparticle aggregation could be caused by the original centrifuge speed being used: 5000 rpm for 5 minutes. This speed was used because it was the speed used to remove the microparticles from oil. In an effort to see if lowering the speed helped a batch of microparticles were sterilized in IPA, and were shaken and then centrifuged at 200 rcf for five minutes. The change in speed did not help the microparticle aggregation problem, but the group did determine that 200 rcf was an adequate speed to pellet the cells at instead of 5000 rpm. Based on these result all other separation of microparticles in any other fluid (IPA, DMEM, and water) will take place at 200 rcf at 5 minutes.

Although 200 rcf for 5 minutes was an adequate speed to separate microparticles from liquid, there was still concern over it causing microparticle aggregation. So the team conducted a test where is placed the microparticle in IPA and shook them up. A sample was removed and some microparticle aggregation was noted. After centrifuging at 200 rcf for 5 minutes another sample was taken and examined under the microscope, and more aggregated microparticle than before were noticed. Although the centrifuge may be contributing to the problem, it is a necessary step to remove the IPA after sterilization and thus will not be removed or altered from the process.

Drying in IPA

Another possible cause of the microparticle aggregation could have been inadequate drying time. To test this, the team sterilized three tubes of microparticles, centrifuged them at 200 rcf for 5 minutes, aspirated off the IPA, and allowed the microparticles to dry in the fridge overnight. The next day, the team checked the microparticles and noted that all the IPA had not dried off, so any residual IPA was then aspirated off in the culture hood and the microparticles were left to dry in the hood without a cap. Then, each tube was filled to the 45 ml mark: one with sterilized water, one with PBS, and one with DMEM. Microparticle aggregation was still occurring.

Trypan Blue Test

In another test, the team attempted to determine if the Trypan Blue stain was the reason that the microparticles did not aggregate initially. The microparticles were stained with Trypan Blue to view them in comparison with cells when plated. It is no longer used to dye the microparticle because they are visible without Trypan Blue and the Trypan Blue leaches out over time, which will kill the cells. In the next test, two amounts of microparticles were measured out. They were each soaked in Trypan Blue for 20 minutes and then rinsed in IPA up to 45 ml mark on tube. One tube was rinsed with cold IPA and the other with room temperature IPA. The tubes were the centrifuged at 200 rcf for 5 minutes and the IPA was aspirated off. Then they were left to sit overnight capped in the fridge. After checking in the morning they were still aggregating in solution.

Amount of Trypan Blue

After the test explained above the team also attempted to determine if it was the amount of Trypan Blue added to the microparticle. The team tested to see if adding more Trypan Blue to the microparticles would make them absorb more and stop sticking. This time microparticles were submerged in 5mL of Trypan Blue for 20 minutes, and then IPA was added up to 45 mL mark. The tube was shaken, but there was too much dye to see the microparticles. The tube was centrifuged at 200 rcf for 5 minutes and the liquid was aspirated off and DMEM was added to the tube to the 45mL mark. The tube was shaken and aggregated microparticles were still noticeable. This test ruled out Trypan Blue as an option to stop microparticle aggregation.

Microparticle Aggregation Due to Size

The group then attempted to determine if the microparticle aggregation of the microparticles was caused by the size of the microparticle. Three batches of microparticles were created: 300 rpm, 500 rpm, and 600 rpm. These created some very distinct sizes; the larger microparticles in the higher lower rpm ranges. Portions of each batch were put on a 60mm plate and submerged in IPA, and examined under the microscope. It was noticed that as size decreased the more microparticles aggregated together in the batch. Size is definitely a factor for microparticle aggregation.

Sieving

Sieving is necessary for retrieving the correct size range of microparticles to be used in culture with the cells. This process could also prevent microparticles from aggregating. Multiple batches have been sieved and it has been noted that this process does not stop the microparticle

aggregation issue. It is possible that the microparticles are sticking during drying. Additionally, the gelatin microparticles stick to the sieves themselves. In upcoming experiments, the gelatin microparticles will be broken up by using a mortar and pestle to determine if this could solve microparticle aggregation and sieving problems.

Residual Oil

A few times when cleaning microparticles it was thought that residual oil was the cause of microparticle aggregation because residual oil was noticed in a few batch. This is seen under the microscope because the microparticles are the color of the oil and the consistency of the powder is tacky and not fine. In an effort to clean the microparticles better, they would need to be left to dry with acetone present. However, oil and packing as still a problem. In an effort to eliminate packing the team began to dry them in a layer of acetone in a beaker so they are spread out and do not pack. This helped with proper drying, and stirring the microparticles with acetone while still in oil improved the amount of oil removed. Overall, the team made sure they were more careful in the cleaning process to remove all the oil, even if it required spinning them more times. Removing residual oil helped stop some of the microparticle aggregation, but was determined not to be the major reason for microparticle aggregation. There are times where some microparticles still contain oil, as seen in Figure 25, but a majority of the batch should not contain any oil if cleaned properly.

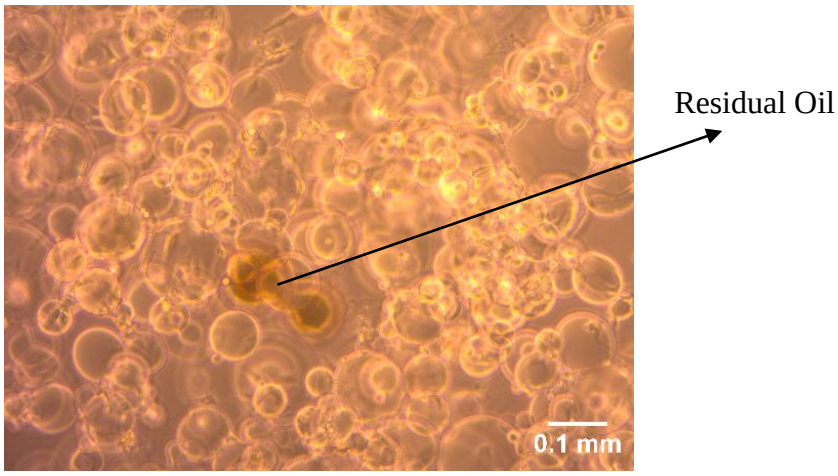

Figure 25: Microparticle with Residual Oil after Cleaning (10x)

Suspension Solution

Through all the previous testing the team noticed that the microparticles would aggregate more in some liquids than others. Through microscope examination the team found that the microparticles do not aggregate in acetone, and minimally aggregate in IPA. There is a little more aggregation in water, and even more aggregation in DMEM. The suspension solution definitely contributes to the microparticle aggregation, but the team has decided to try to break up the aggregation in DMEM since that is the solution is need to be in to plate with cells and to create rings.

Altering Emulsion Temperatures

In a review of past background material for making microparticles through oil and water emulsions, a team member noticed a discrepancy in the temperature at which the oil and gelatin was heated to create gelatin microparticles. The team was heating the gelatin to 60^{o}C to melt it in sterile water, but from the literature, it was noticed that the gelatin should be the same temperature as the oil, 40^{o}C. As a test, the team attempted to melt the gelatin in sterile water at

40°C in a 40°C water bath, after which a water in oil emulsion at equal temperatures was performed. Through this test, it was found that the gelatin dissolves in the sterile water at 40°C. The team also found that microparticle production is better when the emulsion is performed at equal temperatures. This is noted through the appearance of the microparticles in the oil emulsion. They stay as individual microparticles instead of combining together and there are more microparticles that are produced because there is no malformed gelatin that sticks to the bottom of the bottle. In connection with this, it should also be noted that the temperature of the oil needs to be maintained at 40°C and not rise too high or it melts the gelatin microparticles in the emulsion. This prevents the formation of individual microparticles. Once the team determined that the size and amount of microparticles are the reason for microparticle aggregation, the team needed to perform various tests to solve this aggregation issue.

Sonicator

Since shaking the microparticles in the tube vigorously does not help break them up the team attempted to use a sonicator. The team submerged three tubes of microparticles in DMEM in a sonicator for 20 minutes. One tube was the >250 μm range, one was the 120-250 um range, and the other was the 53-120 um range. Samples from each tube were examined under the microscope, and a lot of v was still visible. The sonicator did not prevent the microparticle aggregation.

Vortex

After the testing above, the team decided to research other methods to stop or break up the microparticle aggregation. In some articles, it was found that for polymer microparticles they

used vortexing to help with microparticle aggregation. In a test, batches of microparticles in DMEM were vortexed at the highest speed for about 10 minutes. Then samples were plated and examined under a microscope and it was noticed that vortexing helped with the microparticle aggregation. It was also noticed through shift the plate that some microparticles were not aggregated, but instead the solution was too concentrated. In realization of this, the microparticle samples in the tube were diluted down, vortexed again, and examined again under the microscope. The team noticed that there is still aggregating, but it is on a more manageable level where there are fewer aggregated microparticles. From this test it was decided that the microparticles will be diluted to a manageable level and vortexed to help with the aggregation problem before the concentration is calculated for plating with cells and making rings.

Determining Cell Size

In an effort to determine the correct microparticle size for plating and creating rings, the team decided to determine the average cell size of rat smooth muscle cells. Through research it was determined that microparticles are of adequate size and amount to allow for cellular interactions (Hayashi and Tabata 2011). To determine the cell size, a sample of cells was taken from a trypsinized plate and images of 100 different cells were taken. Then using ImageJ the diameter of these cells was measured and an average cell diameter was calculated from the values. The average cell diameter was determined to be about 17.65±2.89 μm in diameter, which means that the microparticles created need to be around this general size. The distribution of cell size can be seen in Figure 26.

