Creation of Perfusable Tissue Engineering Constructs through Biological Assembly

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Abstract

One of the biggest barriers in creating large tissues is the lack of oxygen and nutrient transport required for cell growth and tissue development in the interior region. The post-implantation cell survival in large tissue engineering constructs can be assisted by prevascularization. In this work we used a bottom-up approach to prepare large, prevascularized tissue constructs through perfusion culture of porous, cell-laden hydrogel constructs biologically assembled from smaller gel modules. The small gel modules had a controlled shape and were laden with HUVECs and hMSCs. They were packed in a bioreactor for perfusion culture, during which capillary formation inside and between individual gel modules led to the assembly of the small modules into a nearly centimeter sized porous construct. Viable cells and hollow lumen-like structures were observed throughout the porous construct, while in a nonporous control construct with similar dimensions viable cells were only observed in a peripheral layer several hundred micron thick. This modular assembly approach allows for creation of prevascularized large tissue constructs through the biological assembly of gels, which was difficult previously.

Table of Contents

List of Figures

Chapter 1: Introduction

1.1 Tissue Engineering

1.1.1 Purpose of Tissue Engineering

Tissue engineering is the combination of engineering and life sciences with the purpose of recreating natural cell environments or developing biological substitutes that can recreate, restore, or improve the function of tissues or organs [1]. These biological substitutes can help advance medicine in several ways. First, they can be used to replace diseased or damaged tissue *in vivo*. While there has been great success in restoring function through organ transplantation, it can be difficult to find compatible donors, leading to long wait times that can result in deterioration or death of patients while waiting for the needed tissue to become available[2,3]. For example, there are over 95,000 patients on the waiting list for an organ, and over 6,300 people die waiting for an organ to save their life [2]. If these needed organs could be created *in vitro*, not only could it greatly reduce the number of deaths, but the organ could be made more suitable for the specific patient. But in addition to this use, tissue engineering constructs could also be quite useful for *in vitro* testing. The study of organs and biological systems currently relies on live human subjects, which have limited use for ethical reasons, animal subjects, which again have ethical limitations as well as limitations in similarity to human anatomy, or post-mortem analysis which is limited in scope. One way to ethically deepen the understanding of the human body and how it is effected by diseases would be to recreate normal or diseased systems with tissue engineering [4,5]. A third important potential use of tissue engineering is to create platforms to test novel disease treatment methods.

Currently, one of the most common methods is animal testing, which has been shown to be a very effective as a drug screening method and resulted in many medical breakthroughs. But even animals with biological systems closest to humans still have important differences in specific signaling pathways that may be crucial for treating human diseases. With effective tissue engineering constructs, new drugs can be more easily assessed with human tissue without endangering live patients, leading to safer and more effective drugs and therapies [6,7].

1.1.2 Current Applications

There are several examples of medical breakthroughs resulting from progress in tissue engineering. One of the largest success stories is the creation of skin grafts to treat burns and other wounds. As far back as 1962, it was shown that a synthetic substitute for skin could be created [8], and in 1979, a skin substitute was actually used to treat a patient, leading to the first commercial skin graft product [9]. Since then there have been significant advances not only in skin grafts but also in the replacement of damaged cartilage [10]. Beyond commercial applications there have been significant advances that have culminated with the implantation of bladder[11], trachea[12], and bone[13]. There has also been significant progress in the development of other organ constructs such as heart valve [14], liver[15], and pancreas[16] tissue, to just name a few significant examples. The field of tissue engineering has shown remarkable advances in the short time it has been around, and has already had a huge impact on how the medical field approaches treatment [17].

1.1.3 Important Factors in Tissue Engineering

While there have been significant developments in tissue engineering since its inception, there remain many difficulties, largely due to the complexity and interdependence of natural tissues. There are many factors that are crucial to the creation of a tissue engineering construct that can effectively replace human tissue. First, mechanical properties of the construct are crucial for cell survival [17]. Different types of tissue require different mechanical properties - tissues such as bone require stiffer substrates to properly develop, while nerve regeneration benefits from a less stiff substrate [18,19]. Even similar cell types can react differently to the same mechanical stimuli; it was shown that MSCs not only react to different mechanical stimulation, but MSCs extracted from bone react differently than MSCs extracted from hair when exposed to the same mechanical stimulation [20]. This also leads to the conclusion that cell type as well as cell source are crucial in forming the desired tissue construct. Another very important part of designing tissue engineering constructs is the biochemical factors present in the construct. These can be supplied through the extracellular matrix used to create the construct [21], other cells present in the construct[22], or soluble factors present in media[23]. The concentration, soluble versus immobilized presentation, and gradients of these signals are all important for cell development. For example, the peptide RGD can assist in cell adhesion when immobilized, while the soluble peptide inhibits cell spreading [24]. Gradients in stathmin can effect microtubule formation [25], even to the point where there are measurable differences in concentration between adjacent cells [26]. Another factor crucial for tissue engineering constructs is the porosity of the construct, as

the diffusion of nutrients and oxygen and removal of waste is crucial for cell survival, and pores also play an important role in supporting cell motility. The biological complexity of cells interacting with their microenvironments is what makes biological systems difficult to carefully design.

1.1.4 Current Barriers in Tissue Engineering

As discussed, most biological systems require special material types and concentrations, specific cell types and even cell sources, and soluble factors to correctly form a construct. And to make this more difficult, many tissues are not homogenous structures, meaning that it can require different cell types, mechanical properties, and signaling molecules depending on both position within the structure and at different times as the culture progresses [27,28]. But one of the largest hurdles that must be overcome is the limitation of nutrient and oxygen delivery to cells contained in an engineered construct. *In vivo*, this issue is solved through the circulatory system. Cells are generally only a few hundred microns away from a blood vessel, and the flow of blood through the vessels means that the diffusion distance of oxygen to any cell is only the distance to the nearest capillary. In order for tissue engineering constructs to mimic natural tissues, many still require high concentrations of cells. Without a method of circulating oxygen and nutrients through the system, diffusion distances are much greater, which limits most tissue constructs to about a millimeter in size [29]. The scope of this issue can be seen by looking at which areas of tissue engineering have seen the most success – cartilage and skin. Cartilage has lower metabolic requirements, and therefore lower oxygen demand,

and it has even been shown that hypoxia can be an important signal to cells used for cartilage repair [30]. The success in skin has come largely because of the thickness required - the epidermis of the skin is limited to thicknesses less than 100 microns [31]. This layer has been where most of the success has originated; clinical applications of full thickness skin grafts have proven much more difficult to create, which impedes the treatment of more severe burns and wounds. To achieve the goal of engineering large complex tissues, we require greater ability to provide oxygen and nutrients throughout tissue engineering constructs.

1.2 Hydrogels

1.2.1 Hydrogel Design

A critical element to engineering three-dimensional tissue constructs is the polymer scaffold. Initially, cell culture was performed in two-dimensional plate cultures, and while this proved useful for learning about cells, it is unable to recapitulate the structures found in a three-dimensional organism. The scaffold created by the human body to support cells is referred to as the extracellular matrix, and is composed of many different types of molecules. This scaffold provides varied microenvironments for cells to flourish, and must be replicated in order to make effective tissue engineering constructs. The primary type of material that has been shown to be effective at replicating this matrix is a broad category of polymers known as hydrogels.

Hydrogels are water-swollen, cross-linked polymeric networks, composed of natural polymers or other polymers with similar properties [32]. Different tissue engineering

goals have unique requirements, so there is no single type of scaffold that works best for all constructs. Different polymers have unique properties based on many factors: rigidity of the polymer backbones, types of cross-linking molecules and cross-linking density, as well as gel swelling as a result of hydrophilic/hydrophobic balance [33]. These factors can lead to differences in the adhesion and gene expression of cells seeded within the scaffold [34]. Depending on the application, the speed of gel formation and degradation can also be important. Another important factor when choosing a scaffold for a tissue engineering application is the biological signaling it can provide. Correct cell signaling through either mechanical properties or immobilization of small molecules can assist in cell adhesion, cell division, cell motility, cell shape, and cell differentiation [35,36]. Another important factor is the biocompatibility of the hydrogel with the human immune system. It is desirable to use gels that will not trigger an immune response from the body, which can lead to rejection of the construct. This leads to two broad categories of hydrogels – natural hydrogels, or polymers that are naturally found to compose biological tissue, which generally have the advantage of biocompatibility, and synthetic hydrogels, which tend to be less biocompatible, but more versatile and allow for finer control of mechanical properties and signal presentation.

1.2.2 Natural Hydrogels

There have been a variety of natural polymers that have proven effective for the creation of tissue engineering constructs. One of the most commonly used is the protein collagen, which can form physical gels under physiological conditions. The most

abundant protein in mammals, it can be degraded with cell-produced enzymes and contains peptide sequences that are important for cell signaling [37]. It does have some drawbacks, such as lower mechanical strength, and batch to batch variation in composition, but it has been widely used in reconstructing skin[38], liver[39], blood vessels[40], and small intestines[41].

Fibrin is a natural polymer associated with the clotting of blood during wound healing, which leads to minimal inflammatory response when used in tissue engineering. It is a protein with mechanical properties similar to collagen, and also contains cell signaling peptides such as RGD. It is formed from the chemical cross-linking of fibrinogen in the presence of thrombin under normal cell conditions [42]. Fibrin gels have been utilized to engineer tissues with cartilage [43], muscle cells [44], nerve cells[45], pancreas [46], and blood vessels[47].

Hyaluronate is a glycosaminoglycan (a type of polysaccharide) found in natural extracellular matrices, and is also an important factor in would healing. Isolation of pure hyaluronate has proven more difficult than other natural polymers, and it requires carbodiimide chemistry for cross-linking [42]. It also typically possesses low mechanical strength, but has found uses in many areas including constructs for muscle [48], adipose[49] and skin tissues[50].

Other commonly used natural polymers include alginate, a polysaccharide from brown algae that can easily gel in combination with Ca^{2+} ions. While it is biocompatible, it is severely limited by uncontrolled dissolution of the gel caused by the diffusion Ca^{2+} ions out of the gel. Because of this it has seen more use as a delivery vehicle for cells [51],

although modification has resulted in uses for skeletal muscle differentiation.[52]. Chitosan is another polysaccharide polymer with stronger mechanical properties and stability through gelation caused by chemical cross-linking. On the other hand, it is relatively insoluble at neutral pH, so it is more common to synthesize derivatives for use in tissue engineering applications [53]. It has found uses in applications including nerve and bone constructs [54].

1.2.3 Synthetic Hydrogels

As opposed to the natural polymers, synthetic polymers are more easily controlled and reproduced with specific molecular weights, cross-linking densities, and linkage degradation rates, which can give them a significant advantage in creating desired properties in hydrogels, but the downside is they tend to have lower biocompatibility than the natural polymers. One example of a commonly used synthetic hydrophobic polymer is Poly(ethylene glycol) (PEG), or Poly(ethylene oxide) (PEO). The monomers can be easily cross-linked when modified with acrylate and exposed to UV light in the presence of a photoinitiator [55]. It has been approved for several applications due to its low toxicity both during cross-linking and implantation. It has shown great promise with its adaptability, especially when modified with poly(lactic acid) (PLA)[56] or cleavable peptides[57] that allow for more controlled degradation, whereas the attachment of peptides such as RGD allow for the alteration of cell attachment properties[58]. It has also been proven effective for drug delivery, such as with the delivery of insulin or cancer treatments [59,60].

A widely used example of a hydrophilic polymer is poly (vinyl alcohol) (PVA). It can be chemically cross-linked, but to reduce toxicity, it can also be physically cross-linked using a freeze/thaw method [61]. Because the freeze/thawing method can not be done in-vivo or in the presence of cells, and PVA hydrogels do not degrade in most physiological environments, it has been used as long term scaffolding for treating issues in cartilage and bone[62,63].

Polypeptides are a different type of synthetic polymer – while they are synthesized *in vitro*, they are a biological product and are a crucial part of the natural extracellular matrix, and there has been a significant effort to design synthetic analogs of natural proteins as well as ones with novel function. The assembly of individual peptides through chemical synthesis is limited in accuracy and length, so a common production method uses genetically modified bacteria to express large amounts of the cloned protein [64]. The diversity of peptide building blocks allows for significant variety in products, which can form anything from a silk-like protein to a reversible or physically cross-linked hydrogel [65-67].

1.2.4 Composite Hydrogels

While the examples above are not comprehensive, they show the large range of available options when creating hydrogels for tissue engineering, and researchers have found that further tailoring of the constructs properties is possible by combining multiple types of polymers into a single scaffolding. For example, natural hydrogel compositions of alginate and chitosan were used to assist with cartilage function [68] and a mixture of

alginate, chitosan, and fibrin was used for adipose tissue engineering [69]. Combinations of synthetic and natural polymers have also show promise, granting the ability to have better control of gel properties while still providing important cell signaling, such as with Hyaluronic acid/PEG hydrogels assisting with nerve growth[70] or PVA with natural ECM support assisting with cartilage regeneration[71]. The possibilities created with the use of the many natural and synthetic hydrogels allow for a large range of options when developing tissue engineering constructs.

1.3 Vascular Network Formation

1.3.1 Purpose of Vascularization

As previously mentioned, one of the biggest barriers to creating large tissue engineering constructs is the inability for diffusion to match the cellular requirements for oxygen, nutrients, and waste removal. This is largely due to the limitations of host tissue forming a vascular network in unvascularized implanted tissue – vascular ingrowth from surrounding tissue into a construct can be limited to microns per day, taking weeks to even reach a millimeter of ingrowth [72]. And without a vascular system present to remove waste and supply oxygen and nutrients, constructs must rely on diffusion from the vascular system present initially only in the host tissue. With normal cell densities, this diffusion can only supply oxygen at a distance on the order of hundreds of microns, which limits construct depths to about a millimeter [73]. In order to reduce or eliminate this problem, researchers have found that forming a vascular network in the construct before

implantation can greatly increase the rate at which the host vasculature can perfuse the implanted construct.

1.3.2 Cell Sources

The type of cell most commonly used in developing vasculature is human umbilical vein endothelial cells (HUVECS). HUVECs have been proven to effectively form vasculature *in vitro*, are relatively easily obtained, and can be quickly expanded in large numbers [74]. Other types of endothelial cells (EC) can also be used, providing distinct advantages and disadvantages. For example, mature human EC can form capillary-like structures without addition of angiogenic factors, but they are limited by low proliferation potential, making it the difficult to obtain them in large numbers [75]. Another available source of ECs is embryonic, induced pluripotent, or other stem cells, although there are ethical concerns in the case of embryonic stem cells. And while obtaining higher numbers is not an issue, the difficulty in controlling differentiation can make this a more complex task than HUVEC culture. Another promising new source for autologous ECs is endothelial progenitor cells, which can be found circulating in the blood and are more easily obtained in larger numbers compared to mature ECs, which allows for great proliferation potential [76]. For this study, HUVECs were used in order to be able to compare to the wider body of literature to inure the new culture methods lead to similar results when compared to more established methods.