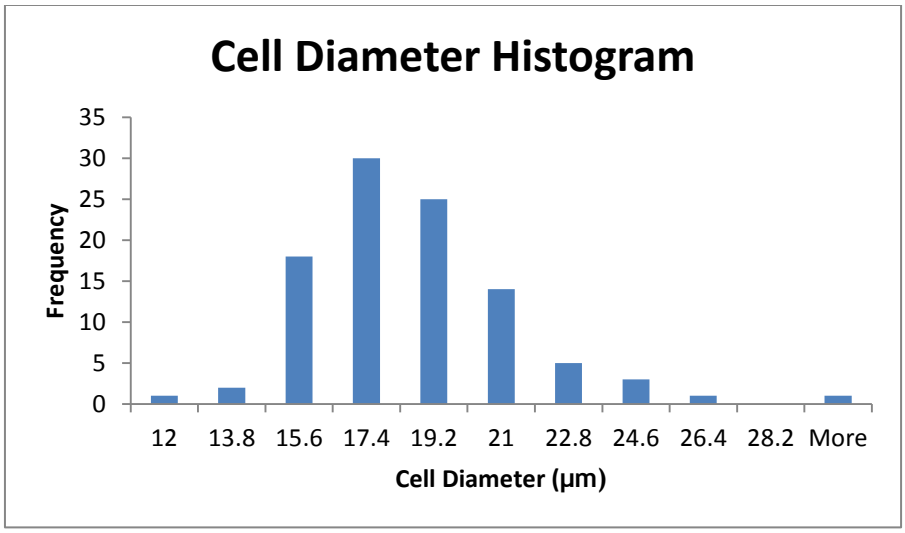

Figure 26: Cell Diameter Histogram (n=100)

Magnetic Stirrer Size

A few times in the creation of microparticles, the team noticed that there was gelatin that was getting stuck to the bottom of certain bottles and would not form into microparticles but rather a gelatinous coat on the bottle. Through observation of bottle size and magnetic stirrer size from multiple batches the team determined that it was due to magnetic stirrer size and bottle size. This gelatinous coat only occurred in bottles with magnetic stirrers smaller than the diameter of the bottle, and did not happen in batches where the magnetic stirrer had a diameter size almost equal to that of the bottle so it covered the entire bottom. From this point on the team has continued to make sure that when batch are made that the magnetic stirrer covers the entire bottom of the bottle.

Sieving Microparticles

In order to obtain a less variable size range of microparticles in each batch, the team has begun using sieves to separate the microparticles into different size ranges. These ranges are

>250 µm, 120-250 µm, 53 – 120 µm, and <53 µm. The three sieves, which designate these size ranges as seen in Figure 27 are stacked together and then the microparticles are placed in the top sieve and shaken through. However, the only problem with this method is that we lose a large amount of the batch because it sticks to the sieves and within the sieves. In addition, due to the sticking and clogging in the sieves the team was not able to collect any particles smaller than the 53 – 120 µm in diameter. However, the sieving has become very advantageous to the project for plating purposes and the creation of rings. Moreover, it makes sure that the team is obtaining more of the microparticles in the range that is required to create stronger tissue ring constructs.

Figure 27: Setup of Sieves to Sort Size Ranges

The following SOP was used to obtain the different size ranges of microparticles previously listed:

1. Remove centrifuge tube of microparticles from fridge
2. Stack sieves smallest to largest from bottom to top (53um, 120 um, 250 um)
3. Pour in microparticles and shack through sieves
4. Once though sieved separated microparticles into proper sizes
    a. 0-53 um (Will not obtain this size with current sieves)
    b. 53-120 um
    c. 120-250 um
    d. >250 um
5. Once separated into centrifuge tubes, store in 4^{o}C fridge

Viability Tests of Gelatin with Cells

Once the viability of gelatin with cells was determined and several factors were altered to produce the most efficient batch of microparticles, more plating was done to determine the optimum ratio of cells to microparticles. Microparticle aggregation in the batch of microparticles was reduced as much as possible, and microparticle to cell ratios of 1:1, 2:1, 1:10, and 1:25 were plated. The cell number stays consistent at 100,000 cells in 100 µl, but the microparticle volume changed per plate to accommodate the desired ratio. Six well plates were used for this plating, and due to available microparticles one well for each ratio was made. Controls consisted of two wells, one with only cells and one with only microparticles. The microparticle control will also help to visualize the gelatin microparticle reaction to the plate, to determine if they stick to the surface or if in fact the adhesion of the microparticles to the cells causes their immobility on the plate. The wells that contained higher cell counts than microparticle counts both demonstrated higher cell-microparticle attachment. This was initially confirmed through the method stated earlier, by shaking the plate and observing microparticle movement. Because fewer microparticles moved in the plates with higher cell counts, it can be confirmed that these ratios have more effective cell-microparticle interactions. Additionally, images were taken using a light microscope at 10x magnification and used to visually characterize the cell-microparticle interactions, and can be seen in the figures below. Both Figure 28 and Figure 29 visually show

vast numbers of microparticles, with little cellular influence, which is not desired in the ring constructs that are currently purely cell based. However, **Error! Reference source not found.** Figure 30 and Figure 31 display microparticles and cells, and more closely represent the interactions the team is trying to achieve. Because of these images, the team further investigates ratios with significantly more cells than microparticles.

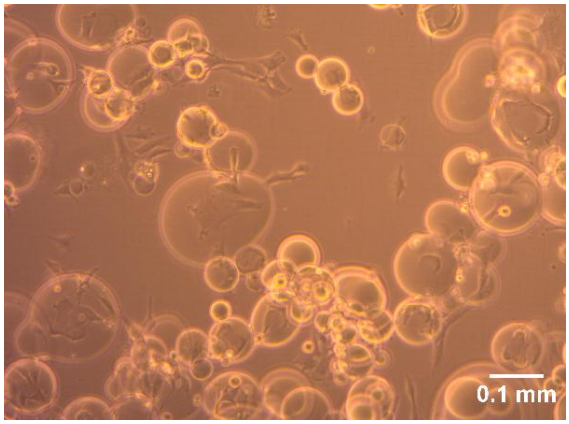

Figure 28: Plating of Cells and Microparticles at a Ratio of 1:1 (microparticles:cells) after 24 Hours of Culture (10x)

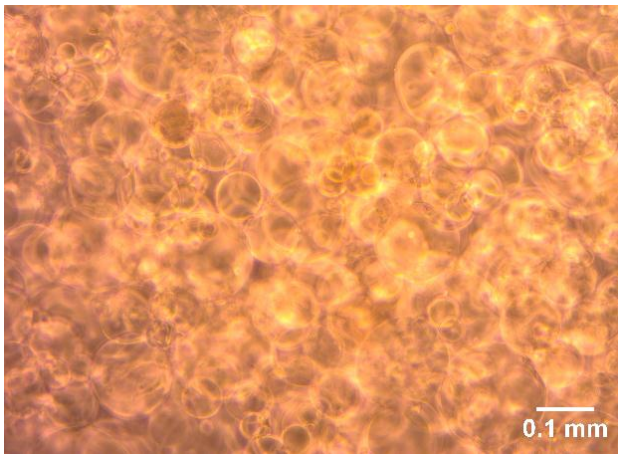

Figure 29: Plating of Cells and Microparticles at a Ratio of 2:1 (microparticles:cells) after 24 Hours of Culture (10x)

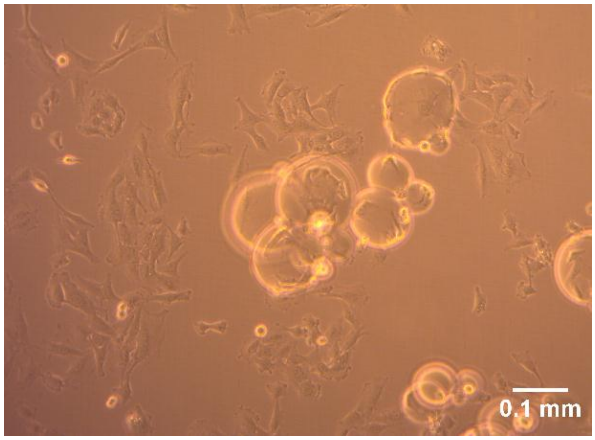

Figure 30: Plating of Cells and Microparticles at a Ratio of 1:10 (microparticles:cells) after 24 Hours of Culture (10x)

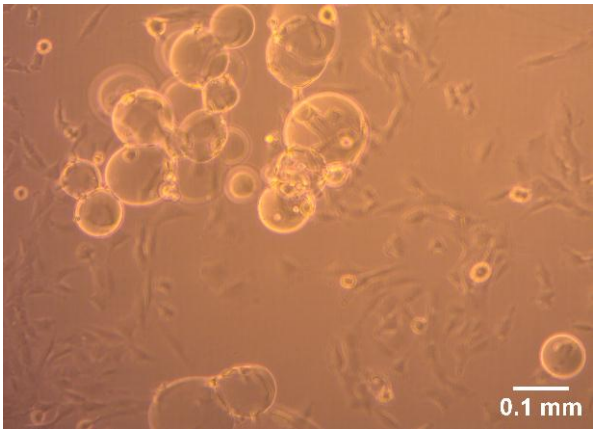

Figure 31: Plating of Cells and Microparticles at a Ratio of 1:25 (microparticles:cells) after 24 Hours of Culture (10x)

Swelling Tests

Since the gelatin microparticles are not crosslinked after they are formed, they swell once they are placed into media. Based on the literature, the team determined that the microparticles need to be of an adequate size and amount when seeded to allow for cell-cell interactions and ring formation (Hayashi and Tabata 2011). The average cell size was 17.65±2.89 µm (n=100) in diameter. In order to determine the level of swelling, the team performed a swell test on the microparticles. This required us to sieve the microparticles to obtain those in the range of 53-120 µm. Then the microparticles were cleaned using isopropyl alcohol (IPA), as seen in Figure 32, and then a sample was imaged under a microscope and ImageJ was used to measure the size

of 100 microparticles. The average size of the dry microparticles in IPA was 14.13±19.25 µm in diameter, the distribution of sizes can be seen Figure 33. After these images were taken, the microparticles were centrifuged and washed in DMEM twice, then centrifuged again, and resuspended in media. A six well plate was filled with a sample of microparticles in media and placed in the incubator for 24 hours. Once warmed, images were taken, as seen in Figure 34, and ImageJ was used to size the microparticles and again an average was found. The six well plate was returned to the incubator and images were taken over a 4 day period to measure microparticle size using ImageJ. From these swell tests, it was concluded that the particles initially swell when incubated in media but do not continue to swell over time. This is a positive result; however, the swelling means the microparticles grow larger than the required size range. The size range of the swollen microparticles was about 78.44±30.9 µm in diameter, and the distribution of sizes can be seen in Figure 35. Overall, the microparticles swell to about six times the cell size, but in the correct amounts they are not so large that they will not allow for cell to cell interactions to form rings, as demonstrated in other experiments. The team believes that crosslinking may solve this problem and keep the microparticles from swelling to larger sizes.

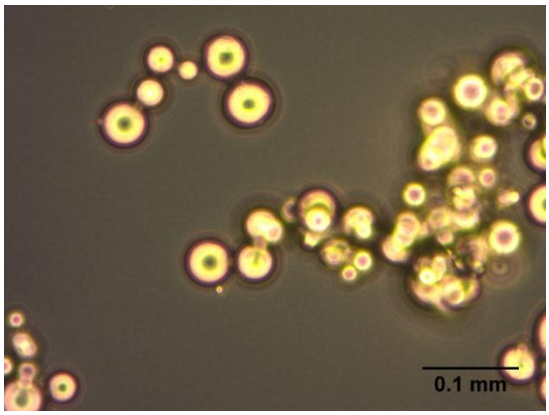

Figure 32: Microparticles in Isopropyl Alcohol (20x)

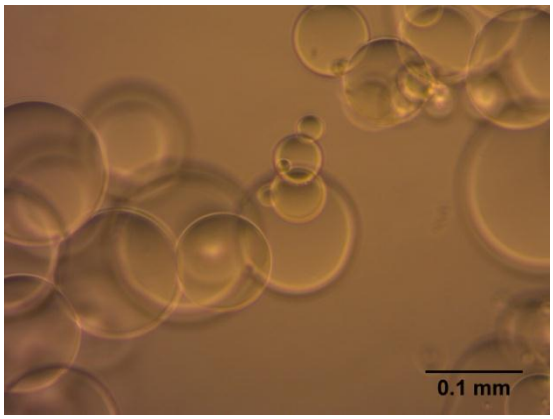

Figure 33: Microparticles Suspended in Media (20x)

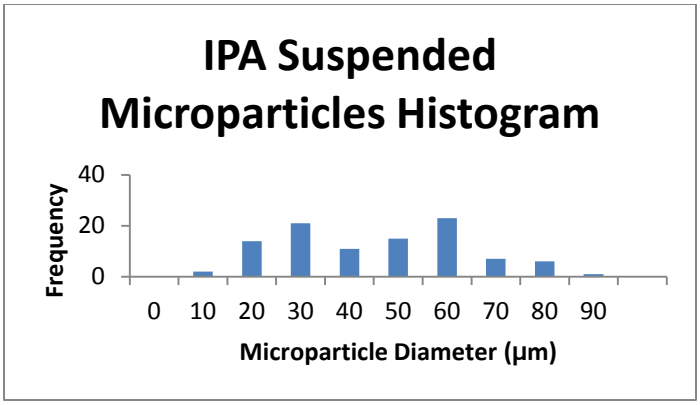

Figure 34: IPA Suspended Microparticle Diameters Histogram (n=100)

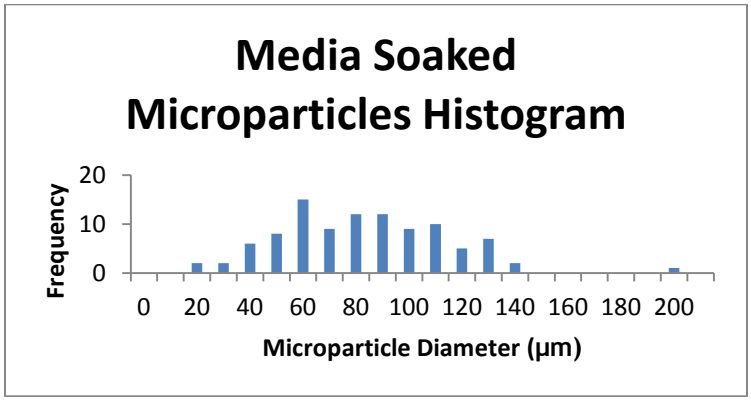

Figure 35: Media Soaked Microparticle Diameters Histogram (n=100)

Microparticle Ratios

After plating various ratios, the group determined that there needed to be higher cell numbers than microparticle numbers when creating the rings so there is proper ring formation. Based on the observations of plating, the team decided to seed rings at a large variety of ratios. When culturing both microparticles and cells in the ring wells, first the desired number of microparticles was added in 50 µl of culture media using a pipette. Following the addition of microparticles, 500,000 cells were added in 100 µl of media. These seeding ratios included a control, which means there were no microparticles and then 1:10, 1:20, 1:50. 1:100, 1:1000 microparticles to cells with 500,000 cells/100µL. These were left to culture for about a week before they were examined for ring formation. When examining these rings the team was looking for rings with uniformity that was smooth and mostly made of cells. Based upon these criteria, the team visually determined the best ratio of microparticles to cells to be 1:100, as seen Figure 38. In observation of the other ratios, the lower ratios had too many microparticles compared to the cells, as seen Figure 39, which is displayed through the large bulges on the side of the rings and the lack of uniformity. The higher ratios displayed little or no evidence of microparticles, as seen in Figure 37, which looks exactly like a control ring, as seen Figure 36, and there are no lighter portions, which show the presence of microparticles. As see in Figure 40, the microparticles had no significant impact on ring formation. Although when evaluated using an ANOVA the p-value was well above 0.05, which means there is no statistical significance it can be clearly viewed the ring formation is not impacted. From all these observations, it was possible to move forward with testing directed toward the particular objectives of the ring system.

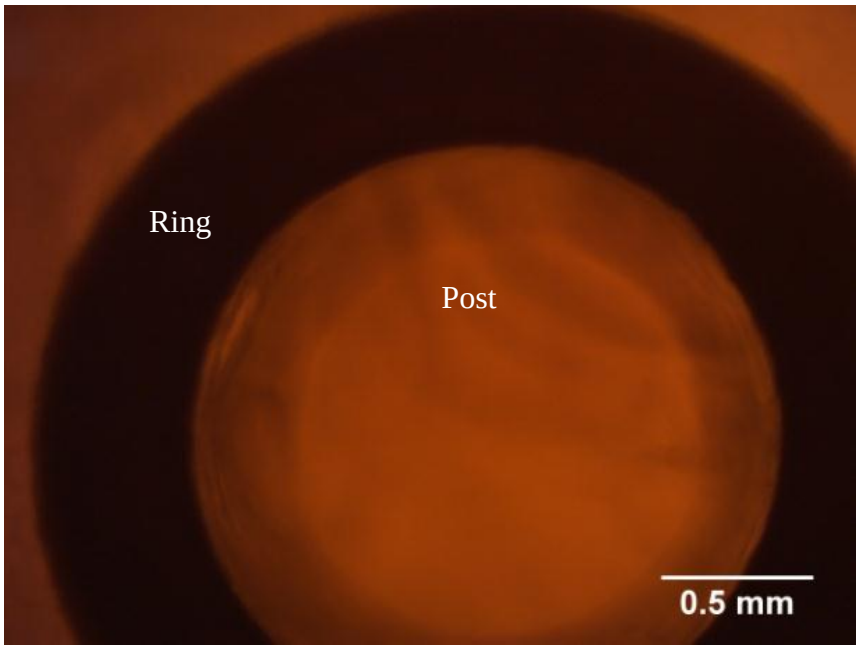

Figure 36: Control Ring Cultured Seeded with 500,000 cells at a Ratio of 0:1 (microparticles: cells) after 7 Days of Culture (4x)

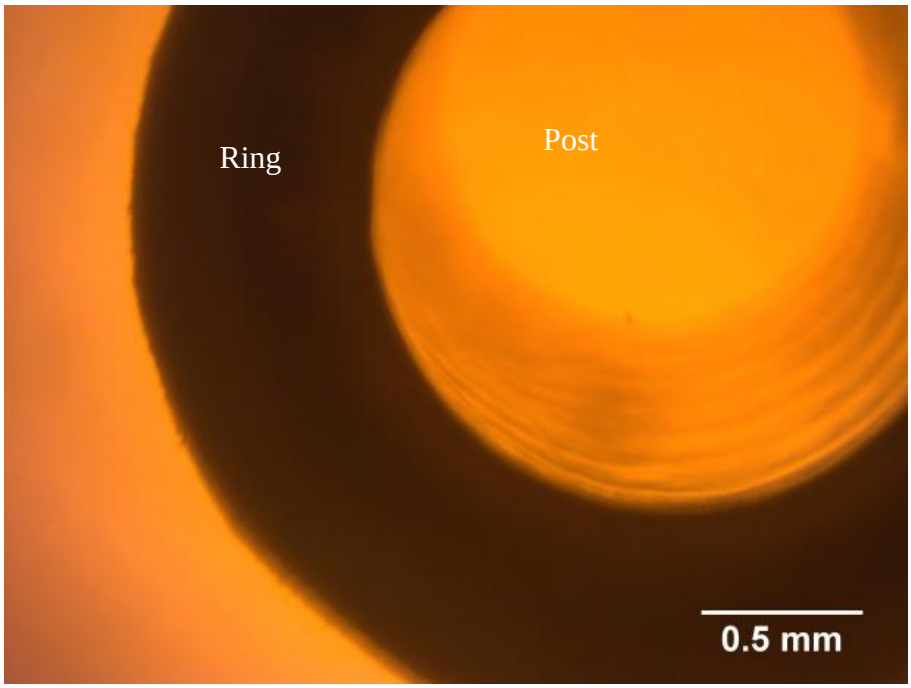

Figure 37: Experimental Ring Cultured Seeded with 500,000 cells at a Ratio of 1:1000 (microparticles: cells) after 7 Days of Culture (4x)