While ECs alone can begin the vascularization of constructs, it has been shown that without support, the networks will quickly begin to regress. By supporting the ECs

with other cells, researchers have greatly enhanced their vascularization potential. Several cell types have proven to be useful in supporting vessel formation, including fibroblasts [77], smooth muscle cells (SMCs) [78], and mesenchymal stem cells (MSCs)[79]. These cells assist in the formation of vasculature by stabilizing blood vessels as they form as well as by releasing angiogenic growth factors [80,81]. While several cell types have been shown to be effective, in a direct comparison MSCs were found to be more effective than fibroblasts, which lead to their use in their project [82]. Specifically, MSCs have been found to become intimately associated with the ECs to support the formation of tubular structures, and cause ECs to upregulate extracellular proteases, specifically membrane-type matrix metalloproteinases [83,84].

1.3.3 Scaffolds

The most common types of hydrogels used to support the development of ECs into vascular networks are the polypeptides fibrin and collagen [47,75,85,86]. Fibrin is especially promising because of its role in wound healing, which requires it to support the formation and repair of vasculature networks during the formation of new tissue. While no other hydrogel has been achieved the same success, there have been some promising results using other natural and hybrid gels. For example, collagen mixed with PEG has shown the development of defined hollow lumens [87], and fibrin ribbons encapsulated in PEG gels also assisted in the formation of vasculature[88]. Gelatin, or denatured collagen, that has been methylated to assist with cross-linking was also used to create a porous structure able to support vessel formation [89]. While there are advantages to modifying

fibrin and collagen or using a hybrid gel, these methods have not proven to be as effective, and since the modifications were not needed, pure fibrin gels were used for prevascularization work here.

1.3.4 Current Research

There are three main methods people currently use to attempt to form vasculature networks in gels. The first involves the creation of a porous scaffold and seeding cells to line the pores which can then form tube structures that are easily perfused [90]. But this method has significant limitations in both the complexity of the network and the size of capillaries formed, as it very difficult to effectively seed cells into small channels without causing blockages. The second method is the creation of vascular outgrowths from cells seeded on a surface. This has been attempted by seeding cells on the surface of the gel and allowing them to grow into the gel [91], or by seeding cells on the outer surface of microbeads, which are then encapsulated inside the gel and can form vascular outgrowths extending from the microbeads [92]. These methods form better vasculature in response to VEGF gradients in addition to requiring support cells. The third method of creating vascular networks seeds the cells throughout the gel and allows the spreading and connection of cells to form a vascular network, and that is the method used for experiments here. There has been a significant amount of research explaining how these networks form *in vitro* with HUVECs and MSCs by Andrew Putnam's group, especially in the area of how support cells assist in the network formation through upregulation of genes in EC's to produce membrane-type matrix metalloproteinases for degradation of

the extracellular matrix [83,84,93]. Usefulness of this method has also been demonstrated *in vivo*, showing the ability of the preformed vascular networks to anastomize once implanted, which greatly decreased the time required for blood to be found circulating within the implanted constructs[85]. The Tranquillo lab has show important advances in creation of aligned constructs with high lumen densities with the assistance of interstitial flow, which more closely mimics natural vasculature [47,94,95]. Significant progress has been made in creating tissue engineered constructs containing a vascular network in order to increase cell viability.

1.4 Perfusion Culture

1.4.1 Bioreactor Design

While a vascular network is important *in vivo*, it is unable to supply nutrients *in vitro* without circulation, and when cells are initially seeded, there is no structure to support convective flow inside the gel. Cells must be supplied with oxygen and nutrients through other methods until the vasculature can develop. In order to create larger constructs where this is possible, researchers have turned to perfusion of porous systems. Instead of relying on diffusion through media and then into the gel, these gels are placed in a system with either intermittent or continuous media flow. This convective flow can act in a similar manner as blood does *in vivo*, making the limiting factor in oxygen delivery the diffusion inside the gel. And with the use of sufficiently porous structures, the liquid is able to flow inside the construct, allowing for the creation of constructs on the centimeter scale that allows all cells to be within a few hundred microns of the

perfusing media, greatly enhancing the cell viability. One example of the advantages of a perfusable system used small collagen gels seeded with cells. Each gel was only a few hundred microns in size, but many of these gels were assembled in a larger tube. Media or whole blood was perfused through this tube, and cell viability remained high throughout all of the gels [96]. Perfusion of stem cells seeded in gels resulted a 50% increase in cells per bead when compared to batch culture, and had the additional benefit of having more consistent nutrient and gene marker levels by controlling nutrient concentrations in the perfusing media[97]. Another example used mammalian cell spheroids and monitored cell densities. In batch culture the cell densities remained unchanged compared to static culture and had wide variations in glucose concentrations, while perfusion cultures showed significant cell density increases and an ability to regulate nutrient levels [98]. Perfusion culture can not only increase cell viability by lowering diffusion distance of nutrients, it also can provide closer control over nutrient levels in order to allow for closer mimicry of *in vivo* environments.

1.4.2 Porous Scaffolds

One common method of creating a 3D perfusable system is to use a porous scaffold. Scaffolds are created from a porous matrix and seeded with cells in order to form a 3D construct. They have been commonly created out of synthetic polymers such as polyglycolic acid (PGA) and polylactic acid (PLLA) because degradation products can be removed by natural metabolic pathways [99,100]. Many natural polymers of proteins and

polysaccharides have also been used, but the most common is collagen, with its many benefits for cell culture.

When making these scaffolds, the most important aspect is the creation of a continuous and complete pore structure so that liquid can perfuse the system. Most initial scaffolds were created by the separation of phases or materials, such as freeze drying collagen to create pores [101], washing away salt crystals entrapped in a matrix[102], or dissolving one of two interwoven polymers [103]. More recently, in order to better control the size and shape of pores in the scaffolds, researchers have been using computational topology design (CTD) and solid free-form fabrication (SFF) methods [104]. With this, the desired matrixes can be designed on the computer and the replicated in lab. But even with this better control, there still remain significant issues with the use of scaffolds. The first is cell uniformity – it is difficult to get even cell distribution throughout a preformed porous construct, and this problem is exacerbated with smaller channels [105]. This method also will only seed cells on the surface of the scaffold. Cells seeded on the surface are exposed to liquid flow through the pores, which can remove secreted cell factors that are crucial for cell development [106]. It can also subject the cells to shear forces, and while in some cases these shear forces can assist in cell developments, other cell types, such as cardiac cells, can suffer significant damage from fluid flow [107].

1.4.3 Modular Assembly

In order to avoid the issues found in scaffolds, while still reaping the advantages of a perfusion culture, we look to the modular assembly of gels. With this method, cells

are seeded inside small gels, and the individual gels are connected to form a single larger construct while still maintaining gaps to form a porous structure that can facilitate the perfusion of media. There have been various methods used to assemble the individual gel modules. One method uses poly(ethylene glycol) diacrylate (PEGDA) to entrap cells and individual modules were gelled with UV light. The individual gels can be assembled in mineral oil due to their hydrophobic nature and then additional UV expose allows unconsumed acryl groups on the gel surfaces to react, forming a larger stable construct[96]. But methods that rely on UV cross-linking are limited by the depth of penetration of UV light, as cells seeded within the gels act as light scatters. And while longer or higher power exposure to UV light can increase the penetration depth, it can also cause damage to encapsulated cells. PEGDA gels have been assembled without the restriction of UV light penetration depth by modifying the gels to allow for a different cross-linking method. In this method, individual PEGDA gels were reacted with a specifically designed protein AC10cys. Multiple proteins were able to react with each gel through the Michael-type addition of unreacted acrylate groups on PEGDA with the thiol on the cysteine residue of the protein. The A group of the protein was able to physically associate to form tetramers at physiological conditions, which allowed assembly of the gels [108]. While this method does not depend on UV light, it requires modification of gels with encapsulated cells, which leads to the possibility of culture conditions that may cause cell damage or inhibit cell development. Also, physical association can be vulnerable to changes in culture conditions that can interrupt the physical connections and lead to dissociation of modules. Additionally, both of these methods use PEGDA, which is not

very good at supporting vascular formation, even in hybrid gels containing collagen or fibrin. An example of a method with a material that does support vascularization uses collagen fibers to connect gel modules. A preformed collagen gel can be connected to other hybrid collagen mixtures through to the nucleation and growth of collagen fibers across the boundary [109]. But while this method can be useful for connecting gels of different compositions, it is unable to connect multiple preformed gels, limiting the number of smaller modules that can be easily assembled into a single construct.

1.4.4 Current Research

Tissue engineering constructs require a constant supply of oxygen and nutrients both *in vitro* and *in vivo*. This can be accomplished through the creation of vasculature to assist *in vivo*, while perfusing the system while *in vitro*. One example of attempts to combine these methods was used to create a full thickness skin graft [110]. While there was no perfusion through the interior of the construct, it was used around the exterior to control liquid height and nutrient concentrations in a bioreactor. It was effective at forming vasculature, but high liquid flow rates did impede the formation of vasculature inside the gel. A different method of prevascularization was able to use artery and vein explants to form a capillary network in a PDMS mold. The capillaries extended from the artery down channels in the PDMS and connected to the explanted vein, and this network was able to assist in the survival of cardiac cells seeded around the network [111]. While the initial vascularization in this case is on a small scale, it is possible constructs could be brought together on a larger scale after capillary formation. Analysis of oxygen diffusion,

cell viability, and cell differentiation of MSC cells has been analyzed using a modular perfusion system, and it showed much higher oxygen concentrations and cell viability inside gels when perfused compared to static culture[112]. Another lab has also created a bioreactor for the study of angiogenesis, combining several larger gel modules for perfusion culture, and specifically looked at different circulating conditions and the effect on cell development [113]. While there are several examples of the importance of perfusion culture and prevascularization, so far there has not been much progress in creating larger constructs using a combination of vascularization and perfusion.[110]

Chapter 2: Biological Assembly of Tissue Modules through Capillary Network Formation

2.1 Introduction

Creating functional tissue constructs is a primary goal of tissue engineering, as they can be used to replace lost human tissue, create better methods for drug testing, and serve as models for research into how human systems work [114,115]. Natural tissues have complex, spatially defined structures that are difficult to mimic *in vitro*. In order to more accurately recapitulate these *in vivo* cell microenvironments, researchers have started using 3D tissue culture as opposed to the traditional 2D cell culture [116,117]. In order to form these larger constructs, approaches using bottom-up methods to integrate smaller gels into a single construct have been attempted. There have been a variety of methods investigated, including photocrosslinking Poly(ethylene glycol) diacrylate (PEGDA) gels under UV light or physically assembling them with oligonucleotides or proteins attached to PEGDA gels [108,118,119]. Another method uses collagen fibers in a preformed gel as nucleation sites to integrate gels [109]. But these methods all have limits – UV light only allows for cross-linking up to a certain depth due to cells scattering light; surface functionalization with other molecules can be complex and may result in non-optimal culture conditions during the modification; collagen fiber assembly cannot be used to link multiple preformed gels.

Another important part of creating tissue engineering constructs is their viability once implanted *in vivo*. One way to assist in oxygen and nutrient transfer post implantation is to pre-form a vascular network from Human Umbilical Vein Endothelial

Cells (HUVECs) and Mesenchymal Stem Cells (MSCs) *in vitro* [79,120]. Once implanted, this pre-formed network can rapidly anastomize with the host vasculature, greatly decreasing the time it takes for blood to be found circulating inside the construct, which increases the overall viability cells within the construct [85]. Without this pre-formed network, it can take weeks to form even a millimeter of vascular ingrowth in a construct from surrounding tissue [72]. And as many types of tissue require blood flow within several hundred microns to maintain viable cells, this severely limits the size of constructs that can be made using cells with high oxygen demands.

Here we have developed an approach to biologically assemble individual gel modules into a single construct through the formation of an endothelial vasculature network both within and between gels. We observed that gels seeded with HUVECs and MSCs can form a vascular network that can bridge the interface between two adjacent gels, while gels seeded with only one of the two cell types or no cells easily fall apart after the same amount of culture time. This provides a simple method of modular assembly for preformed gels that does not interfere with cell growth and development.

2.2 Methods

2.2.1 Cell Culture

GFP-HUVECs (EssenBioscience) were maintained in EGM-2 media (Lonza) and MSCs (Texas A&M HSC) were maintained in αMEM media (Sigma) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological) and 1% Penicillin/streptomycin (Thermo Scientific). Cells were cultured in 37˚C incubator with 5% CO_2. Medium was

changed for GFP-HUVECs every 2 days and MSCs every 3 days. Passage 5 GFP-HUVECs and Passage 4-5 MSCs were used in experiments

2.2.2 PDMS Mold Formation

1.5% w/v agarose (Invitrogen) gel was sliced into rectangular blocks about 2 mm wide, 2 mm deep, and 3 cm long and placed in the bottom of an agarose coated 12-well plate. Silguard 184 elastomer base and crosslinker (PDMS) (Ellsworth Adhesives) were mixed at a 9:1 ratio for 2 minutes and then poured over the agarose forms to make channels. The PDMS solution is placed under vacuum for 40 minutes to remove air bubbles and then allowed to gel overnight at 37˚C. The agarose is removed and the solid PDMS molds are heated to 90˚C for 24 hours to finish cross-linking. Before they are used the molds are suspended in 5% w/v bovine serum albumin (BSA) (Fischer Scientific) for 2 hours and then rinsed with phosphate buffered saline (PBS). The treated molds were placed in the bottom of a tissue culture plate and then dividers cut from a whole piece of PDMS, prepared in a similar manner to the molds, are inserted into the channels in order to create either 2 or 6 chambers within the channel.

2.2.3 Gel Preparation and Culture

For 2 chamber experiments, fibrin gels are prepared by making a solution of 200,000 GFP-HUVECs/mL, 80,000 MSCs/mL, and 7.5 mg/mL fibrinogen (Sigma) in EGM-2. Some gels were prepared with only GFP-HUVECs or only MSCs as controls, and 50 μL of 25 U/mL thrombin (Sigma) was added per mL of fibrin gel to all samples.

This solution was mixed and then pipetted into the left chamber of the mold. A fibrin gel without cells and with 1% of the fibrinogen replaced with Alexa Fluor 546 Conjugate fibrinogen (Invitrogen) in order to visualize gel boundaries was mixed in a similar manner and pipetted into the right chamber of the PDMS molds. Te gels were incubated for 5 minutes at room temperature and then 20 minutes in the incubator at 37˚C to allow the solutions to completely gel. Following this incubation, the PDMS divider was removed and the gels were pushed together, with the PDMS divider reinserted into the end of the channel to insure gels remained adjacent in culture. EGM-2 medium was added to cover the molds and gels, and the gels were cultured in the incubator for 8 days, with media being changed every 2 days. Fibrin gels were prepared in a similar manner for the 6 chamber PDMS channels, except all gel solutions contained cells, and every other chamber replaced 10% of the normal fibrinogen with Alexa Fluor 546 Conjugate fibrinogen instead of 1%.

2.2.4 Gel Staining and Imaging

From the 2 chamber experiments, samples containing only MSC's were incubated with calcetin AM (Invitrogen) and ethidium homodimer 1(Invitrogen) for 2 hours, and then rinsed three times with PBS and incubated for 1 hour in fresh media, changing media at the half hour and manually shaking the dish every 10 minutes before imaging. Samples with GFP-HUVECs were imaged directly. Gels were either imaged with a ×10 objective (N-Achroplan 0.25 NA) on a Zeiss Axiovert Observer inverted microscope equipped with an ApoTome (which allows 3D fluorescence imaging) or on a Zeiss LSM7

LIVE laser-scanning confocal microscope with a 40× Plan-Apochromat 0.95 NA objective.