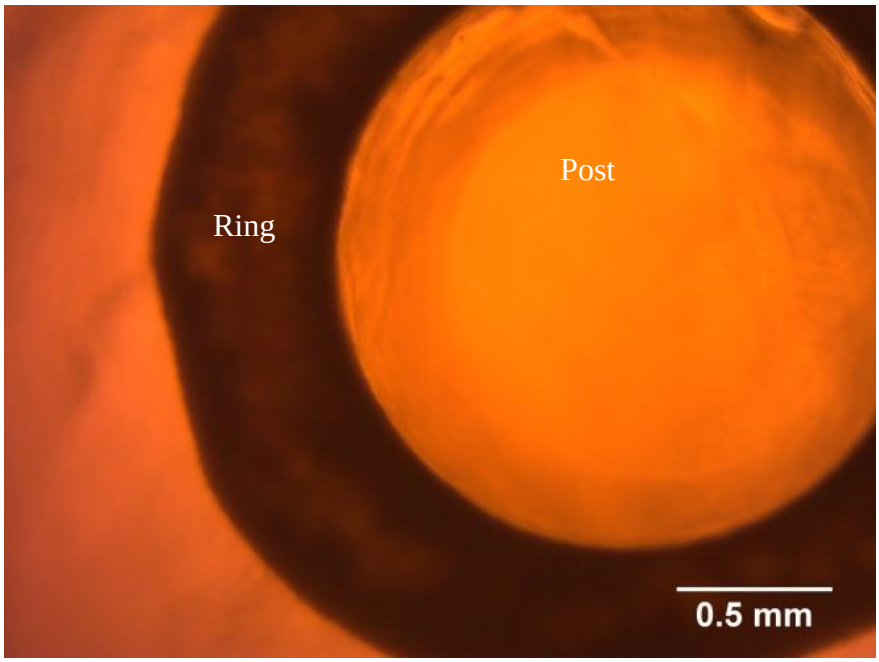

Figure 38: Experimental Ring Cultured with 500,000 cells at a Ratio of 1:100 (microparticles: cells) after 7 Days of Culture (4x)

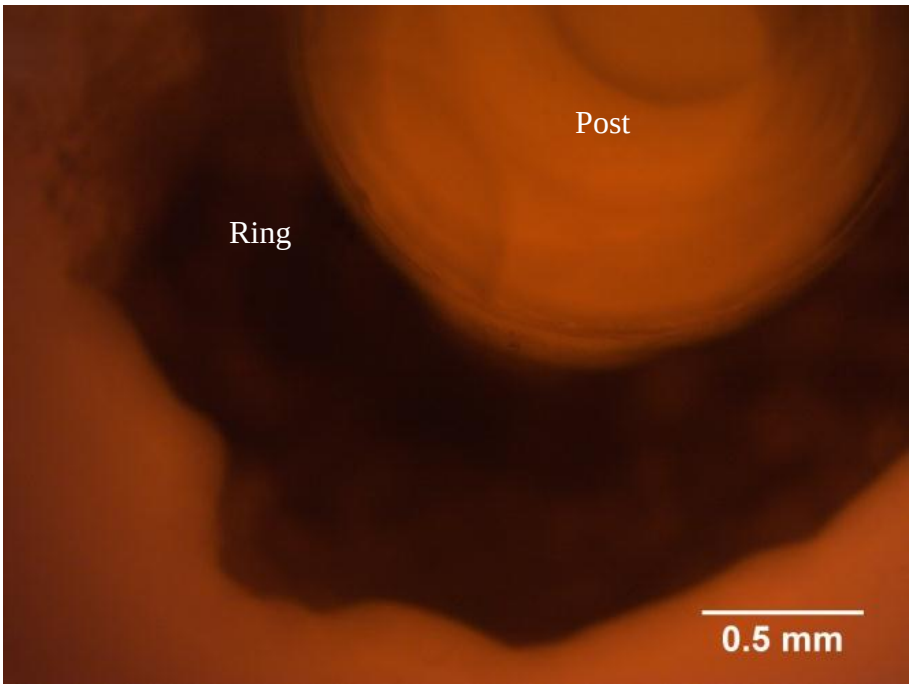

Figure 39: Experimental Ring Cultured with 500,000 cells at a Ratio of 1:10 (microparticles: cells) after 7 Days of Culture (4x)

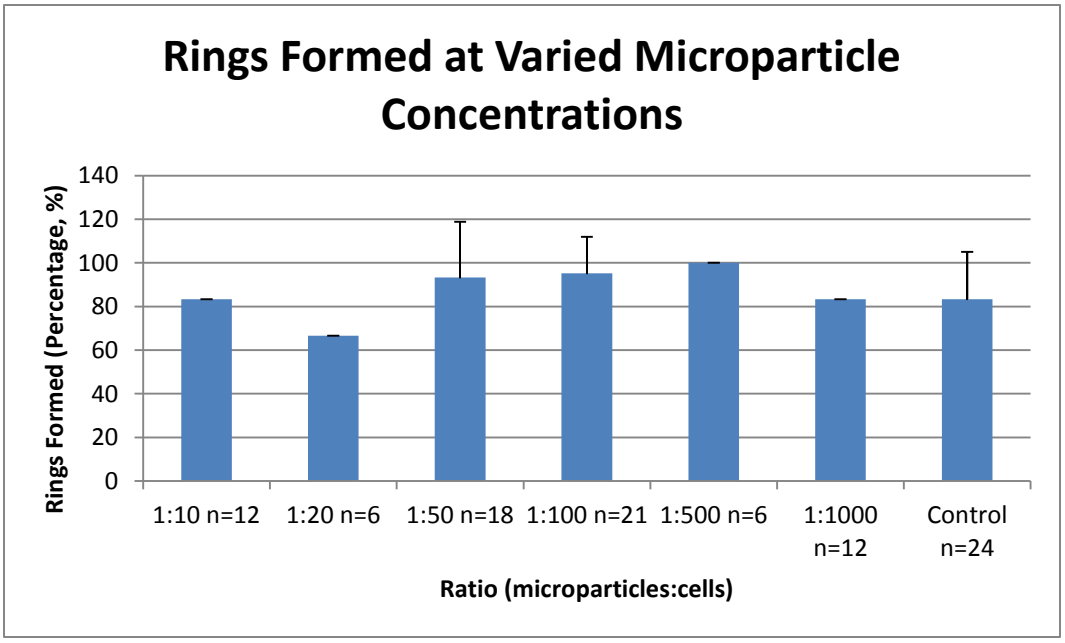

Figure 40: Rings Formed at Varied Microparticle Ratios for Rings Cultured for 7 Days

Ratios that produced rings that visually demonstrated microparticle incorporation and uniform thickness were used for continued experimentation. The 1:100 microparticle:cell ratio demonstrated the most promise for proper ring formation, and was consistently chosen for each experiment. The 1:10 and 1:1000 ratios were also used for comparison in many following experiments.

Cellular Aggregation Time Tests

When the rings are seeded without microparticles the ring formation is sensitive to movement. If the plates are moved before 24 hours the rings can break from the post and will not form properly. In order to determine if the addition of microparticles impacted the stability of forming rings, rings were seeded in 6 well plates (3 controls, and 3 tests rings), and tested at four time points: 6, 12, 18, and 24 hours. This test was done with the 1:100 and 1:50 ratio, each with their own controls. At each time point from the initial seeding the rings were moved and

imaged using a light microscope. From these experiments it was determined that the rings without microparticles are tighter around the posts. Initial formation of the rings is more uniform and smooth for the control rings whereas the experimental rings initially are less uniform and not as tight around the posts as the controls. Based on these observations, the microparticles do not increase the time of ring formation, but they do not hinder the ring formation time either, as seen in Figure 41 and Figure 42 controls and experimental rings both formed at 6 hours.

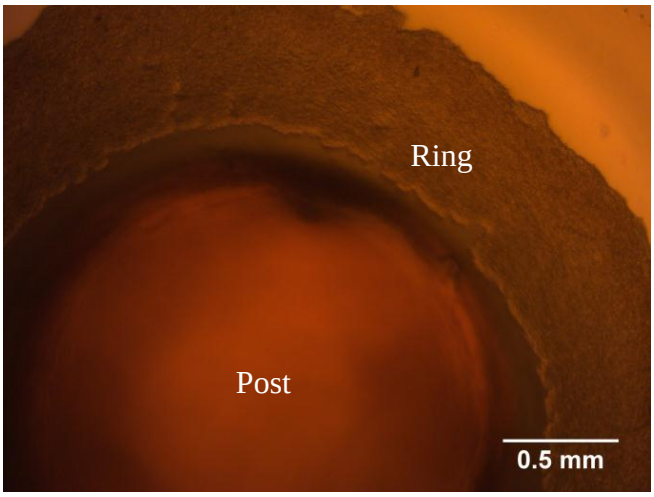

Figure 41: Control Ring Seeded with 500,000 after 6 Hours of Culture (4x)

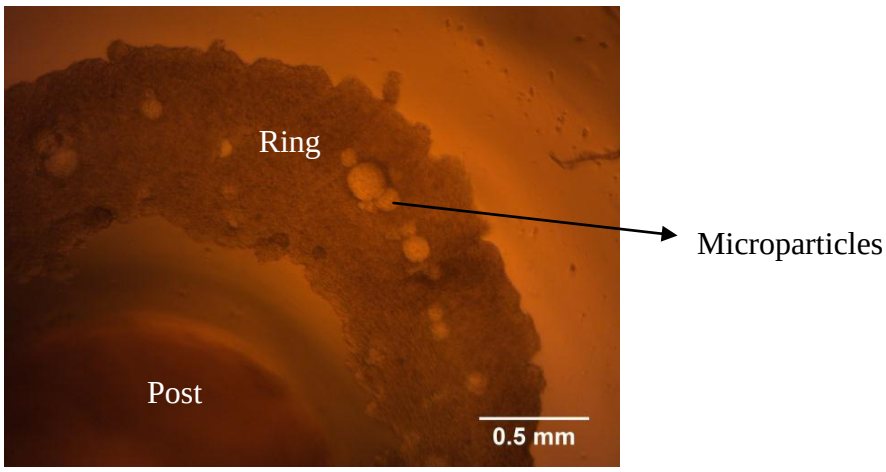

Figure 42: Experimental Ring Seeded with 500,000 at a Ratio of 1:100 (microparticles:cells) after 24 Hours of Culture (4x)

Lower Cell Count Tests

To determine the lowest number of cells needed in the formation of tissue rings incorporated with gelatin microparticles, rings were seeded with a consistent ratio of 1:100 microparticles:cells and decreasing total cell numbers. In 12-well plates, six control rings (containing no microparticles) and rings with 1:100 microparticles:cells were seeded. The first plate contained rings with 500,000 cells. Each subsequent plate had cell counts decreasing by 100,000. In total, rings with 500,000 cells, 400,000 cells, 300,000 cells, 200,000 cells, and 100,000 cells were all seeded, both with and without microparticles. The only rings that formed were the control rings with 500,000 cells, as seen in Figure 43. The lower cell counts had no ring formation with gelatin microparticles, as seen in Figure 44. This demonstrates that it is not possible to form complete rings with gelatin microparticles with fewer than 500,000 cells.

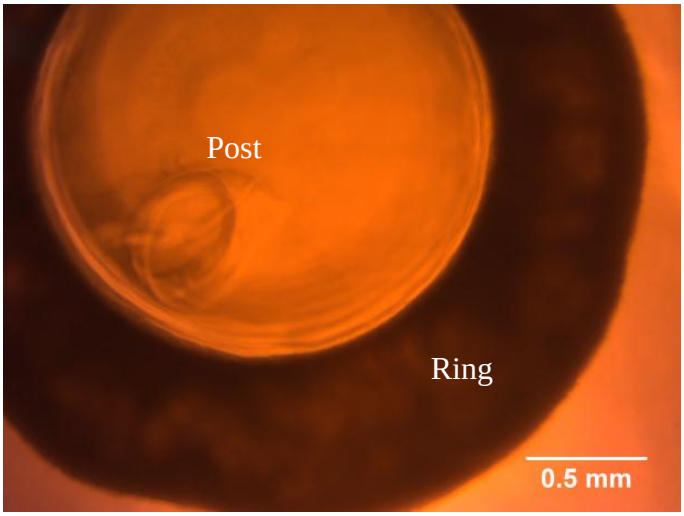

Figure 43: Control Ring Seeded with 500,000 Cells at Ratio of 1:100 (microparticles:cells) after 1 Day of Culture (4x)

Figure 44: Control Ring Seeded with 400,000 Cells at Ratio of 1:100 (microparticles:cells) after 1 Day of Culture (4x)

Tensile Testing

Tensile testing of rings was performed using a uniaxial testing machine (ElectroPuls E1000; Instron). The rings were submerged in PBS and the mounted on two small grips where they were then cyclically loaded before being pulled to failure at 10 mm/min. The load cell used was a 1N (±1mN) and the tare load used was 5mN. Unfortunately, the rings initially tested (controls, 1:50 ratio, and 1:100 ratio) were only 7 days old and did not make it through the pre-loading cycles. This means the rings were not strong enough after 7 days in culture, possibly due to ring characteristics or possibly due to the age of the cells. There were two rings: a control and a 1:50 ratio that made it through tensile testing. However, since a majority of the rings broke too soon more rings were cultured for 14 days, and tested to determine if their strength would increase to allow for a complete test. Ratios tested included 1:10, 1:100, and 1:1000 microparticles to cells. The data produced from the testing of the rings at 14 days can be seen in Table 10. This table shows the analyzed data of the engineering stress and the grip-to-grip strain. The analysis was done through MATLAB (The MathWorks Inc., Natick, Mass., USA) and provided the ultimate tensile strength (UTS). The data in this table include collected data from all rings that made it through the eight pre-cycle loads place on the rings. Any ring that did not

make it through the pre-cycles was not considered statistically relevant because the ring was not strong enough. From this analyzed data, the mean and standard deviation of the ultimate tensile strength were calculated for each specimen tests as seen in Table 11. Based on this data a graph of the results was generated, as seen in Figure 45. The ultimate tensile strength of the ratios of 1:10 and 1:1000 is less than that of the control. The ratio of 1:100 did have a larger ultimate tensile strength, but this data is from one ring. Overall, an ANOVA was performed on all the ultimate tensile data to determine statistical significance between the control rings and the experimental rings. The difference in data collected is not statistically relevant because the p-value is above 0.05. Due to this overall, the microparticles did not increase the strength of the tissue rings, but instead decreased the strength.

Table 10: Mechanical Properties of 2 mm Tissue Rings Cultured for 14 Days

Mechanical Properties of 2 mm Tissue Rings				
Raw Specimen #	**UTS (MPa)**	**MTM**	**Failure Strain**	**Toughness**
Control 2	0.091	0.152	1.097	0.033
Control 3	0.116	0.165	1.218	0.054
Control 4	0.101	0.118	1.470	0.048
Control 5	0.085	0.138	1.113	0.029
Control 6	0.122	0.143	1.246	0.057
1:10-1	0.098	0.144	1.180	0.038
1:10-4	0.098	0.117	1.432	0.048
1:10-5	0.081	0.134	1.181	0.029
1:10-6	0.076	0.142	1.089	0.024
1:100-4	0.118	0.142	1.396	0.062
1:1000-1	0.105	0.135	1.283	0.044
1:1000-2	0.078	0.161	1.038	0.022
1:1000-3	0.125	0.124	1.440	0.057
1:1000-4	0.089	0.131	1.135	0.032
1:1000-6	0.062	0.166	0.876	0.015

Table 11: Average Mechanical Properties of 2 mm Rings Cultured for 14 Days

Average Mechanical Properties of 2 mm Rings		
Specimen	Mean UTS	Standard Deviation (mm, ±)
Controls	0.103	0.016
1:10	0.088	0.011
1:100	0.118	0.000
1:1000	0.092	0.024

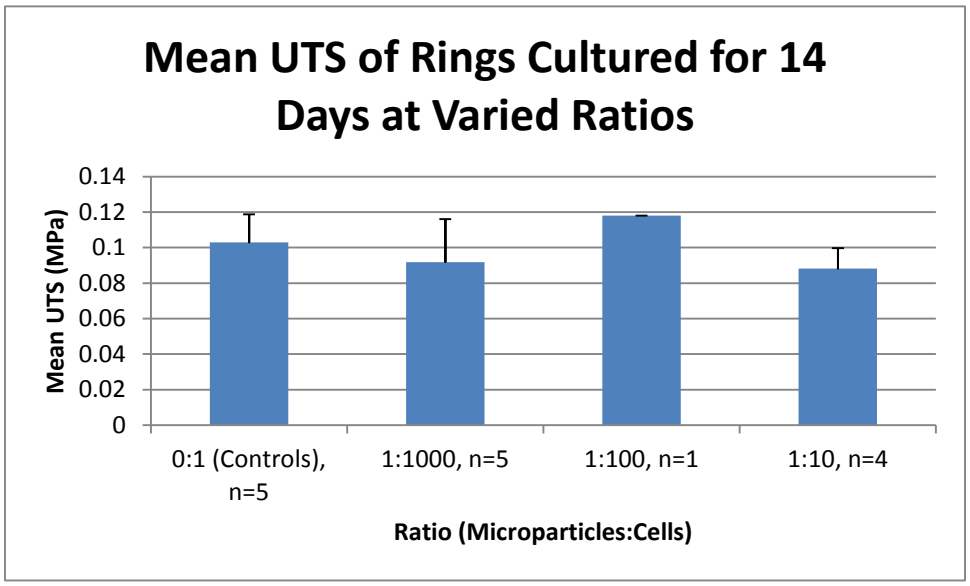

Figure 45: Mean UTS of Rings Cultured for 14 Days at Various Ratios

Ring Thickness

Before tensile testing, the thicknesses of the rings were determined as seen Table 12. The data from this table was then combined to determine the mean and standard deviation of each specific set of specimen, as seen in Table 13. From this table and Figure 46, the thicknesses are all between 0.70 mm and 1.00 mm in thickness. Overall, there is not too much variation as seen by the mean thicknesses and their standard deviations. An ANOVA was performed on the thickness measurements data. Based on this test, the data is not statistically significant. While not

statistically significant, it can be clearly seen that the thickness is not affected by the addition of the microparticles.

Table 12: Rings Thickness Measurements after 14 Days of Culture

Specimen Thickness Measurements						
Specimen	Thickness (mm)				Mean (mm)	Standard Deviation (mm, ±)
Control 1	0.720	0.810	0.880	0.680	0.773	0.078
Control 2	0.690	0.870	0.800	0.720	0.770	0.070
Control 3	0.850	0.960	0.900	0.890	0.900	0.039
Control 4	0.770	0.740	0.890	0.870	0.818	0.064
Control 5	0.770	0.880	0.840	0.860	0.838	0.041
Control 6	0.850	0.820	0.870	0.770	0.828	0.038
1:10-1	0.980	1.020	0.980	0.990	0.993	0.016
1:10-2	0.720	0.790	0.910	0.580	0.750	0.119
1:10-4	0.980	1.020	0.930	0.860	0.948	0.060
1:10-5	0.920	0.930	0.830	0.850	0.883	0.043
1:10-6	0.810	0.820	1.000	0.820	0.863	0.079
1:100-1	0.740	0.540	0.900	1.030	0.803	0.183
1:100-2	0.720	0.820	0.960	0.740	0.810	0.094
1:100-3	0.710	0.850	0.990	0.680	0.808	0.123
1:100-4	0.870	0.800	0.840	0.710	0.805	0.060
1:100-5	0.770	0.940	0.870	0.770	0.838	0.072
1:100-6	1.110	0.930	0.680	0.560	0.820	0.214
1:1000-1	0.750	0.730	0.920	0.800	0.800	0.074
1:1000-2	0.960	0.860	0.750	0.830	0.850	0.075
1:1000-3	0.890	0.900	0.940	0.870	0.900	0.025
1:1000-4	0.880	0.740	0.770	0.900	0.823	0.069
1:1000-6	0.720	0.810	0.930	0.770	0.808	0.078

Table 13: Average Ring Thickness Measurements after 14 Days of Culture

Average Thickness Measurements		
Specimens	Mean Thickness (mm)	Standard Deviation(mm, ±)
Controls	0.821	0.048
1:10	0.887	0.092
1:100	0.814	0.013
1:1000	0.836	0.041