2.2.5 Gel Mechanical Testing

From the 6 chamber experiments, fibrin gels were removed from the PDMS molds and were tested for cohesion first in liquid and then by suspension in air. Gels that remained intact were then manually stretched with tweezers to test extension length before breaking into multiple pieces. Tensile tests were performed for constructs that could be effectively removed from the molds in a PBS bath on an Instron-Sacks Planar Biaxial Soft Tissue Testing System equipped with a 5 N load cell. The samples were stretched at a rate of 50 μm/s until the gel completely separated into 2 pieces.

2.3 Results

2.3.1 Biological Assembly of Fibrin Gel Modules

The ability of cells to migrate and develop across the boundary between two adjacent gels was tested using GFP-HUVECs, which have previously been shown to be effective at forming vascular networks at concentrations of fibrin from 2.5 mg/mL to 10mg/mL with the support of MSCs. One fibrin solution was seeded with cells and gelled in one chamber of the PDMS mold, while another gel was formed containing 1% red fluorescent fibrinogen and no cells, and the two solutions were not allowed to come in contact until they were completely gelled (Figure 2.5.1, A). This allowed for the visualization of the boundary showing single cells are initially seeded in the left non-

fluorescent gel, while the fluorescent gel is initially cell free (Figure 2.5.1, B). After 8 days of culture in the mold, the GFP-HUVECs, with support of MSCs, have formed large tubule-like structures throughout the gel initially seeded with cells. These structures also crossed the original boundary between the two gels, and continue to migrate and form tubule-like structures well into the initially cell-free gel (Figure 2.5.1, C). It was also seen that the two gels became connected, and were able to be removed from the mold as a single piece.

2.3.2 Endothelial Network Formation between Gel Modules

Higher magnification images revealed the endothelial capillary networks present in the connected gels contained tubule-like structures having hollow spaces. These structures were present in both gels and could even be seen crossing the boundary between the fluorescent and non-fluorescent gels (Figure 2.5.1, D). This was significantly different from gels seeded with only one cell type or no cells at all. Gels seeded with GFP-HUVECs alone had very different structures from the cultures also containing MSCs, as most cells remained rounded single cells and there was almost no tube-like structure formation (Figure 2.5.1, E). While single MSCs are able to form longer extensions, they do not form multi-cell tubule structures and do not contain hollow spaces – they are unable to pre-vascularize a construct without GFP-HUVECs (Figure 2.5.1, F) In both cases, very few cells migrated across the boundary, and the gels were not firmly connected as was seen in the samples containing both cell types.

2.3.3 Mechanical Properties of Assembled Constructs

While these experiments show the ability of cells to cross between and connect fibrin gels, these connections were tested further to examine the strength of the assembly. To make sure that several gel modules could come together to form a single construct without additional cross-linking, smaller gels, all of which were seeded with either HUVECs, MSCs, or both cell types were formed in 6 chamber PDMS molds. Every other gel of the six contained 10% red fluorescent fibrin so there would be a visible color difference between gels after the gels had formed and been pushed adjacent (Figure 2.5.2, A). After 8 days of culture, constructs containing both cell types had formed tubule-like networks crossing gel boundaries, in the same manner seen in the 2 chamber gels. The entire construct was then completely removed from the well, and it remained a single piece when held aloft using a forceps (Figure 2.5.2, B). This was not the case for gels prepared with only GFP-HUVECs (Figure 2.5.2, C), only MSCs (Figure 2.5.2, D), or no cells, which when removed from wells split into smaller sections. To further test the strength of assembled constructs containing GFP-HUVECs and MSCs, they were stretched with tweezers by pulling one end of the gel while the other was held in place. The gels containing both cell types would elongate over 20% without breaking into multiple pieces, while gels with only one cell type would easily separate into individual modules (Figure 2.5.2, E). To test this more precisely, gels with both cell types were uniaxially stretched at a rate of 50 μm/s. The stress–strain relationship reveals an average strain to failure of 117 ± 26%, tensile strength of 2208 ± 83 Pa and Young's modulus of

2548 ± 574 Pa based on three samples (Figure 2.5.2, F). Gels with only one type were unable to even be loaded into the testing system without breaking.

2.4 Discussion

Vascularization is critical to the survival of cells in most tissue engineering applications. While initial methods relied on ingrowth of blood vessels from the host tissue, this process is relatively slow and can lead to low cell viability in the implanted construct because without circulation through blood vessels, oxygen and nutrient transport to cells in the construct is limited by diffusion from the blood vessels in the host tissue[121]. But newer methods have greatly increased the speed at which blood begins to circulate inside constructs by using endothelial cells in coculture with support cells to form vasculature inside a construct before implantation. The vasculature can anastomize with the host vasculature, allowing for much faster perfusion of the construct by the hosts blood supply. But when forming larger constructs, adding cells that can form vasculature to a gel is insufficient. The limitations of oxygen diffusion will still cause the death of cells at the center of the construct during the *in vitro* culture, only allowing for the formation of vasculature around the exterior of the construct. This means that statically cultured constructs must be small enough to not exceed the limitation of this oxygen diffusion or else have low enough oxygen demand within the construct to not cause significant cell death.

In order to make larger tissue engineering constructs, we designed a method to modularly assemble smaller gels with the ability to support vasculature. Using fibrin

seeded with both HUVECs and MSCs, which others have shown to support the formation of vasculature, we were able to create gel modules that form a vascular network not only within the modules initially seeded with the cells, but also in adjacent modules that were initially cell-free. These vascular structures assisted in the assembly of the modules into a single construct, as shown by the inability of gels containing only one cell type to remain a single construct when moved. While there was some connection formed between the gels containing only a single cell type, likely caused by the migration of the HUVECs or MSCs into adjacent gels, only the gels with both cell types resulted in the formation of tubule-like structures and strong connections, indicating that the vascularization process is the reason for the strong connection between individual gel modules. The formation of vasculature also has the secondary benefit of assisting in anastomosis and therefore cell viability post implantation. The formation of an endothelial network that connects the gel modules holds even more promise here, as an endothelial network that extends to connect an entire construct is likely to be more effective for anastomosis than forming smaller networks inside the individual gel modules.

Also important in assembled constructs is the strength of the assembly. Here we found a strain to failure of 117 ± 26%, a tensile strength of 2208 ± 83 Pa and a Young's modulus of 2548 ± 574 Pa, which are comparable to pure fibrin gels [122]. While the ultimate tensile strain was lower than values found for whole fibrin gels, the strength of the connection was similar, indicating that the modularly assembled gel constructs could be used in similar applications as a single large construct while maintaining the useful properties of modular assembly.

This method shows great promise for the modular assembly of even larger constructs. This modular assembly is advantageous for assembling smaller gels into a larger, porous construct instead of a non-porous gel. Connections between individual modules can be controlled to have boundaries that remain unattached to neighboring gels, which allows for the formation of gaps betweens gels that create a porous construct. Perfusion of the construct in combination with high porosity allows for a flow of media through the interior of the construct, which in turn will allow nutrients to be supplied by convection even in the center of larger porous gels, which means nutrients need only diffuse from the perfused media into the small gel, not from the exterior of the entire construct. This allows cells throughout the gel to remain viable and able to form endothelial networks throughout constructs on a much larger scale. If nutrient delivery still remains an issue because pore size is not large enough to accommodate flow, the size and number of the pores can be adjusted by using different shapes and sizes of fibrin gels. This biological assembly method also can be tested using alternate scaffold types, such as collagen or hybrid gels.

Another advantage of creating a large gel through the biological assembly of gel modules is the ability to more carefully design the larger construct. Instead of making a single large gel with the same types and concentrations of cells, small gels can be seeded with different cell types or numbers. They can then be arrayed in desired patterns or layers, depending on what is required for individual applications. While here fibrin gels of different colors were assembled, they could contain different cells types or different immobilized signaling molecules to allow for the creation of a heterogeneous construct.

The ability to create larger prevascularized networks that not only cross the boundary between distinct gels, but also allow those gels to form a single cohesive construct is a significant step towards being able to create larger and more diverse constructs for tissue engineering applications.

2.5 Figures

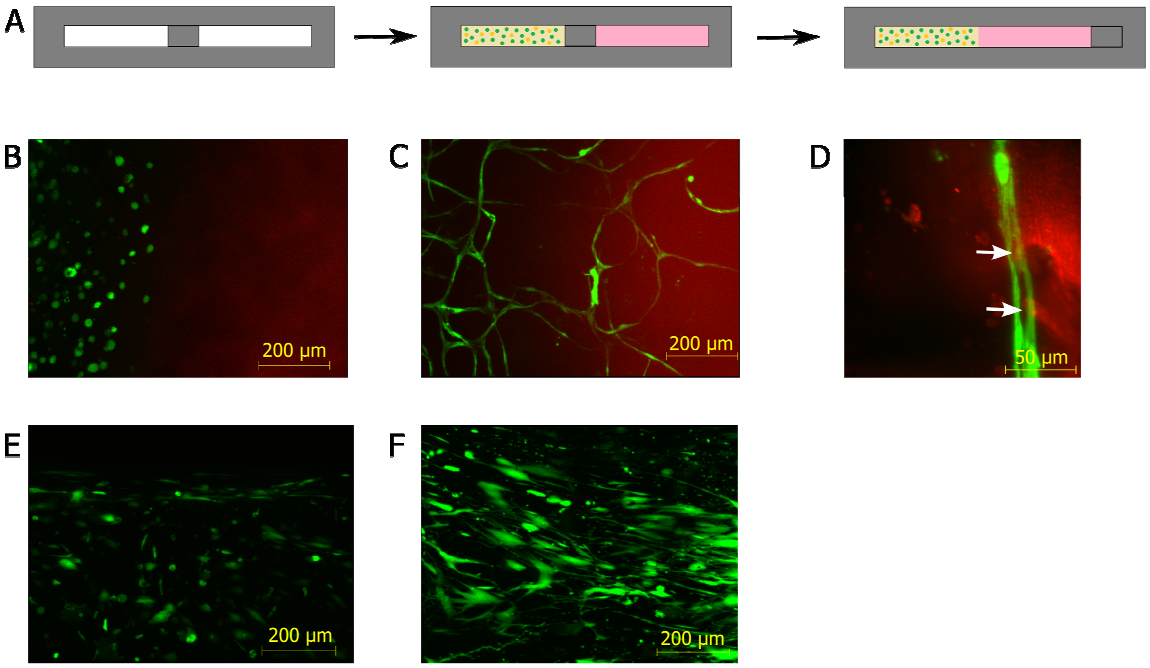


Figure 2.5.1: Endothelial network formation. (A) Fibrin gels were prepared in chambers and then pushed adjacent. (B) GFP–HUVECs and MSCs were seeded only in the left gel while the right cell-free gel contained red fluorescent fibrin. (C) After 8 days of culture, endothelial capillary networks formed and grew across the gel boundary between gels, assembling them into a single piece. (D) The networks had lumen-like structures containing hollow spaces (arrows) surrounded by endothelial cells in both gels and crossing the boundary between them. (E,F) When only GFP–HUVECs (E) or MSCs (F) were seeded in the gel, no capillary network was able to form after 8 days of culture.

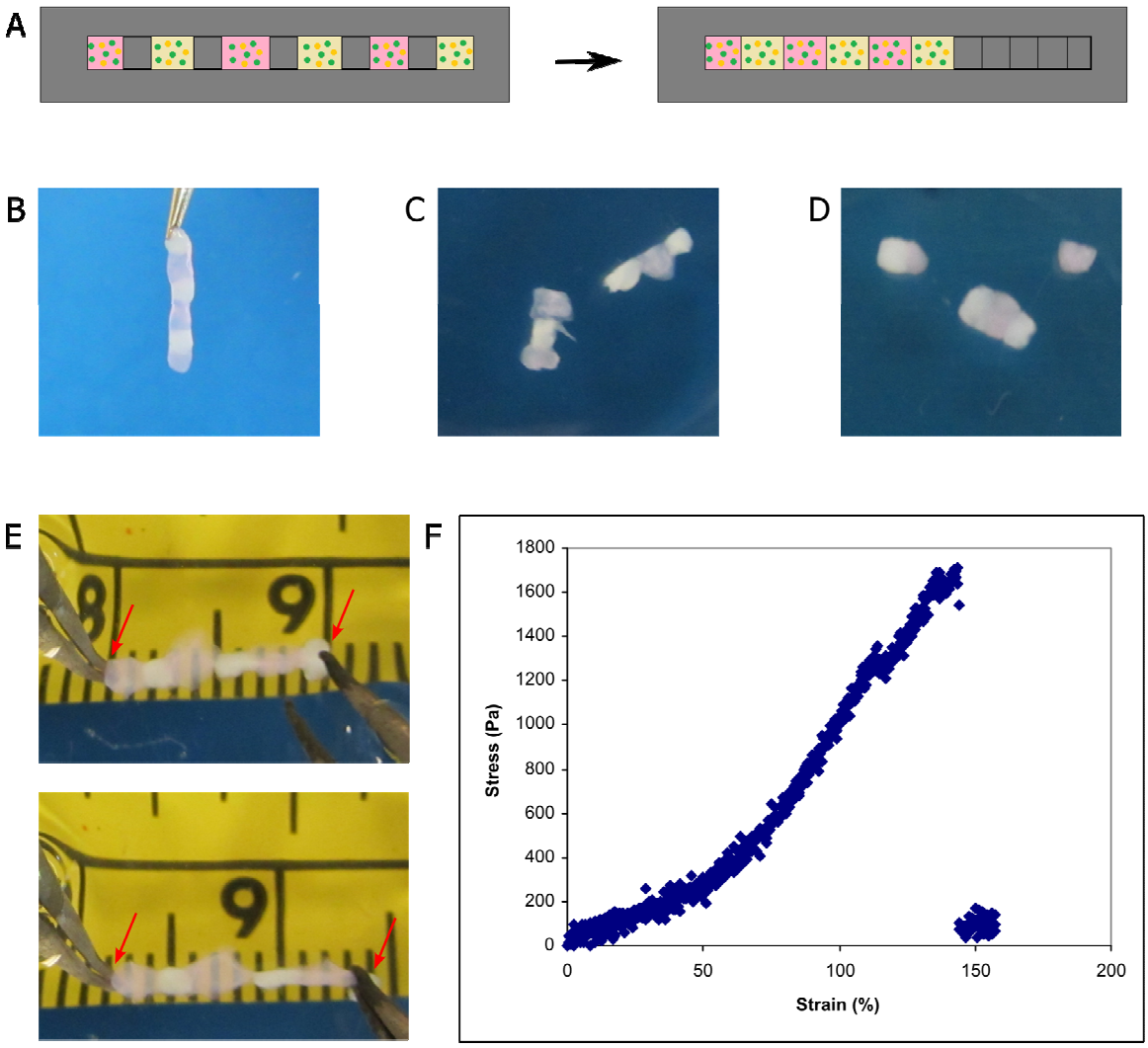


Figure 2.5.2: Robust assembly through endothelial capillary network formation. (A) Six fibrin gels containing cells with alternating gels containing fluorescent fibrin were gelled in individual chambers before being placed adjacent to each other. (B) Gels with both GFP–HUVECs and MSCs, which can form endothelial capillary networks, were firmly assembled into a single piece after 8 days of culture and could be suspended in air. (C, D) Gels containing only GFP–HUVECs (C) or MSCs (D) would fall apart even when handled carefully. (E) Constructs integrated from the gels containing both GFP–HUVECs and MSCs could be stretched by hand to over 20% longer than the initial length. (F)

Mechanical testing showed the stress–strain relationship for an integrated construct composed of gels containing both GFP–HUVECs and MSCs; the strain to failure was 117 ± 26%, based on measurements of three samples.