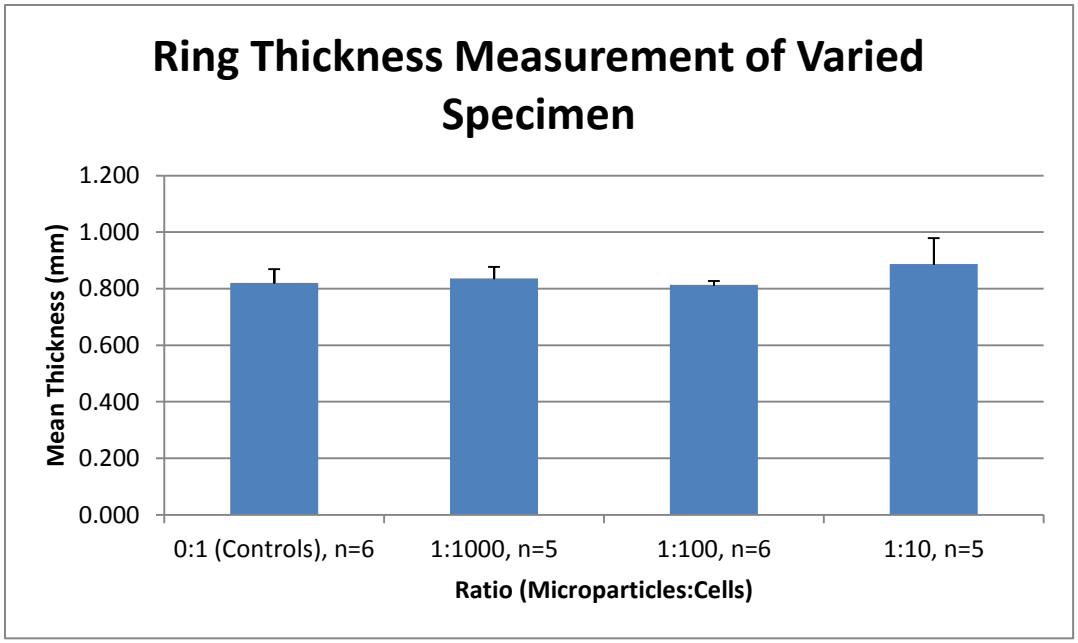

Figure 46: Ring Thickness Measurements of Varied Specimen after 14 Days of Culture

Histology

In order to verify the presence of the microparticles in the rings, histology was performed on rings at one day for controls and ratios of 1:50 and 1:100. Rings were also stained at seven days for controls and a ratio of 1:100. First the rings were fixed in a 10% neutral buffered formalin and embedded in paraffin. Then 5um sections were cut and adhered to slides. The sections were stained with Hoechst, hematoxylin and eosin, and fast green/picrosirius red. Hoechst images were obtained using a fluorescent microscope, and the other staining images were obtained using a light microscope. For the Hoechst images, the nuclei of the cells fluoresce in blue. These images show little presence of microparticles because it is only for the nuclei of cells, as seen in Figure 47and Figure 48. In Figure 49, however, there is a larger black circular gap that might indicate the presence of microparticles. The lack of microparticles is further evident in Figure 50 and Figure 51. On the other hand, when viewing the other stains the presence of microparticles is more evident. As seen in Figure 52 and Figure 55, the cells stain a

deep purple color. In Figure 54 and Figure 56 the microparticles stain almost a pink color. As demonstrated by Figure 53, microparticle presence varies throughout the ring. However, the stained samples provide evidence to support gelatin microparticle presence in the rings. The final stain a fast green picrosirius red shows the cells in blue, as seen in Figure 57 and Figure 60. Gelatin is a derivative of collagen; the microparticles will stain a deep red as seen in Figure 61. Again the variability of microparticles within the rings is displayed in the three Figure 58 and Figure 59. These images again show that the microparticles are present within the ring, but display the uneven distribution and variable amounts.

Hoechst

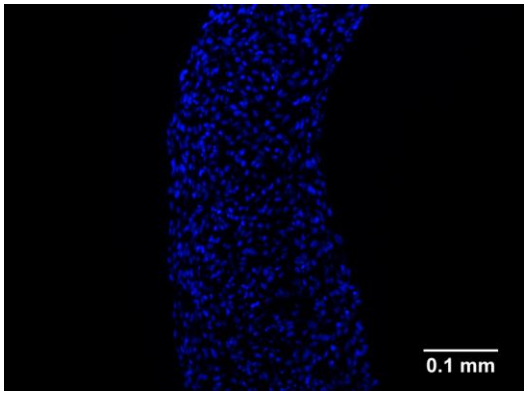

Figure 47: Control Ring (no microparticles) after 1 Day of Culture Hoechst Stain (20x)

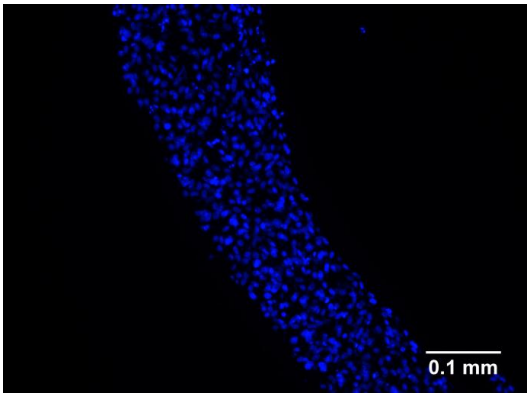

Figure 48: Experimental Ring at a ratio of 1:50 (microparticles:cells) after 1 Day of Culture Hoechst Stain (20x)

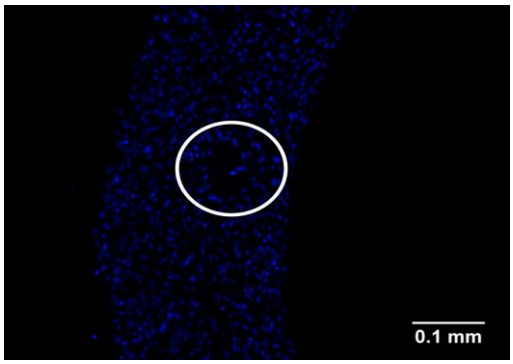

Figure 49: Experimental Ring at a ratio of 1:100 (microparticles:cells) after 1 Day of Culture Hoechst Stain (20x)

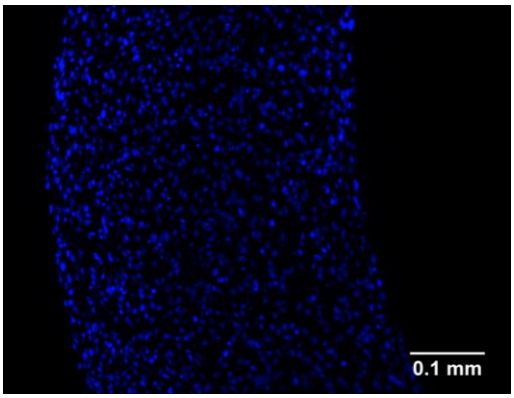

Figure 50: Control Ring (no microparticles) after 7 Days of Culture Hoechst Stain (20x)

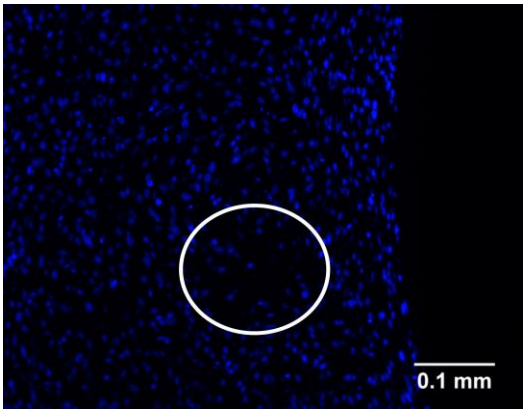

Figure 51: Experimental Ring at a ratio of 1:100 (microparticles:cells) after 7 Days of Culture Hoechst Stain (20x)

Hematoxylin and Eosin

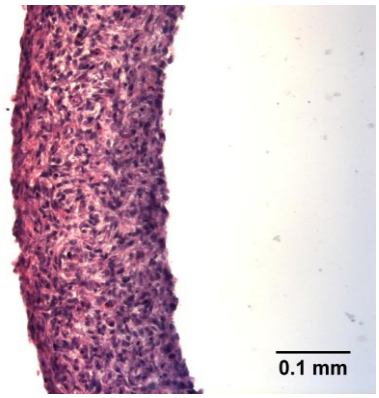

Figure 52: Control Ring (no microparticles) after 1 Day of Culture Hematoxylin and Eosin Stain (20x)

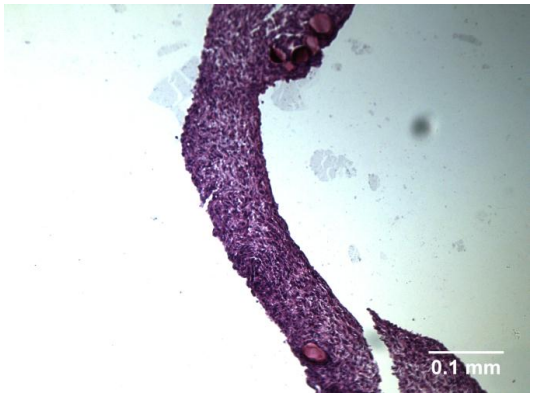

Figure 53: Experimental Ring at a ratio of 1:50 (microparticles:cells) after 1 Day of Culture Hematoxylin and Eosin Stain (20x)

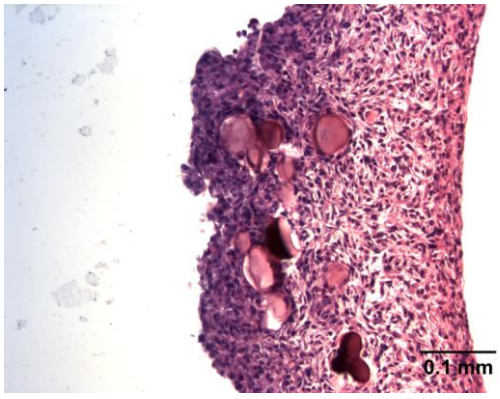

Figure 54: Experimental Ring at a ratio of 1:100 (microparticles:cells) after 1 Day of Culture Hematoxylin and Eosin Stain (20x)

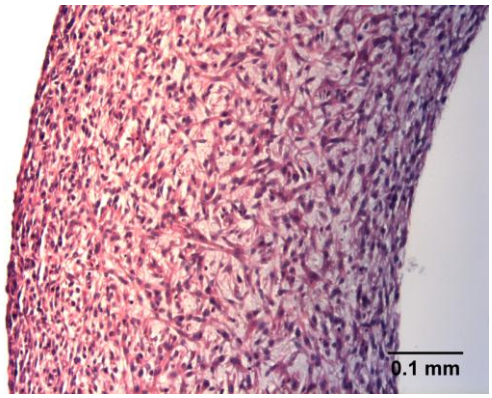

Figure 55: Control Ring (no microparticles) after 7 Days of Culture Hematoxylin and Eosin Stain (20x)

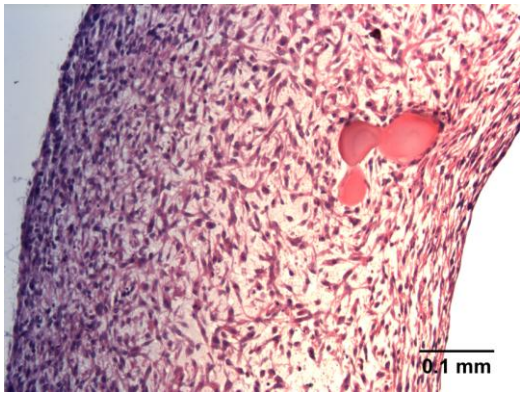

Figure 56: Experimental Ring at a ratio of 1:100 (microparticles:cells) after 7 Days of Culture Hematoxylin and Eosin Stain (20x)

Fast green/ picrosirius red

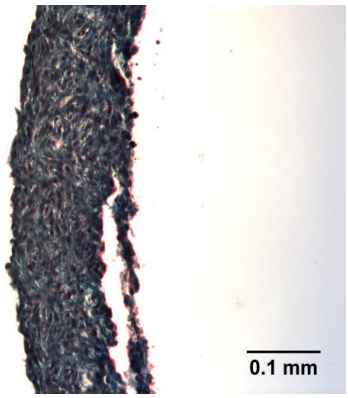

Figure 57: Control Ring (no microparticles) after 1 Day of Culture Fast Green Picrosirius Red Stain (20x)

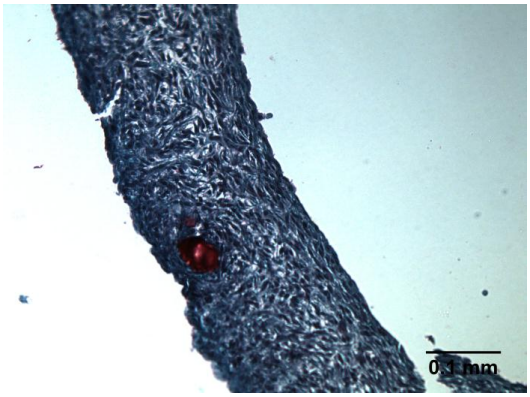

Figure 58: Experimental Ring at a ratio of 1:50 (microparticles:cells) after 1 Day of Culture Fast Green Picrosirius Red Stain (20x)

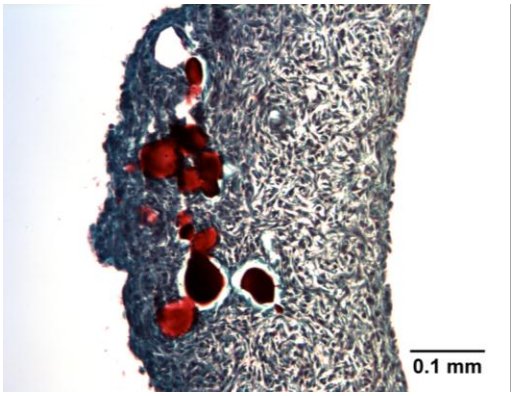

Figure 59: Experimental Ring at a ratio of 1:100 (microparticles:cells) after 1 Day of Culture Fast Green Picrosirius Red Stain (20x)

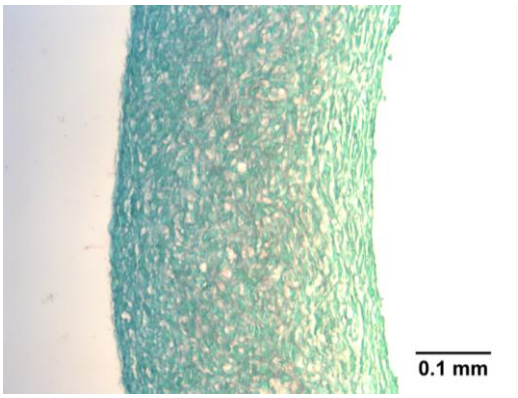

Figure 60: Control Ring (no microparticles) after 7 Days of Culture Fast Green Picrosirius Red Stain (20x)

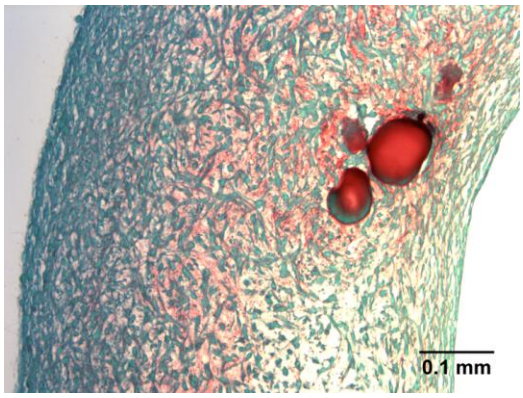

Figure 61: Experimental Ring at a ratio of 1:100 (microparticles:cells) after 7 Days of Culture Fast Green Picrosirius Red Stain (20x)

6: Discussion

The focus of this project was the development of an adhesion system that would enhance cellular aggregation in the creation of smooth muscle cell tissue rings. Multiple conceptual designs were developed, and each was evaluated accordingly. The final adhesion system chosen was the addition of gelatin microparticles to the cell suspension upon seeding. This system was designed to meet the desired objectives; including creating a system that would create a ring, be inexpensive, and produce consistent results. The overall system was also designed to include the objectives desired for the produced tissue ring, including increasing the aggregation speed and strength of each ring, requiring fewer cells at seeding, and decreasing cell death within the ring. In addition to the consideration of each objective, both social and ethical concerns must be addressed, as this produced system may contribute to advances in medical research and patient care.

6.1: Adhesion system design

6.1.1: Create a Ring

As demonstrated the presence of microparticles has no significant impact on ring formation. It is possible to create rings with the presence of microparticles at different ratios. Depending upon the ratio these rings are not always uniform and smooth, but their formation is possible. This means that the microparticles do not hinder the ring formation.

6.1.2: Be inexpensive

The use of this adhesion system involves adding gelatin microparticles to the currently used ring formation method, and therefore any additional cost can be attributed to creation of the

gelatin microparticles. This process includes gelatin, extra virgin olive oil, and acetone solution. In total, it costs approximately $3.50 to make a batch of gelatin microparticles. Cost breakdown can be seen in Table 14.

Table 14: Cost Breakdown for One Batch of Gelatin Microparticles

Material	Unit Cost	Amount Needed per Microparticle Batch	Cost per Microparticle Batch
Gelatin	$40/100g	1g	$0.40
Oil	~ $7/17oz	150ml	~$2
Acetone	~$53/5gal	~400ml	~$1.10

6.1.3: Be consistent

Consistency, in the scope of this project, is defined as the ability to recreate rings with the same characteristics when produced under the same conditions. Rings produced with the same microparticle:cell ratios demonstrate similar characteristics, as shown by previous data's standard deviations. Because the characteristics of rings produced in the same conditions, but in different batches, are comparable, it can be concluded that the methodology followed results in consistent rings.

6.2: Ring Produced

6.2.1: Decrease aggregation time

This objective as not met, the microparticle system did not decrease the aggregation time. It did not however, hinder the aggregation of the rings, even as early 6 hours.

6.2.2: Increase aggregation strength

This objective was also not met. The microparticles within the system did not increase the aggregation strength of the ring. Again based on the data collected it also seems that they did not decrease the aggregation process either.

6.2.3: Require fewer cells at seeding

Based on the results, it is not possible to seed rings with fewer cells. This objective was also not met. If the cell count is lowered to less than 500,000 cells there is no ring formation. The addition of gelatin microparticles hinders the ability of the cells to aggregate into complete rings if fewer than 500,000 cells are seeded.

6.2.4: Decrease cell death

Due to time and budget constraints, the team was not able to address the issue of cell death in the interior of the cell rings. This objective was not able to be addressed through experimentation, and should be considered in future plans for microparticle incorporation into cell rings.

6.3: Economic Impact

The economic impact is related to the benefits that could result from the use of this technology. The success of creating improved tissue rings that can be formed into functional vascular structures is beneficial for both research and patient graft purposes. Any success for either of these purposes could outweigh the potential increase in cost associated with the use of this adhesion system. This cost difference associated with adding gelatin microparticles to the ring culture system, as seen by previously mentioned cost analysis, is low.

6.4: Environmental Impact

Environmental impact is minimized in the production of this adhesion system with the use of sterilizable and reusable materials wherever necessary. Chemicals such as acetone and oil used are recycled as appropriate, and with the exception of only pipette tips and centrifuge tubes all glassware needed in the production of gelatin microparticles can be reused.

6.5: Societal Influence

This adhesion system was developed with the potential to be used in vascular research and possibly the production of vascular grafts. This allows the system to potentially aid in the treatment of patients in need of vascular replacements, and therefore the system could have a beneficial impact by increasing the quality of life for these patients.

6.6: Ethical Concerns

There are minimal ethical concerns associated with the use of this adhesion system. This system is used in conjunction with the current ring method to create *in vitro* tissue constructs, which could potentially reduce the need for graft donors in the treatment of vascular tissue replacements. These rings could be made from the patient's muscle cells after biopsy, a customization that would reduce any incompatibility responses. Initially, the use of this technology may need to be tested in animal subjects before any treatment could occur in humans. This raises ethical concerns in the treatment of animal as medical test subjects. However, the success of this system may lead to treatments that could save patient lives, which may justify the use of animals in a test trial as long as treatment follows the proper protocol as outlined by the Institutional Animal Care and Use Committees (IACUC).

6.7: Health and Safety Issues

The overall ring system has been designed for the future purpose of creating vascular tissue grafts to be used in patients and for research into vascular issues. Extensive animal and clinical trials would need to be conducted according to FDA regulations to ensure the safety of this technology before human use. In addition, this system also has the potential for patient-derived vascular tissue grafts, which would decrease the rejection of the replacement graft after implantation in the patient. This adhesion could ultimately lead to improved patient treatment options.