Chapter 3: Perfusion Culture of Porous Tissue Engineering Constructs

3.1 Introduction

Creating functional tissue engineering products for the replacement of natural human tissue is an important goal of tissue engineering[1,115,123], but the number of commercial applications for tissue constructs so far has been mostly limited to thinner tissues or tissues with lower metabolic demands, such as skin and cartilage[124]. This is because creating most functional, clinically relevant tissues requires the creation of larger, 3D constructs, and a large obstacle to forming these constructs is the limited mass transfer of oxygen and nutrients [124,125]. The concentration of oxygen and nutrients are critical for cell survival and development, but their transport in large constructs is limited to diffusion from the exterior construct surfaces [126]. In order to overcome this barrier *in vitro*, 3D constructs containing pores allow for the perfusion of liquid through the interior of the construct, reducing the diffusion distance from the media to cells [127]. One common method of forming these constructs *in vitro* is to seed cells onto preformed porous scaffolds [107,126,128]. But this method leads to non-uniform cell distribution throughout the scaffold that can inhibit cell development, and the seeded cells become directly exposed to the perfusion media with associated shear stresses that may damage cells and can remove important cell secreted factors.

These drawbacks can be eliminated through modular assembly of smaller hydrogel constructs seeded with cells packed into a bioreactor while leaving pores to facilitate perfusion with media [96]. The gel modules can be uniformly seeded with cells, and the gel itself can protect the cells from shear stresses caused by the liquid flow. The

modular assembly of smaller gels into a single construct has been attempted using several methods including UV cross-linking and the physical assembly of gels modified with complementary proteins, but these methods come with limitations [108,129,130]. UV light penetration into gels is limited in depth because cells scatter the light, and modifications of gels can create environments that inhibit cell growth.

Also, in order to increase the viability of cells once implanted, it is preferable to form a vascular network within the construct so that the *in vivo* diffusion barrier can be more quickly eliminated by anastomosis of the pre-formed vascular network with the host vasculature [85]. So far the most effective gels for forming this network have been natural gels such as fibrin and collagen, and while there is progress in hybrid gels of PEG with fibrin or collagen, they are not as effective [87,88], so a modular assembly method with fibrin or collagen would be desirable.

We report a method to biologically assemble a large, porous, 3D, prevascularized fibrin gel construct. Fibrin gel modules seeded with Human Umbilical Vein Endothelial Cells (HUVECs) and Mesenchymal Stem Cells (MSCs) have shown the ability to form endothelial networks between individual gel modules, connecting them into a single prevascularized construct[131]. Here we use this method to create a larger gel construct by rationally designing small modules that can biologically assemble to form a single porous construct. By perfusing this construct in a controlled bioreactor, cells can be provided with oxygen and nutrients through convective flow inside the construct instead of relying on diffusion through the gel alone. In this way the entire construct can support higher

viable cell densities and form a vascular network *in vitro*, which in turn could greatly increase cell survival for constructs post implantation.

3.2 Materials and Methods

3.2.1 Cell Culture

HUVECs (Lonza) and GFP-HUVECs (EssenBioscience) were maintained in EGM-2 media (Lonza). MSCs (Texas A&M HSC) were maintained in αMEM media (Sigma) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological), 2mM l-glutamine (Thermo Scientific), and 1% penicillin/streptomycin (Thermo Scientific). Cells were cultured in 37˚C incubator with 5% CO_2. Medium was changed for HUVECs and GFP-HUVECs every 2 days and MSCs every 3 days. Passage 4-5 HUVECs, passage 5 GFP-HUVECs and passage 4-5 MSCs were used in experiments.

3.2.2 PDMS Mold Preparation

The desired final gel shape was initially created using 20% w/v 3.4 kDa poly(ethylene glycol) diacrylate (PEGDA) (Laysan-Bio) with 1% v/v of a 1.2M solution of 2,2-Dimethoxy-2-phenyl-acetophenone (Sigma) in 1-vinyl-2-pyrrolidinone (Sigma) as a photoinitiator. The PEGDA was photocrosslinked between glass slides separated by a 1 mm glass spacer in an ELC4001 Light curing unit (Electro-Lite Corporation) for 55s under a photomask designed using Autocad containing cross shapes with a leg length of 1.8 mm and a leg width of 1 mm. The resulting cross shaped PEGDA gels were then moved into a 12 well plate coated with a thin layer of 1.2% w/v agarose and allowed to

dry for 20 min at 37˚C. Silguard 184 elastomer base and crosslinker (PDMS) (Ellsworth Adhesives) were mixed at a 9:1 weight ratio and then poured over the PEGDA gels. This was placed under vacuum for 40 minutes to remove air bubbles and then allowed to gel overnight at 37˚C. The agarose and PEGDA were removed and the PDMS molds were heat treated at 140˚C for 5 days. These PDMS molds were then used as masters to create additional PDMS molds by filling them with excess 1.2% w/v agarose and covering with a glass coverslip before cooling at -20˚C for 1 min. Once the cross shaped agarose gels had solidified, they were moved to an agarose coated 12 well plate and used to create additional PDMS molds in the same manner as the original masters (Figure 3.5.1, A).

3.2.3 Fibrin Gel Preparation

The molds for fibrin gels were prepared by autoclaving the PDMS and then treating with plasma in a PDC-32G plasma cleaner for 5 min. They were then submerged in a 1% w/v solution of Pluronic F-127 (Sigma) in PBS for 1 hour and rinsed once with PBS before adding fibrinogen solution. The 7.5 mg/mL fibrinogen (Sigma) solution was prepared from a 30 mg/mL stock solution of fibrinogen in sodium chloride irrigation (Baxter) mixed with 2 million HUVECs (Lonza) or GFP-HUVECs/mL (Essen Bioscience) and 400,000 MSCs/mL (Texas A&M) suspended in EGM-2 media. This was mixed well before 50 μL of 25 U/mL thrombin (Sigma) in sodium chloride irrigation was added per mL fibrinogen solution. This was again mixed thoroughly before either completely filling 1 mm deep cross shape holes in PDMS molds with leg width of 1 mm and length of 1.8 mm (Figure 3.5.1, B) or pipetting 2.5 mL into a 3 mL syringe to make a larger non-

porous gel. The fibrin was allowed to gel for 5 minutes at room temp and then for 20 minutes in the incubator. The gels were removed from PDMS molds using a tweezers and 100 μL of gels were placed into a 12-well plate with 1 mL of EGM-2 media, which was changed every 2 days. Gel modules were statically cultured for 8 days and imaged with a 2.5× objective (EC Plan-Neofluar 0.075 NA) on a Zeiss Axiovert Observer inverted microscope, while gels for perfusion culture were statically cultured in plates for the first 3 days before being moved to a bioreactor.

3.2.4 Bioreactor Preparation

A media reservoir was made by drilling 3 holes into the plastic cap of a 50 mL glass bottle. The holes were covered with o-rings (5/64 ID) and held in place with adhesive (DP420, Ellsworth adhesives). PDMS plugs to seal the top of the bioreactor were created by removing the plunger from a 3 mL syringe (1180300777, Covidien) and inserting a plastic transfer pipette. PDMS was prepared as described previously and gelled around the pipette in the syringe. It was then cut into hollow rings that would form a seal between the valve and syringe at the top of the bioreactor. Most parts of the bioreactor were sterilized by autoclave before use, including the peristaltic pump tubing (1/16 ID, AY242002 Pharmed PBT), connective tubing (1/32 ID, ACF00001 Tygon), cotton balls, filter paper (09-795D, Fischer Scientific), plastic pump fittings (13-876-1, Fischer Scientific), PDMS plug, and the reservoir. Valves (WU-30600-06, Cole Palmer) were sterilized by shaking in a tissue culture hood with 70% ethanol overnight, then washed with PBS 3 times and dried under UV light in the tissue culture hood.

3.2.5 Perfusion Culture

The bioreactor was assembled by inserting a piece of cotton into 3 mL syringe and covering it with a layer of filter paper. This was pushed to the bottom of the syringe and served to hold gels in the bioreactor (Figure 3.5.2, A,C). The peristaltic pump tubing was attached to connective tubing by the pump connectors, and this was used to connect a valve on the bottom of the bioreactor to the pump and then to the reservoir containing 30 mL media (Figure 3.5.2, B,D). The bioreactor was filled with 2 mL EGM-2 media and then 2.5 mL fibrin gel statically cultured for 3 days was moved from tissue culture plates into the bioreactor, either as a single piece of gel or many small cross shaped gels. The top of the syringe was sealed with a valve inserted into the PDMS plug, and this was connected to the reservoir with tubing for circulating media through the bioreactor at a flow rate 120 μL/min using a low flow peristaltic pump (control company). The reservoir lid was not tightened to allow for venting. The whole system except the pump was placed into a 37 ˚C incubator and then connective tubing was inserted into the reservoir and hooked up to a gas tank with 95% O2/5% CO2 (Fischer) which was slowly bubbled into the reservoir to insure sufficient oxygen was present in the media inside the reservoir. Medium was changed in the reservoir every 2 days for at least an additional 5 days of perfusion culture.

3.2.6 Staining and Imaging of Assembled Construct

Constructs were removed from the bioreactor and sliced about 3 mm from the top of the approximately 7 mm construct using a Vibrotome 3000. Samples containing HUVEC and

MSC's were incubated with calcetin AM (Invitrogen) and ethidium homodimer 1 (Invitrogen) for 2 hours, and then rinsed three times with PBS and incubated for 1 hour in fresh media, changing media at the half hour and manually shaking the dish every 10 minutes before imaging. These stained samples or unstained samples with GFP-HUVECs were imaged on a Zeiss LSM7 LIVE laser-scanning confocal microscope with the 5× EC Plan-Neofluar 0.16 NA and 40× Plan-Apochromat 0.95 NA objectives.

3.2.7 Mechanical Testing

Tensile tests were performed for constructs in a PBS bath loaded into an Instron-Sacks Planar Biaxial Soft Tissue Testing System equipped with 5 N load cells. The samples were prestressed by stretching then contracting the constructs 6 times for 10 seconds at a rate of 55 μm/s. Samples were then stretched at a rate of 50 μm/s until they separated into two pieces. Strain at failure was recorded as the point at which the first large drop in stress without recovery occurred.

3.2.8 Construct Perfusion

To test perfusion of the porous constructs, they were prepared as previously described, except 1% of the fibrin was replaced with Alexa Fluor 546 Conjugate fibrinogen (Invitrogen). After gels had been cultured for 8 days (3 static and 5 perfusion), the medium was replaced with 20% w/v Bovine Serum Albumin (BSA) with 1% v/v 0.5 um, yellow-green fluorescent carboxylate-modified Fluospheres (Invitrogen). This suspension was circulated through the perfusion system for 5 minutes at room

temperature and then 200 mM TCEP was combined with the BSA suspension at a 1:2 ratio in order to break disulfide bonds in BSA. After 10 minutes of additional circulation, the fibrin construct in BSA suspension was moved to a 37 ˚C incubator to allow the BSA to gel. After the BSA had gelled, the construct was removed from the bioreactor and placed into a solution of 10% neutral buffered formalin (Sigma) for 5 days before being sectioned on a Leica CM 1900 cryosectioner and then imaged on the confocal microscope.

3.2.9 Viability Assay

Three sets of assembled and non-porous 2.5 mL fibrin constructs cultured under the same conditions were removed from perfusion culture and sliced into sections less than 1 mm thick by hand and then each slice was further cut into quarters. Assembled construct pieces were evenly distributed between 6 wells of a 6-well plate, while non-porous construct pieces were evenly distributed between 4 wells, with 2 mL media added to each well. A standard curve from cells seeded on a plate and two types controls (freshly prepared fibrin gels seeded with cells and cell-seeded fibrin gels statically cultured for 8 days) were used. The standard curve of 7,000 to 224,000 cells was created in triplicate using freshly seeded HUVECs and MSCs at a 5:1 ratio in a 96-well plate and incubated for 40 minutes in 120 μL media before use. Two sets of freshly prepared cell-laden fibrin gels with volumes of 50 or 120 μL were made as individual cross-shaped fibrin gel modules and as single non-porous fibrin gels (50 μL gels were cast in 96-well plates and 120 μL gels cast in 48-well plates). After the gels had formed they were moved

into 6-well plates with 12 μL of media added per μL of fibrin gel. The four types of fibrin gels were also prepared in the same way in duplicate and statically cultured in 12 well plates with 1 mL media changed every 2 days for 8 days before being moved to 6 well plates and adding media at the same ratio. For all samples, AlamarBlue reagent (Sigma) was added at 10% of the media volume. All samples were incubated for 2 hours at 37˚C with gentle shaking. 100 μL of the liquid from each sample was moved to a black-lined 96-well plate, and the florescence was read with a Synergy HT Multi-Detection Microplate Reader (Bio-Tek) with 530ex/590em filters.

3.3 Results

3.3.1 Controlled Gel Preparation

We created small, cross shaped microgels that can be assembled into a larger porous construct in a perfusion culture system. In order to decrease the packing density of these gels in the bioreactor, the gels were cast in the shape of a cross (Figure 3.5.1, A). The maximum size of gel modules was determined by finding that HUVECs and MSCs could survive and form tube-like structures could form up to 600 μm deep in a non-porous gel; therefore each gel was made to be no more than 1 mm thick to limit the longest diffusion distance from surface to center at 500 μm. Gels seeded with HUVECs and MSCs undergo significant changes in size and shape when removed from PDMS molds, so the gels were monitored in static culture for 8 days. It was found that gels in the shape of a cross made in molds with a depth of 1 mm and a leg length of 1.8 mm and width of 1 mm would best maintain their initial shape for the duration of the 8 day culture

while being easily removed (Figure 3.5.1, B), as longer legs were difficult to remove from a mold without breaking. As the thickness of each gel was less than 1 mm, the distance from the top or bottom to the center of the gel would be under 500 μm. During the first three days, in which the majority of the shrinking occurred, the distance from the vertex of the cross legs to the center of the construct from went from 800 μm to 600 μm, and the gels still maintained most of the initial cross shape over this time period (Figure 3.5.1, C-D). This shrinking continued and after an additional 5 days of static culture that distance shrank from 600 μm to 550 μm, still maintaining some of the initial cross shape (Figure 3.5.1, E).

3.3.2 Construct Assembly and Mechanical Properties

In order to limit the amount of channeling and increase porosity in the perfusion cultures, gels were maintained in static culture conditions for 3 days before moving into the bioreactor for perfusion culture. After 5 additional days in perfusion culture for a total culture time of 8 days, the gels had assembled to form a large construct that could be removed from the bioreactor as a single piece (Figure 3.5.3, A). This was only seen when the cells were viable – constructs with gels that did not contain viable cells would break into smaller pieces as it was removed from the bioreactor. In order to test this further, the constructs were uniaxially stretched, and from three samples it was found that they would reach strains of 72 ±18% before they would begin to tear apart (Figure 3.5.3, B-C). It was found that even after the construct had begun to tear (noted by a drop in force by the

force transducers), or even after complete separation into two pieces, individual gel modules still remained a part of the larger construct (Figure 3.5.3, D).

3.3.3 Cell Viability in Assembled Construct

In order to compare our construct of biologically assembled gels to a non-porous gel, 2.5 mL of fibrin gel precursor containing HUVECs and MSCs was cast either as small gels in PDMS molds or as a single gel in a 3 mL bioreactor, then cultured for 8 days – 3 days in a tissue culture dish and then perfused with EGM-2 media in a bioreactor for an additional 5 days. The samples were sliced with a vibrotome and images of cross-sections at the top and near the center of the approximately 7 mm gels were taken to visualize cells survival throughout the sample. Live/dead staining revealed that in non-porous gel constructs, cells were viable at the top and exterior of the gels cast in 3 mL syringes (Figure 3.5.4, A), but cells were only viable within a distance of about 600 μm from the outer surface where the gel was in contact with media (Figure 3.5.4, C). On the other hand, when the construct was composed of biologically assembled fibrin gel modules, cells were viable through the center of constructs with diameters and heights over 7 mm, and were similar to cells present in the exterior of the constructs (Figure 3.5.4, B,D), indicating nutrient transport throughout the assembled constructs and not just around its exterior. To verify that the cells in the center of the construct were viable and tube-like structures were developing in a similar manner to static culture, GFP-HUVECs with MSCs were used to visualize tube formation. After 10 days of culture (3 static and 7 perfusion), GFP-HUVEC cells were viable throughout the center and exterior of the gel

(Figure 3.5.5, A,D). Images acquired at higher magnification indicated that tube-like structures formed at the center of cross-sections taken from the middle of the construct (Figure 3.5.5, B). These tube-like structures appeared to be similar in size and structure to those seen at the exterior of a non-porous construct and throughout statically cultured gel modules.

We expected that this difference in tube-like structure formation was the result of an increase in porosity in the biologically assembled construct, and designed a method to show the perfusion of the construct. In order to visualize gel boundaries with the fluorescent microscope, fibrin gels were prepared with 1% of the normal fibrin replaced with fluorescent fibrin before being cultured for 3 days in static culture and 5 days in perfusion culture. The porous construct was the perfused with a solution of BSA containing green microbeads unable to penetrate the gels. TCEP was used to reduce disulfide bonds within the BSA and allowed the perfusion liquid to form gel by forming intramolecular disulfide bonds between BSA molecules. Sectioning of the resulting solid showed the presence of significant amounts of gelled BSA inside slices 3 mm below the top of the construct, indicating perfusion of the construct by liquid flowing through the bioreactor (Figure 3.5.5, C). This explains higher viability in assembled constructs, as the perfusion leads to lower diffusion distances between the media and cells than that in a non-porous gel.

We then used the AlamarBlue reagent to quantitatively compare cell viability between non-porous constructs and assembled constructs as well as statically cultured gel modules. A linear standard curve for the AlamarBlue signal was created with 7,000 to

224,000 cells incubated in 120 μL of media with an additional 10% AlamarBlue for 2 hours. The viability ratio of cells inside freshly prepared gels to 2D cultures was found to be 96 ± 2% for 50 μL single gels, 103 ± 17% for 50 μL of gel modules, 92 ± 5% for 120 μL single gels, and 93 ± 3% for 120 μL of gel modules. These results showed very similar values for cell viability when comparing freshly prepared 2D and 3D cultures. 3D gels statically cultured for 8 days resulted in consistent but significantly lower cell viability at 47 ± 6% for 50 μL single gels, 44 ± 8% for 50 μL of gel modules, 45 ± 6% for 120 μL single gels, and 40 ± 4% for 120 μL of gel modules. The cells in gels statically cultured for 8 days on average had only 44% viability compared to freshly prepared samples, which could have several causes including changes in cell metabolism of AlamarBlue as they form capillary tubules, shrinking of the gels possibly leading to exclusion of cells from the gel, and some cell death (although at 8 days few dead cells were seen during staining). Analysis of perfused constructs after 8 days of culture revealed lower cell viability, as the cells in perfused porous constructs had only 30 ± 3% viability compared to the 2D standard curve or 68% viability compared to individual gel modules statically cultured 8 days. This is likely a result of both some cell death from incomplete perfusion and cell loss as liquid flowed through the bioreactor. But the constructs made of biologically assembled gels showed a much higher viability than non-porous fibrin constructs, as non-porous constructs had only 13± 4% of the 2D standard curve or 31% of the viability of gel modules statically cultured for 8 days. Overall, the cells in non-porous constructs had less than half the cell viability of the cells in

biologically assembled constructs, indicating greatly improved oxygen and nutrient delivery in the porous construct (Figure 3.5.5, E).

3.4 Discussion

In recent years there have been many advances in the development of 3D tissue engineering constructs, but there still remain barriers to their effective implementation. One of the most important issues in this area is the viability of cells seeded in larger constructs, which is greatly influenced by availability of oxygen and nutrients. *In vivo*, convective blood flow in microvasculature reduces the required diffusion distance, but in a large, non-porous, statically cultured hydrogel the only transport process is diffusion, which is insufficient to allow for cell viability for most tissues on a scale larger than a few hundred microns [132]. The most effective method of reducing this problem for constructs to be implanted *in vivo* is to create a microvascular network inside the construct pre-implantation [87, 88,133]. It has been shown that a prevascularized construct can anastomize with host blood vessels to greatly enhance the speed at which an implanted construct can be perfused with blood from the host, which increases the survival rate of cells within the implant[85,134,135]. Increasing *in vitro* viability on the other hand has been focused on perfusion of porous constructs, with convective flow reducing the required diffusion distance and therefore increasing cell viability inside the construct [96]. By modularly assembling gels seeded with cells instead of adding cells to a preformed scaffold, cells can be uniformly seeded and are not exposed to shear stresses, which can impede cell growth and development[107,108,128]. So by combining modular assembly for *in*

vitro culture with vascularization strategies that improve *in vivo* culture, we form the basis for creating larger tissue engineering constructs with high cell viability.

We have been able to create a large construct containing an endothelial network through the biological assembly of smaller fibrin gels, a method that uses natural cell development and migration to form connections between gel modules [131]. This method of gel assembly is not limited by the depth of UV light penetration, an issue that arises in the assembly of PEGDA gels. It does not require modification of the hydrogel which can expose cells to non-optimal conditions and interfere with cell viability and vascular development. It also does not rely on synthetic hydrogels, which can make vascular network formation more difficult. But while fibrin has been shown to be effective at supporting vascular formation, it does undergo significant change in shape and size when seeded with HUVECs and MSCs [86,134]. In order to maintain pores within the final construct that allow for perfusion as the gels change shape, the gels had to be carefully designed. As we saw in non-porous constructs, capillary tubule formation is limited to 600 μm from the surface of the tissue construct, so we designed small gels that limited the distance of any encapsulated cell to media to about 550 μm. In order to maintain the porosity of the final construct with these gels, we used high concentrations of fibrin to reduce shape change while still supporting vascular development, and additionally used fibrin gels in the shape of a cross to decrease the packing density once placed in perfusion culture [108]. We also delayed assembling the gel modules until after most of the gel shrinking had occurred, which should increase the porosity of the final construct and reduce the channeling of the liquid around the outside of the construct.

After 5 days of perfusion culture the individual gel modules formed a single connected construct, with the gel modules remaining connected even when the construct was sectioned through the center for imaging or stretched uniaxially, indicating biological assembly occurred effectively throughout the entire construct. On the other hand if insufficient oxygen was supplied to the reservoir during perfusion, there was limited cell viability and the gels would not form a single construct, but instead broke into individual modules when moved, indicating it is the cells themselves that allow for the construct formation. So therefore this construct cohesion can only be explained by the perfusion of the gel, as non-porous gels with no interior media flow had cell viability limited to 600 μm from the exterior surface, while the assembled constructs had viable cells forming lumen-like structures over 3 mm from the outer surface of the construct. This perfusion was also shown by the presence of green microbeads in the center of crossections of the porous construct after only 15 minutes of circulation. As seen in the Alamar-blue assay, the viability in the porous constructs is over twice as high as the viability of the cells in a non-porous construct, indicating much better delivery of oxygen and nutrients. As the porous constructs only had 69% of the cell viability when compared to statically cultured cells, the construct may benefit from additional porosity, but this could also be explained by fluid flow removing small pieces of fibrin from the construct, which lowers the volume available for cell growth. But because of the dependence on cells to assemble the gels, as long as the construct allows sufficient delivery of oxygen for tube-like structures formation this method can be scaled to create much larger constructs.

This new method for creating porous constructs is an effective means of creating larger constructs with greatly increased cell viability. Instead of being limited to hundreds of microns, constructs with uniform cell viability can be created on centimeter and larger scale as the diffusion of oxygen and nutrients is not the limiting factor if the porosity of the construct is maintained. This method also allows for other possibilities, such as the creation of heterogonous constructs by creating unique tissue modules seeded with different cell types or signaling factors. Individual modules could even initially be allowed to assemble in static culture on a smaller scale and then later assembled into separate layers or areas inside a bioreactor when forming a final construct in order to create defined spatial heterogeneity. Biological assembly should prove to be an effective method for creating large constructs that overcome oxygen limitations currently present in both *in vitro* and *in vivo* culture.

3.5 Figures

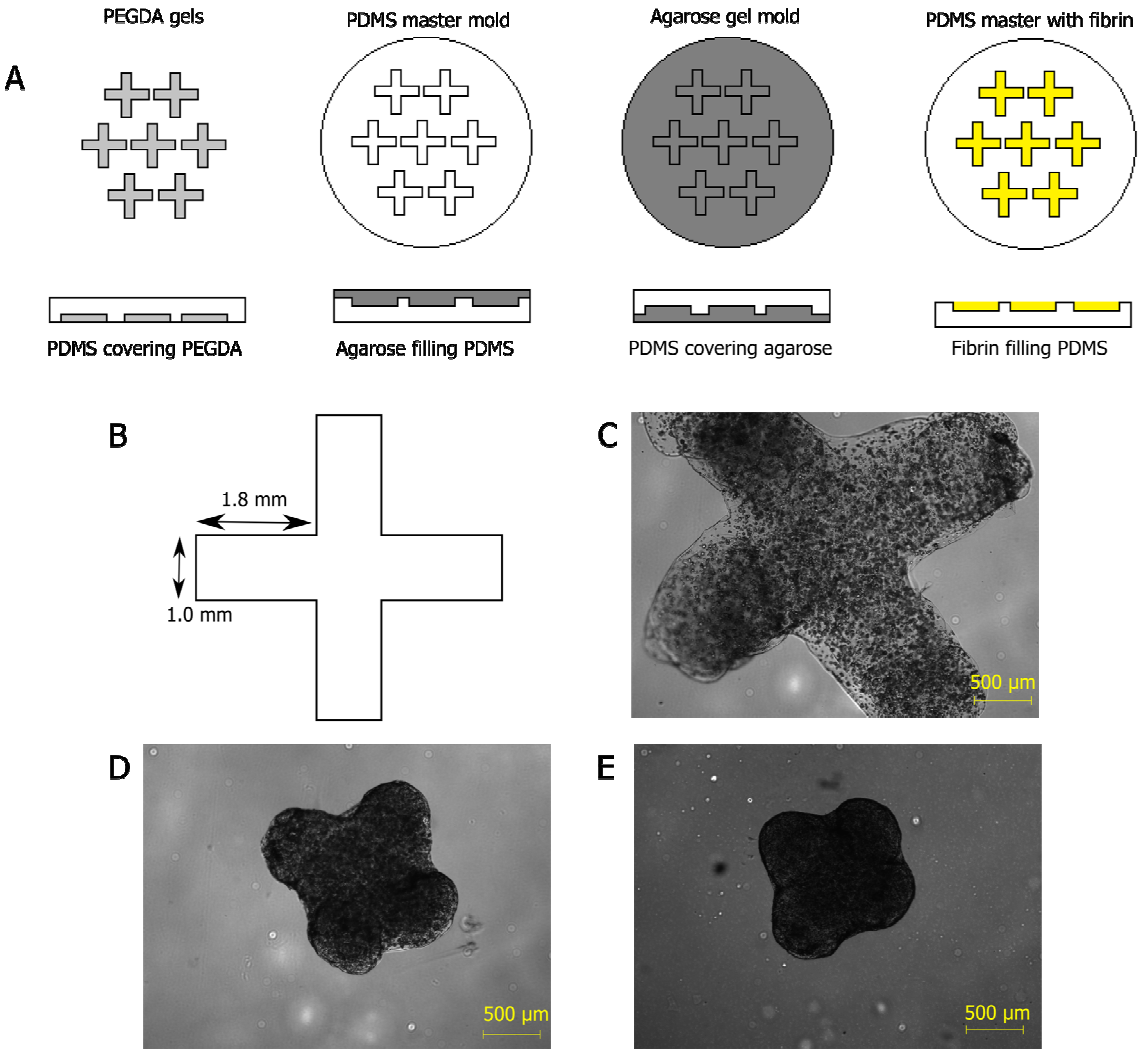


Figure 3.5.1: Preparation of shaped fibrin gels. (A) The schematic shows the process of making molds for casting fibrin gels. PEGDA crosses are used to make PDMS master molds. The original PDMS molds are then filled with agarose that can be used to more quickly make additional PDMS molds. (B) Each cross shape is 1 mm deep and has four legs, each with a length of 1.8 mm and width of 1.0 mm. (C) Fibrin gels cast in these molds are removed as a cross shape. (D,E) The gels shrink, but still maintain some of the initial shape over 3 (D) and 8 (E) days of static culture.