6.8: Manufacturability

The production of gelatin microparticles consists of readily available materials and machinery. Materials, such as the gelatin itself, can be purchased from a commercial source, and the needed machinery is found in most laboratories, such as a centrifuge. The production of the gelatin microparticles takes a total of approximately 15-20 hours, including microparticle fabrication, washing, and drying. After the microparticles are produced, they can be stored for extensive periods of time at 4^{o}C without damage. Adding the microparticles to the cells when seeding into rings takes approximately 5 minutes, including counting the microparticles and combining them with the cell solution. The process of producing the microparticles is simple, and minimal skill is required beyond proper laboratory training.

6.9: Sustainability

Waste is minimized throughout this process by using reusable containers and recycling materials as appropriate. Due to the nature of this adhesion system, the chemicals needed to

produce and clean the microparticles (oil, acetone, IPA) and plastic materials (pipette tips and centrifuge tubes) eventually get discarded after use, though materials are reused and recycled whenever appropriate. Glass containers and other reusable materials are taken advantage of whenever possible. Additionally, the biological products such as gelatin and cells are natural and renewable.

Chapter 7: Final Design and Validation

The final adhesion system design choice was the fabrication of gelatin microparticles made using a water-in-oil emulsion. These microparticles are added to the wells, followed directly by cell seeding, and allowed to culture with the cells to form tissue rings as seen in Figure 62.

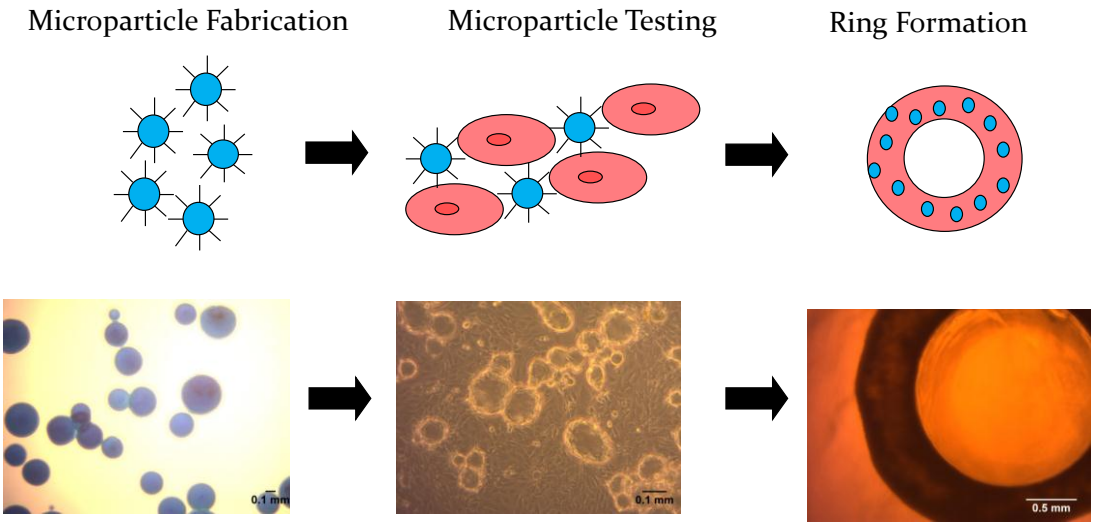

Figure 62: Final Design Approach

Microparticle Fabrication

Gelatin was chosen for this design because it adheres to cells, is degradable, and is non-toxic. This material was readily available and also significantly cheaper than alternatives, making it ideal for experimental testing.

Water-in-oil emulsion was chosen as the fabrication method. This method is simple and can be altered to customize the resulting microparticles as seen in Figure 63. The final method used in the creation of this adhesion system involves adding a solution made of 1g of gelatin to 9ml of water into olive oil at 40^0C, stirring at 500rmp for ten minutes, cooling to -4^0C, acetone washing, and air drying. Following microparticle production, the gelatin microparticles are sieved to collect appropriately sized microparticles, sterilized in IPA, and rehydrated in both

DMEM and full media before addition to cells for ring culture. This process is simple, reproducible, and customizable, and resulted in microparticles that can be easily handled and stored.

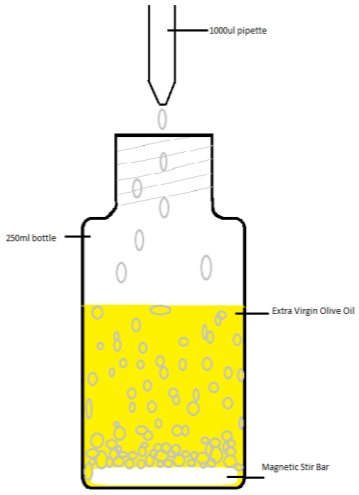

Figure 63: Water in Oil Emulsion Schematic

Microparticle culture with cells

As seen in Figure 64, the microparticles were seeded with RSMCs on a culture plate. This image demonstrates the cells interacting with a microparticle; as the cells appear alive and unaffected by the microparticles and no contamination is present, it can be concluded that the microparticles are biocompatible and have no toxic effect on the cells.

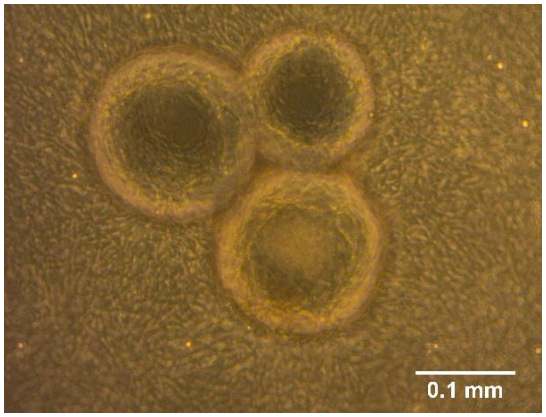

Figure 64: Cells and a Microparticle (10x)

Seeding with Rings

These microparticles are added to the ring culture wells prior to the addition of cells, simply adding one step to the culture process. Fifty microliters of microparticles were added to the wells at appropriate ratios to achieve desired microparticle:cell ratios. Consistent with the current ring system, 500,000 cells are seeded following the microparticles.

Validation

The gelatin microparticles were visualized using light microscopy. Shape and size of these microparticles were determined based on images and software measurement. These examinations proved microparticles formed at 500rpm were appropriate for RSMCs.

The biocompatibility of these microparticles was verified through the plating experiments. Continued cell proliferation and incorporation around the microparticles confirmed the biocompatibility of the gelatin microparticles.

Gelatin microparticles were seeded with 500,000 cells at varying ratios to confirm they were integrated into the produced ring. As seen Figure 65, it is possible to see red stained gelatin microparticles that have been incorporated into Figure 65B.

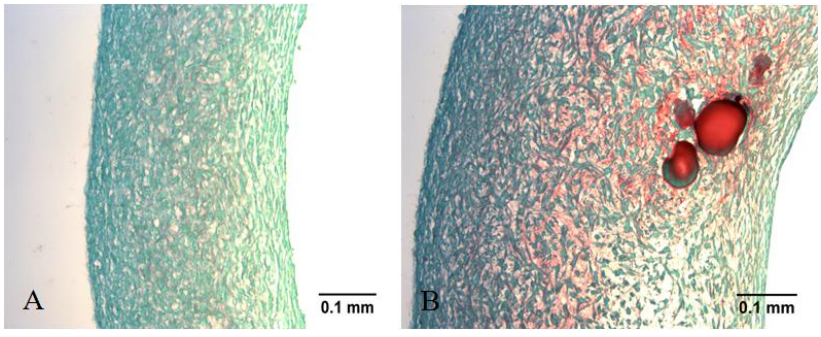

Figure 65: Fast Green Picrosirius Red Histology Ring Culture for 7 days - A. Control Ring with no Microparticles, B. Ring Seeded with Microparticles at a Ratio of 1:100

The consistency of these rings was determined by comparing the percentage of fully formed rings to the total attempts, determining thickness, and performing histology on cross-

sections of formed rings. This analysis proved rings could be consistently created with the incorporation of gelatin microparticles.

In addition to determining ring consistency, aggregation speed, strength, and required cell count of each ring was tested. Experimentation proved that the addition of gelatin microparticles did not hinder formation of the rings, but it did not increase these characteristics as expected: mechanical strength of the rings with incorporated gelatin microparticles was comparable to the controls after 14 days in culture, both controls and rings with microparticles began to form after 6 hours, and reduced cell count tests proved 500,000 cells were required to make complete rings, with or without microparticle addition.

Chapter 8: Conclusions and Recommendations

The main objective of this project was to design an adhesion system that would aid in the increased aggregation of rat smooth muscle cells in the formation of tissue rings. After formulating design alternatives and considering potential materials, the team decided to evaluate the addition of protein microparticles to the ring culture. Gelatin, the most readily available material that successfully produced microparticles, was chosen in the final design. Through bench top tests the gelatin microparticle design was validated. This successful validation is proof-of-concept, suggesting that the microparticle system could be used to successfully improve aggregation of cells in the ring culture method.

Future work should include the perfecting of both material choices and design made in the creation of this microparticle adhesion system. Due to time constraints, the team was only able to fully produce and test gelatin in the form of microparticles. The team suggests pursuing research into additional materials that could be used, such as fibronectin, collagen, PLLA, PLLA coated with either gelatin or collagen, or other materials mentioned in the conceptual design portion of this report. Alternative materials may provide improved aggregation and may be produce more uniformly. Because of these possibilities experimentation may be beneficial.

Adding more consistency and control to the microparticle fabrication process is also recommended. This could potentially be achieved through the use of machinery such as a homogenizer. Using a machine, such as the homogenizer, could allow for the creation of smaller particles and a more uniform microparticle batch, decreasing any waste created in the current fabrication process. Finding a better sieving method could also improve production, as the gelatin sticks to many materials and a significant loss of product is involved in the current

process. Experimentation using metal sieves is suggested. Microparticle aggregation and swelling of the gelatin microparticles in solution continues to be a problem as well, and though progress has made in compensating for these issues, further testing should be continued. Crosslinking is recommended for the reduction of microparticle aggregation and swelling. By reducing microparticle aggregation, determining effective concentrations of microparticles to cells can be refined. Additionally, by reducing the swelling of the microparticles size can be more accurately controlled.

Mechanical strength of the rings formed with the addition of gelatin microparticles must also be improved. The mechanical strength of rings with gelatin microparticles was only comparable to controls. Finally, the team recommends additional experimentation to perfect the ratio of needed microparticles to cells in culture and the concentration of this solution to maximize efficacy.

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