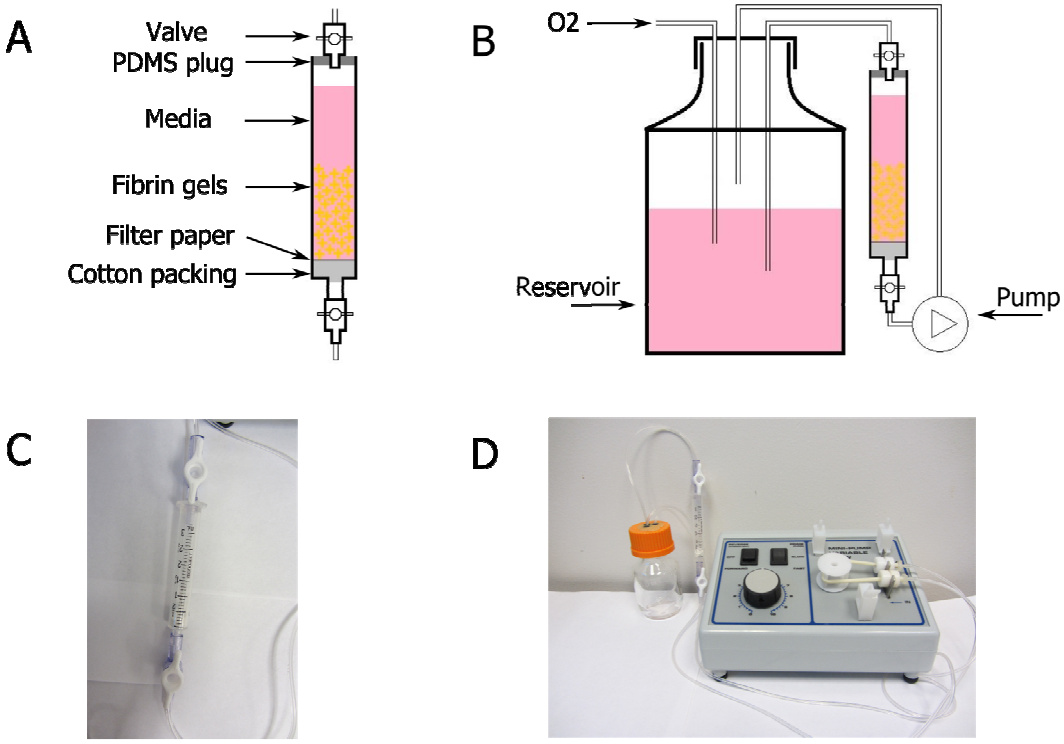


Figure 3.5.2: Perfusion bioreactor setup. (A) The schematic shows cotton and filter paper are packed into the bottom of a syringe and connected to a valve and tubing to hold fibrin gels in place, while the top valve is sealed in the syringe with a ring of PDMS. (B) The bioreactor is then connected to a reservoir containing media through tubing and a peristaltic pump, while the reservoir is also connected to a flow of oxygen. (C,D) Actual bioreactor and reservoir setup is also shown.

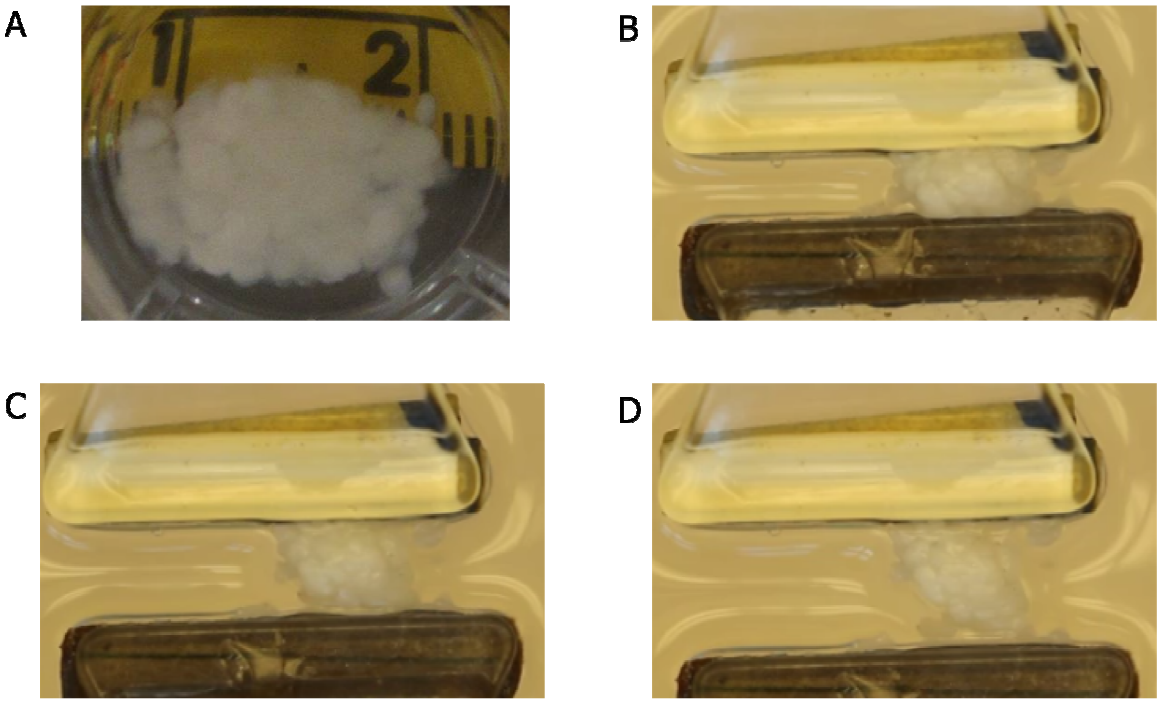


Figure 3.5.3: Mechanical stability of constructs. (A) After 3 days of static culture and 5 days of perfusion culture, the porous construct can be removed as a single piece from the bioreactor. (B-C) These constructs were loaded into an Instron-Sacks Planar Biaxial Soft Tissue Testing System (B) and stretched uniaxially (C). (D) The constructs were stretched until a noticeable drop in stress occurred, reaching strains of 72 ±18 without constructs breaking into individual modules even as the construct was pulled into two pieces.

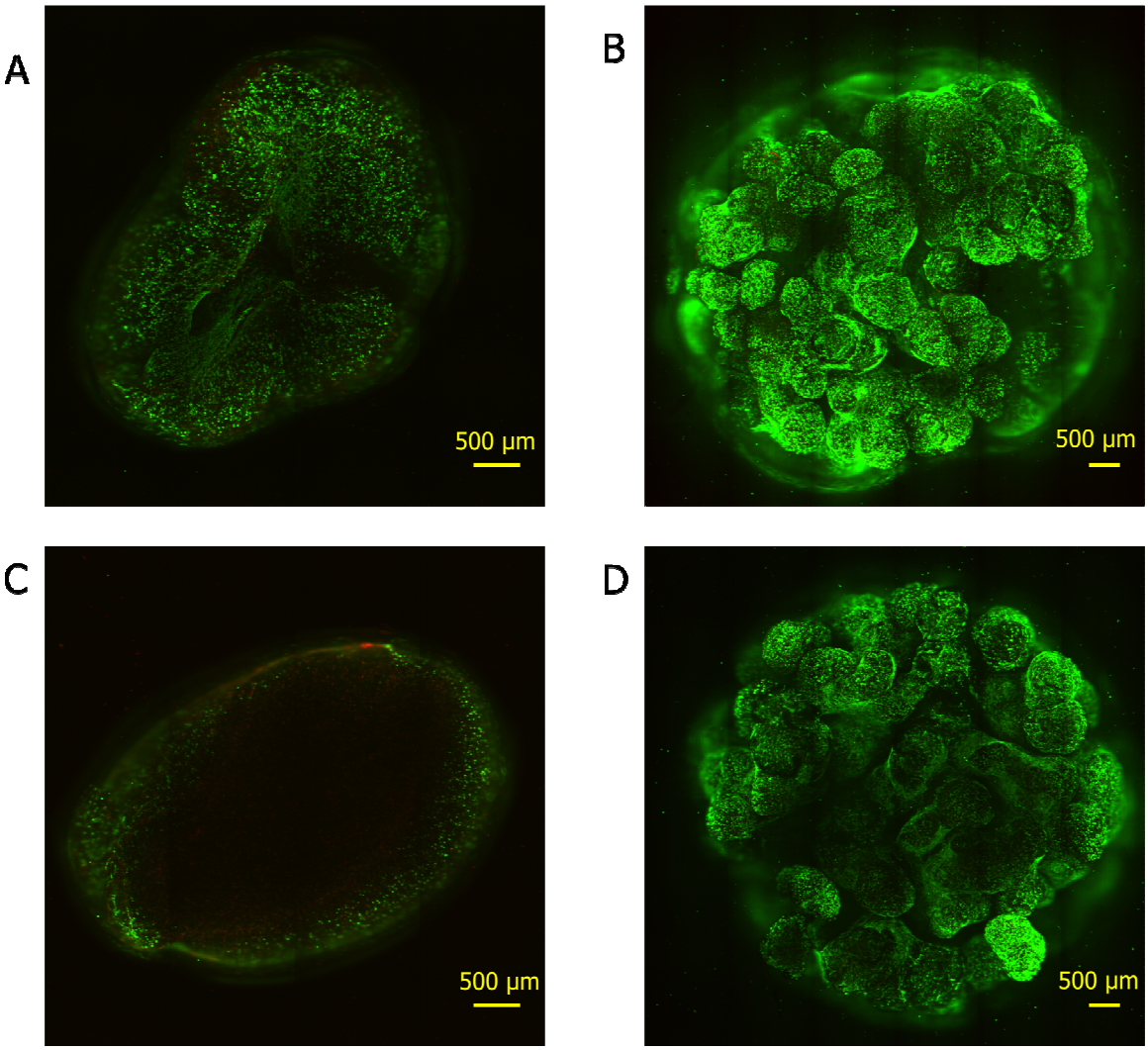


Figure 3.5.4: Live/dead staining of constructs. (A,C) Staining shows that after 3 days of static culture and 5 days culture in a 3 mL bioreactor, MSCs and HUVECs are alive at the exterior of a non-porous gels (A), but the viability is limited to about 600 µm from the exterior (C). (B,D) On the other hand, a biologically assembled construct cultured in a 3 mL bioreactor showed good cell viability on the top (B) and throughout a slice about 3 mm from the top of the approximately 7 mm construct (D).

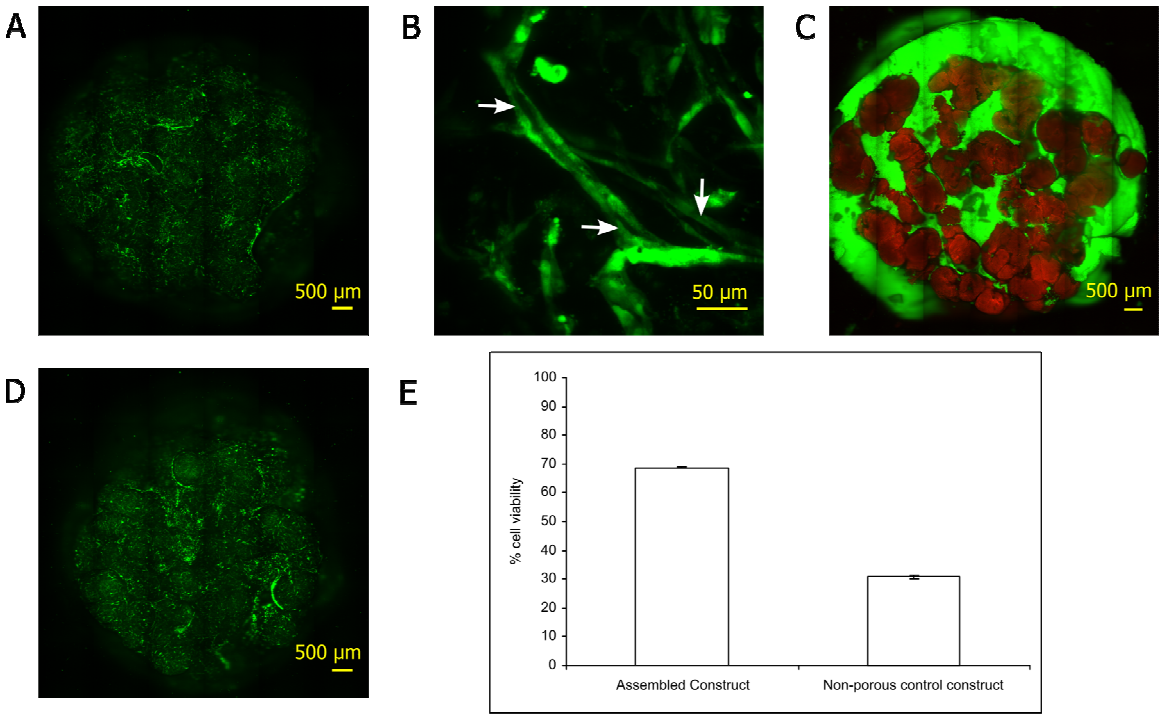


Figure 3.5.5: Tube-like structure formation, porosity, and viability in constructs. (A, D) GFP-HUVECs showed similar development at the top (A) and throughout a slice about 3 mm from the top (D) of an assembled construct after 10 days of culture. (B) The center of the slice contained connected cells with lumen–like structures (arrows). (C) Perfusion of red fibrin gels with green microbeads showed significant porosity at the center of the construct. (E) The cell viability in perfused porous constructs is over twice as high as that in non-porous constructs based on the AlamarBlue assay. The values are normalized to the average viability of cells statically cultured for 8 days in all four types of individual fibrin gels (50 and 120 µL gel modules and single gels).

Chapter 4: Hyaluronic Hydrogels cross-linked with self-assembling polypeptides

4.1 Introduction

Hydrogels have been widely used to assist in the differentiation, growth, and expansion of cells in tissue engineering. These gels generally attempt to mimic the extracellular matrix naturally found *in vivo*. Both two-dimensional (2D) and three-dimensional (3D) hydrogels have been created using either proteins or polysaccharides, but there has been much less study of the benefits to using both types of polymers to form a single hydrogel [136-138]. Polysaccharides with attached proteins, called glycoproteins, are a natural part of the extracellular matrix. One example of a polysaccharide naturally modified by proteins is hyaluronic acid (HA), a gylcosoaminoglycan, which is a part of the extracellular matrix in cartilage, skin, and brain. It is important support for cell attachment, growth, nourishment, and migration [139]. It plays a critical role in wound healing, immune system regulation, and nerve regeneration [140].

Proteins are commonly attached to hyaluronic acid, and they assist in cell support and can also provide cell signaling [141]. In the brain, hyaluronic acid binds to several proteins including versican, aggrecan and the glial HA-binding protein, and their appearance during development causes significant changes in the extracellular space [142]. Studies of natural polypeptides found in the extracellular matrix, such as fibrin and collagen, have shown that small peptide sequences, such as RGD and IKVAV, are crucial for cell growth and differentiation [143]. Through cloning and protein expression these peptides can be isolated and inserted into synthetic proteins. The ability to create these

synthetic polypeptides has proven useful, as they allow for the creation of unique hydrogels, and also provide a higher degree of control over gel properties [108,144].

Here we have created a hydrogel by modifying hyaluronic acid with a synthetic polypeptide that can physically associate into tetramers, which allows for the creation of a physical hydrogel. As physical hydrogels can reversibly form and disassemble, they do not require degradation of the hydrogel to allow for cell mobility. This combination provides a way to more closely mimic the natural glycoproteins found in the extracellular matrix, allows for mimicry of physical stabilized microenvironments, and also can present peptide cellular signals such as RGD. Here we show hydrogels can support cell spreading in two dimensions as well as the three-dimensional cell development, such as cyst formation and neurite outgrowth, specifically because of the incorporation of the RGD sequence in the protein crosslinker.

4.2 Methods

4.2.1 Thiolated Hyaluronic Acid Synthesis

Thiolated HA derivative was synthesized and characterized according to a procedure reported previously [145,146] and first prepared and analyzed in our lab by Bo Liu. In summary for a basis of 1 mL, 10 mg Hyaluronic acid (HA, sodium salt, MW 234 KDa) (Lifecore Biomedical Co) was reacted in water with 12 mg 3,3'-dithiobis(propanoic hydrazide)(DTPH) prepared from 3,3'-dithiodipropionic acid. This was done at room temperature for 20 minutes at a pH of 7.0. The pH was adjusted to 4.7 before adding 9.6 mg EDC (1-Ethyl-3-[3-(dimethy-lamino)propyl]carbodiimide) to the solution, which was

stirred for 10 minutes. The pH was increased to 8.5 and 50 mg dithiolthreitol was added and the solution was stirred for 24 hours. The pH was adjusted to 3.5 and the solution was dialyzed against 100 mM NaCl at pH 3.5 for 4 days and then against pH 3.5 water for 1 day before lyophilizing to get HA-SH as a white solid that is stored at -20 ˚C under nitrogen. The structure and degree of substitution of HA-SH were detected by ^{1}H NMR and free thiol groups on the side chain of HA-SH were measured by the Ellman (DTNB) method with the assistance of Bo Liu. The calculated degree of substitution for thiolated HA in this study was 42% and the free thiol content per 100 disaccharide units was 32%.

4.2.2 Synthesis and Purification of Polypeptides

The genes encoding the polypeptides AC_{10}cys and AC_{10}RGDcys, were each constructed in the Qiagen pQE9 vector (Figure 4.5.1, B), In these proteins, A is a tetramer forming leucine zipper domain, C_{10} is a hydrophilic random coil, RGD is a cell-signaling peptide and cys is a cysteine residue. The polypeptides were expressed in the *E.coli* strain SG13009 according to a procedure reported previously [147]. Briefly, E. coli containing the desired vector was grown at 37 ˚C in 2xYT media until an optical density of 600 nm was reached. Protein expression was induced with 1 mM Isopropyl-b-D-thiogalactopyranoside (IPTG, EMD Chemicals, Inc) and then incubated for an additional 4 h. Cells were harvested by centrifugation and lysed in 8M urea (Sigma). The cell lysate was centrifuged and cell debris was removed. The supernatant was reduced with 2-mercaptoethanol (0.1% V/V, Sigma) and the protein was purified through nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography, dialyzed, and lyophilized

to give a white powder. The polypeptides were examined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.3 Preparation of Polypeptide-DEGDA Conjugates

The polypeptides previously synthesized were conjugated with di(ethylene glycol) diacrylate (DEGDA) through the Michael-type addition reaction. A 0.5 mM solution of AC10cys or AC10RGDcys was prepared in 8 M urea containing 2 mM TCEP at pH 4.5 to break disulfide bonds. The solution was stirred at room temperature for 1 h and the pH of the solution was adjusted to 8.0. DEGDA was added to the polypeptide solution at a 50:1 molar ratio (DEGDA/polypeptide), and the mixture was stirred in the dark at room temperature for 12 h. The sample was dialyzed against deionized water at 4 °C to remove unreacted DEGDA and then lyophilized to remove water, resulting in a white powder.

4.2.4 Cell Culture

Madin-Darby Canine Kidney Epithelial (MDCK) Cells (ATCC) were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC) supplemented with 10% fetal bovine serum FBS and 1% Penicillin/streptomycin. Cells were cultured in 37˚C incubator with 5% CO_2 and medium was changed every 2 days. Embryonic day 7 (E7) chicken eggs (Generous gift from Paul Letourneau) were dissected and the dorsal root ganglia (DRG) were placed in F-12 media supplemented with 2mM L-glutamine, 16μM B27, 160μM sodium pyruvate, and 8mM glucose buffered to pH 7.4 with 10 mM HEPES for a

short time until placed into hydrogels. Chick DRGs were cultured in hydrogels containing the same media with the addition of 40ng/mL NGF and 4μg/mL laminin [148,149].

4.2.5 Preparation of Hydrogels and Cell Seeding

To prepare hydrogels, solutions of 1.5% w/v thiolated HA and 6% w/v polypeptide-DEGDA (AC_{10}RGD-DEGDA or AC_{10}-DEGDA) or 10 kDa PEGDA (Laysan Bio) were made at pH 7.4 in the medium of the cells to be cultured in that hydrogel. For 2D MDCK cultures, these stock solutions were mixed with additional media to make solutions with 0.4 to 1.0% w/v HA-SH and 1.4% to 2.0% w/v protein or PEGDA and the pH was checked to be at 7.4 to allow solid gel formation. MDCK cells were detached from culture flasks using 0.05% trypsin, centrifuged, and resuspended in media. After an hour for the hydrogel to solidify, 6,000 MDCK cells in 100 uL media were seeded on top of the 100 μL hydrogel in a 96 well plate and cultured for 3 days, changing media every other day. For 3D MDCK cultures 15,000 cells in media were combined with stock solutions to prepare 100 μL of gel with desired HA-SH and protein concentrations. After gels had set for an hour, 100 μL of medium was added on top of the gel, and the MDCK cells were cultured for up to 10 days, changing media every other day. Similarly for DRGs, 150 μL hydrogel solutions prepared with F-12 media for DRG culture were pipetted into a 48-well plate. The explanted DRGs were completely submerged in the hydrogel solution, and the solution was then allowed to gel. After 24 hours, 150 μL of

medium was added and the gels were incubated for 2 days and then imaged for axon extension from the DRG.

4.2.6 Cell Staining and Imaging

Gels containing MDCK cells were stained at a 1:1 ratio with 0.1 mg/mL Hoechst 33342 (Thermo Fischer) in PBS for 20 minutes in the tissue culture incubator and then rinsed with PBS in order to visualize nuclei. All gels were imaged on a Zeiss Axiovert Observer inverted microscope equipped with ApoTome (which allows 3D fluorescence imaging) using the 10X (N-Achroplan 0.25 NA), or 20X (LD Acroplan 0.4 NA) objectives.

4.3 Results

4.3.1 Hydrogel Preparation

The first step in preparing the glycoprotein system was to create a method to attach proteins to a polysaccharide backbone, and for this we chose to use HA, a naturally occurring polysaccharide. In order to attach desired proteins to this polysaccharide the carboxylic acid groups on the backbone were reacted with DTPH to present thiol groups. Of the carboxyl groups available on the HA, 42% were converted to thiols, with 32 free thiols per 100 disaccharide units, as some reacted to form disulfide bonds. In order to allow for physical hydrogel formation, HA-SH was reacted with the proteins AC_{10}cys and AC_{10}RGDcys. In these proteins, the A groups are leucine zippers that have the ability to physically associate into tetramers, allowing for physical cross-linking and

hydrogel formation [147]. Once the proteins were produced, they were modified with DEGDA through Michael-type addition reaction of the thiol present in the cysteine residue with only one acryl group on DEGDA by having a large excess of DEGDA. The other acryl group on protein-DEGDA could then be attached to the thiol of HA-SH through a similar reaction, and so a solution of the two was able to form a physically-assosiated glycoprotein hydrogel at neutral pH (Figure 4.5.1, A). It was observed that for a wide range of concentrations for both AC10-DEGDA and AC10RGD-DEGDA (1.0% to 2.2% w/v) with modified polysaccharide (0.4% to 1.5% w/v) the solution would form a solid gel within twenty minutes, capable of holding its shape without flowing. Gels composed of HA-SH without the protein would flow even after 24 hours, indicating that the attached physically-associating protein groups were required for the formation of the solid hydrogel.

4.3.2 Cell Adhesion on 2D Hydrogels

To test the ability of the hydrogels to support cell growth and present the RGD signaling peptide, MDCK cells were seeded on top of hydrogels composed of 1% w/v HA-SH and 1.6% w/v protein or PEGDA and cultured for 3 days in order to observe cell spreading in 2D culture. The hydrogels prepared with AC10RGD-DEGDA crosslinker were able to support 2D growth quite well. The cells quickly spread and covered the entire surface of the hydrogel (Figure 4.5.2, A). Significantly different results were seen when a crosslinker without the signaling peptide RGD was used - gel prepared at the same concentrations with the protein AC10-DEGDA, which lacks the RGD peptide, did

not support cell spreading on the surface of the gel. Instead cells formed a single cluster which shrank as cells lifted and died (Figure 4.5.2, B). This cell response was also seen in HA gels chemically cross-linked with PEGDA, which also lacks the RGD peptide (Figure 4.5.2, C).

4.3.3 Epithelial Cyst Development and Neurite Extension in 3D Hydrogels

Gels were also tested for the ability to support epithelial cyst formation in 3D cultures. MDCK cells initially were seeded inside hydrogels with the same concentrations (1.0% HA-SH and 1.6% AC10RGD-DEGDA, AC10-DEGDA, or PEGDA) previously tested with 2D cultures, but there was not any significant difference between the samples. By testing 0.5% - 1.0% HA-SH and 1.6 -2.0% Ac10RGD-DEGDA, it was found that lower HA concentrations but higher protein concentrations allowed for the formation of large aggregates, specifically with 0.8% HA-SH and 2.0% AC10RGD-DEGDA. These gels allowed for formation of large, spherical structures after 6 days of culture (Figure 4.5.3, A). This was further analyzed using the Apotome microscope feature to look at crossections of the spherical structures. Crossections at the top and bottom of the sphere showed complete cell sheets, while crossections closer the center of the sphere reveal a circular ring of cells (Figure 4.5.3, B,C). This indicates the cells formed a hollow sphere, or cyst-like structure. Higher HA-SH concentration (1.0%) resulted in smaller cell aggregates instead of large cyst-like structures (Figure 4.5.3, D). In hydrogels containing 0.8% HA-SH and 2.0% AC10-DEGDA (lacking RGD), smaller cell aggregates also

formed, but they are more irregularly shaped and crossections through the center of the aggregates did not reveal any hollow centers (Figure 4.5.3, E,F).

HA and either protein or PEGDA crosslinkers were also tested for creating hydrogels that support chick DRG neurite extension. Lower concentrations of both HA-SH (0.4%) and AC10RGD-DEGDA (1.4%) proved effective at assisting in the extension of neurites from the explanted DRGs (Figure 4.5.4, A). Again, the presence of RGD was crucial as gels with 1.4% AC10-DEGDA or PEGDA had much lower levels of neurite extension from the DRG's when compared to gel made with AC10RGD-DEGDA (Figure 4.5.4, B-C). Also, the importance of gel stiffness was seen as higher concentrations of AC10RGD-DEGDA, specifically 1.8% or 2.2%, resulted in much lower amounts of neurite extension when compared to 1.4 or 1.6% (Figure 4.5.4, D-F).

4.4 Discussion

There are numerous types of hydrogels that are currently used for tissue engineering applications. Some are based on polysaccharides, such as chitosan and alginate gels, which have been shown to be useful in creation of scaffolds and microbeads for cell encapsulation [150-152]. Others are protein based, and can be naturally occurring, such as fibrin and collagen, or synthetically prepared for a specific purpose, and have been shown provide important signals to cells [153]. Here we attempt to create a hydrogel that can effectively integrate both polysaccharides and synthetic proteins into a single glycoprotein hydrogel through physical associations within the protein. We decided to mimic the naturally occurring extracellular matrix material HA, as it is naturally modified

with proteins *in vivo*. Using this polysaccharide backbone and synthetically prepared proteins would give the system a very versatile structure useful for many applications.

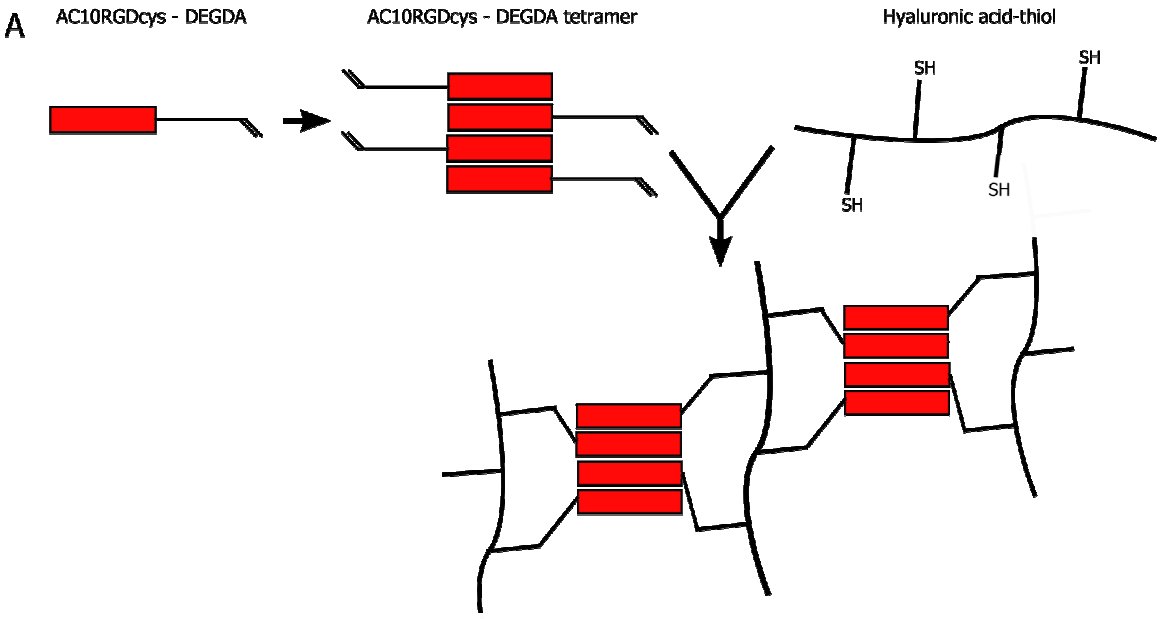
Another advantage of the system we developed is the physical connections that create the hydrogel. Most currently used hydrogels are chemically cross-linked, creating junctions that must be cleaved before cells can migrate within the gel. This cleavage can cause material degradation within the gel and lead to undesirable changes in the mechanical properties of the gel. The ability to create physically cross-linked gels is also a way to more closely mimic some natural biological systems to assist in cell development. For example, the ability of basement membrane to support the rapid migration of leukocytes are the result of physically stabilized bonds [154].

We also have demonstrated the importance of signaling that can be supplied by synthetic peptides. Removal of the RGD peptide did not appear to influence the physical structure or mechanical properties of the gel, but cells reacted very differently. In two dimensions, they were unable to attach effectively to the gel surface; while in three dimensions they showed no development of cysts from MDCK cells and limited neurite extensions form DRGs when compared to proteins containing the RGD peptide. And RGD is just one example - other small peptides such as IKVAV and YIGSR have been shown to provide crucial signals to cells. Because of the independent preparation of HA and proteins, these or other signals can be provided through cloning of the peptide sequence. By creating multiple peptide-DEGDA molecules with unique signals , multiple peptides could be presented within the hydrogel. The ability to control the signaling from the protein in the hydrogel gives this hybrid gel great flexibility in possible applications.

And while signaling to cells through the RGD ligand proved to be very important, the concentrations of polymers, and thus mechanical properties of the gel, was also crucial for creating the correct environment for cells. Higher crosslinker concentrations of AC10RGD-DEGDA up to 2.2% w/v significantly impeded the outgrowth of neurons from the clusters of DRG implanted inside the gel when compared to 1.4% w/v. And while higher concentrations of HA-SH and protein (0.8% w/v and 2.0% w/v respectively) were used to support the formation of cysts compared to neurites, even higher HA-SH concentrations (1.0% w/v) impeded cell motility and cyst formation. This hydrogel has shown significant flexibility in mechanically properties to support different cell structure formation.

By combining polysaccharides and polypeptides, we have created a physically cross-linked hydrogel that supports cells by mimicking the glycoproteins of the extracellular matrix. Through the use of modified hyaluronic acid and recombinant proteins gels at conditions commonly used in cell culture, this hydrogel can effectively support cell culture in both two and three-dimensional gels. It also provides important signaling through small peptide sequences in the protein, which can be easily adapted for the expression of other signaling molecules. This novel hydrogel system can support cell culture through signals presentation, physical crosslinking, and controlled mechanical properties.

4.5 Figures



B

AC_{10}cys:MR**[6H]**DDDDKWA**[A]**IGDHVAPRDTSYRDPMG**[C_{10}]**ARMPTSMGGC

AC_{10}RGDcys:MR**[6H]**DDDDKWA**[A]**IGDHVAPRDTSYRDPMG**[C_{10}]**ARMPTSYAVTG**RGD**SPASSTSM GGC

Abbreviations for peptide domains:

[6H]: GSHHHHHHGS

[A]: SGDLENEVAQLEREVRSLEDEAAELEQKVSRLKNEIEDLKAE (self-assembling leucine zipper)

[C_{10}]: $[AGAGAGPEG]_{10}$ (hydrophilic random coil)

Figure 4.5.1: Illustration of hydrogel formation. (A) AC10RGDcys is reacted with DEGDA and can form tetramers by physical cross-linking. The acryl groups on the protein can react with thiol modified hyaluronic acid to form a physically stabilized gel. (B) Sequences of proteins used are presented indicating the important domains.

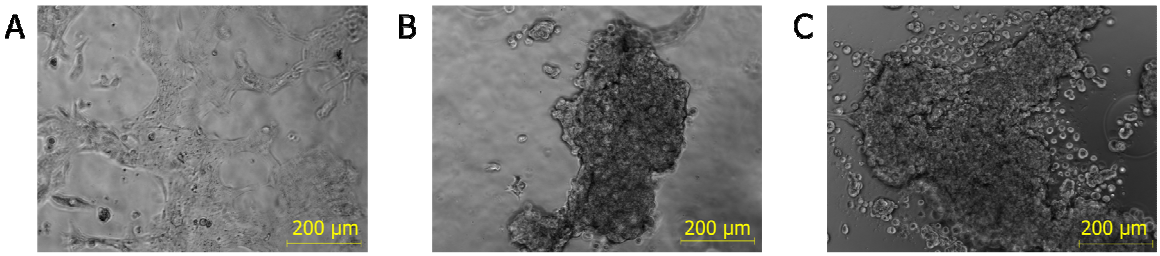


Figure 4.5.2: Effects of crosslinker on 2D MDCK spreading. (A) Solutions of 1.0% w/v HA-SH with 1.6% w/v concentrations of AC10RGD-DEGDA allowed for MDCK cells to spread. (B,C) Samples with the same concentration of AC10-DEGDA (B) and PEGDA (C) show that without the RGD peptide, cells clump and detach form the gel surface.

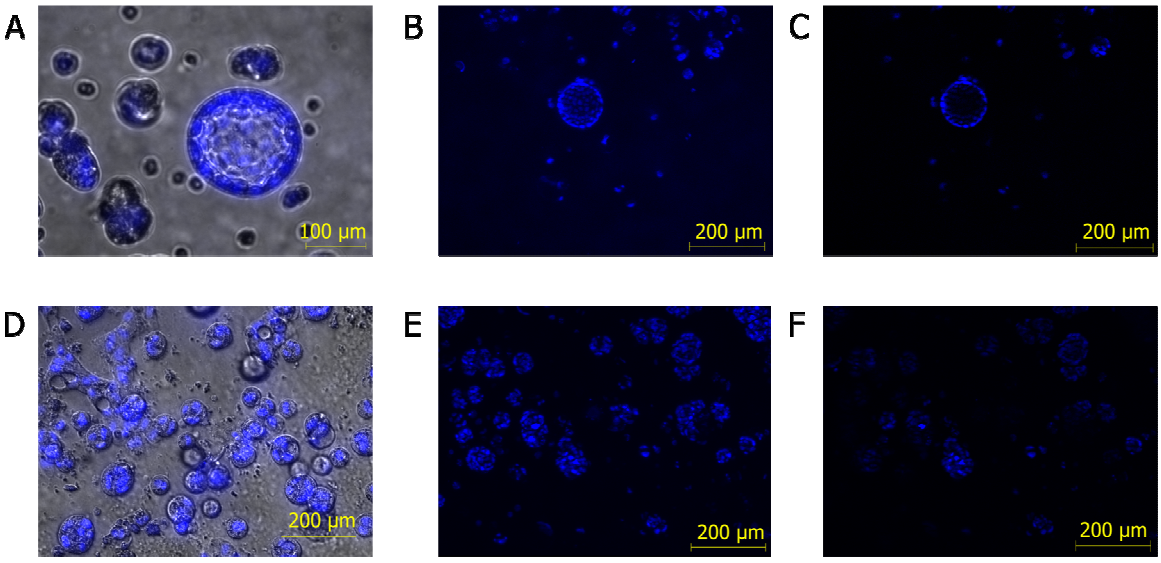


Figure 4.5.3: Effect of HA-SH and crosslinker on 3D MDCK cyst formation. (A) MDCK cells in gels composed of 0.8% w/v HA and 2.0% w/v AC10RGD-DEGDA formed large cell aggregates over 6 days of culture, as seen with an overlay of bright field and fluorescent 20X images after staining with Hoechst 33342. (B, C) Images of the same gel using a 10X objective and Apotome to create a z-stack of 29 images separated by 4.76 µm showed a spherical aggregate (B), while a single image at the center of the stack reveals a cell-free center (C), indicating cyst formation. (D) When concentration of HA-SH was increased to 1.0% w/v without changing protein concentration, large hollow structures were absent. (E, F) Cells encapsulated in 0.8% w/v HA-SH gels with 2.0% w/v AC10-DEGDA (lacking RGD) form smaller aggregates, and when a z-stack of 28 images (E) was analyzed, single slices did not show any large cell-free cavities (F).

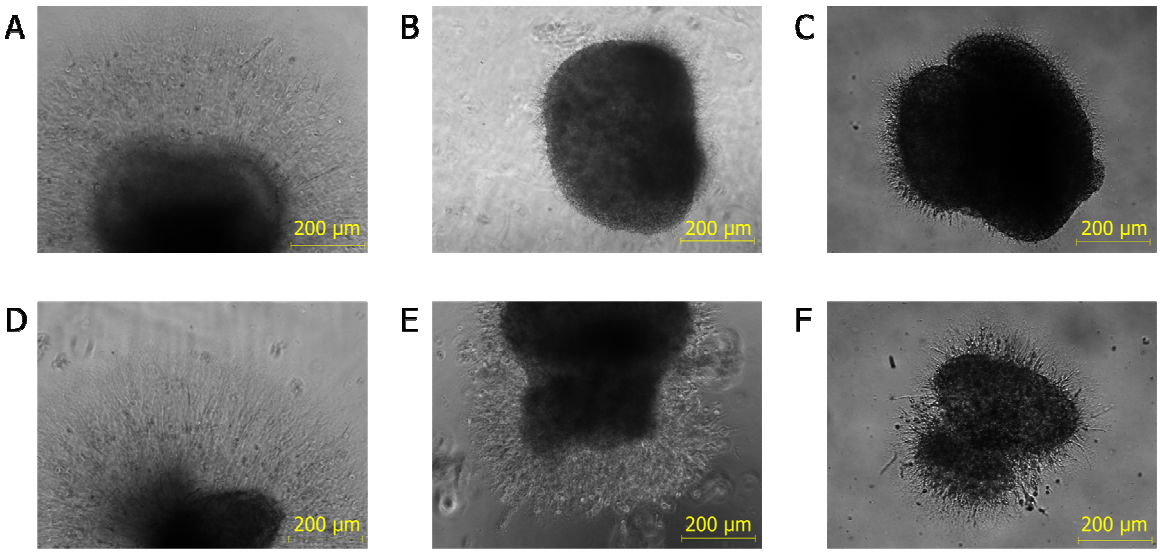


Figure 4.5.4: Effects of crosslinker on neurite extension. (A) Solutions of 0.4% w/v HA-SH with 1.4% w/v AC10RGD-DEGDA allowed for significant neurite extension from chick DRG's. (B, C) Samples containing AC10-DEGDA (B) or PEGDA (C) had greatly decreased extension of neurites. (D-F) Mechanical properties of the gel also proved important, as increasing the concentration of AC10RGD-DEGDA to 1.6% (D), 1.8% (E) or 2.2% w/v (F) results in significantly less neurite outgrowth in chick DRG's as the protein concentration increases.

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

5.1.1 Biological Assembly of Tissue Modules through the Formation of Endothelial Networks

Individual fibrin gels were modularly assembled though the development of an endothelial network. Cells seeded in a single gel were able to migrate across the interface into an adjacent, initially cell-free gel, and after 8 days of culture an endothelial network that crossed the interfaces had assisted in connecting the modules. This assembled construct behaved as a cohesive unit and could be manipulated without loss of individual modules. Both GFP-HUVECs and MSCs were crucial for this assembly, as either cell type alone did not develop an endothelial network and resulted in a weak construct that would easily break apart. Constructs assembled from 6 gels could be stretched to over twice their original length before breaking, and in general had similar mechanical properties to a whole fibrin gel, indicating a very strong connection was formed between individual modules through network formation. The experiments shown here are an important first step in using modular assembly to create porous, 3D hydrogels that can support cell growth throughout the construct by reducing the limitations of oxygen and nutrient diffusion.

5.1.2 Perfusable Construct Formation through Biological Assembly

Large, porous constructs were assembled from smaller fibrin modules containing HUVEC and MSC cells. Cells initially seeded in small, cross shaped fibrin gels were

used to biologically assemble the individual modules into a single construct when cultured in a perfusion bioreactor. This single, cohesive piece of gel could be removed from the bioreactor and stretched over half of its initial length without loss of individual modules from the construct. While cells in non-porous fibrin gels only survived and formed vascular structures up to 600 um from the edge of the construct, the cells in the biologically assembled construct proved to be viable over 3 mm from the exterior of the gel and showed the ability to form hollow, tube-like structures even at the very center of the porous gel. This is a strong indication that medium was able to perfuse the construct, further shown by the ability of BSA containing green microbeads to flow through the interior of the construct. This perfusion reduces oxygen diffusion distances, and results in cells being twice as viable in the porous, modularly assembled constructs compared to nonporous gels. The increased viability and development of a capillary network indicate that this method is an effective way to create larger tissue engineering constructs without the need for more complicated and less effective methods of modular assembly. This biologically assembled, porous, vascularized construct is an effective way to reduce barriers to cell survival and development present in larger tissue engineering constructs.

5.1.3 Cell Development in Synthetic Glycoprotein Hydrogels

By combining polysaccharides and synthetic polypeptides, we have created a physically associated hydrogel that supports cells by mimicking the glycoproteins of the extracellular matrix. The specific use of tetramer forming polypeptide A domains allows for the formation of this physically connected gel. The mixture of modified hyaluronic

acid and recombinant proteins gels can effectively support both cell spreading on top of the gel as well as the development of cell structures such as cysts and neurite extension for cells seeded inside the gel. The synthetic polypeptides used also have shown the ability to provide important signaling to the cells through the addition of the RGD peptide. This novel hydrogel system shows promise for use in a wide range of tissue engineering applications because it combines signaling control through the use of modified HA and a synthetic peptide that can be adapted to express important cell signaling peptides with mechanical control from physical cross-linking and wide gelation concentrations.

5.2 Future Direction

While this biological assembly method showed significant promise in the development of large, perfusable, prevascularized constructs, there are still challenges to overcome, but also opportunities for new developments. In order to create specific types of tissues, additional cells would need to be seeded in gels, which can change oxygen demand. As there was already some loss of cell viability from gel modules when creating the larger construct, closer analysis of the shape, size, and culture time of gel modules may be needed in order to further optimize the perfusion of the construct after the addition of other cell types. Biological mechanisms were able to assemble gel modules after three days of static culture, but it is possible that this mechanism for connecting gels could be just as effective even after longer static incubation times. As there was still some shape and size change in gels after 3 days, it may be easier to control porosity of

constructs if even longer static culture times were attempted before assembly. Also, in order to more completely understand the uses of this method, implanting the construct and comparing *in vivo* angiogenesis to constructs without preformed vasculature would be useful.

5.2.1 Creating Larger Tissue Engineering Constructs

This assembly method could lead to *in vivo* assembly of gel modules, which would be beneficial from the standpoint of not having to deliver a single, large construct, but instead smaller gels that could possibly be injected to form the desired structure. But even if the biological assembly is limited to the initial stages of vascular formation, this method should still be able to create larger constructs. One of the main limitations is the difficulty in forming the many small gel modules, so improving the method of fibrin gelation would make assembling larger constructs easier. There have been methods for quickly removing gels from PDMS molds that have worked with larger gels, so it is possible that with some adaptation, the gel preparation can be simplified for the creation of the smaller fibrin modules [155]. Assembly of larger constructs in a larger bioreactor would need to be careful of excess channeling caused by gel shrinking, but as long as the gel porosity can be maintained it should still be an effective method on a larger scale.

5.2.2 Creating Heterogeneous Tissue Engineering Constructs

Another opportunity arises through the controlled assembly of the individual gel molecules. Most tissues are very complex, and can require different cell types, cell

signals, or mechanical properties. This spatial control can be mimicked through the controlled assembly of individual tissue modules – each module can be created with different cell concentrations, cell types, or statically cultured with different signaling molecules. The module themselves can be gelled with different concentrations of polymer to change mechanical properties, or if effective hybrid hydrogels are found with the ability to support vasculature, modules can even be created from different types of polymers. Individual modules cultured in different conditions can then be assembled into heterogeneous constructs. One option would be to first assemble small numbers of these gels to develop small constructs before further assembling these small constructs into a single larger construct. Another method could use more careful construction of the construct, possibly with the assistance of a dissolvable barrier to seed unique individual gels into desired patterns within a heterogeneous construct.

5.2.3 Alternate Proteins for Cell Development in Glycoprotein Gels

While RGD was found to be successful at supporting two-dimensional spreading as well as cyst formation in MDCK cells and neurite extension in chick DRG's, there are many other proteins or peptides that could be tested with this type of hydrogel. For example, collagen or fibrin could be used in conjunction with HA to create an environment that could be more effective at forming prevascularized gels. Alternately, synthetic proteins with different or even multiple signaling peptides could be used. An example of this method would be to add a mixture of modified AC10 protein with another synthetic protein B containing a different cell signaling peptide. As the A and B

proteins can form a physically linked dimmer, this could allow for colocalization of different signals, such as α5β1 and α6β1 integrins which have been proven to be important in stem cell differentiation when spatial proximity is controlled [156]. In order to make the gel more closely mimic the natural cell environment, proteins that mimic the function of versican, aggrecan or the glial HA-binding protein, could be used to change cell behavior within the gel [142]. The adaptability of synthetic polymers leaves great potential for creating effective tissue engineering hydrogels.

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