R03 NIH Grant Proposal

3-Dimensional Scaffold-Tissue Construct Bioprinting

Chemical Engineering 590B

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Specific Aims

A paramount issue addressed by the tissue engineering field is the limited supply of donor organs and tissues. Manufacturing techniques that can produce devices that closely mimic the functionality of a particular organ are being explored, one of which is three-dimensional bioprinting. 3D bioprinting is the positioning of biochemicals, biological materials, and living cells in discrete patches and patterns. Currently, bioprinted constructs are limited in thickness to a few hundred microns due to the lack of microvasculature, and the incorporation of vessels is necessary if printed biological devices are to be further advanced. In a recent study by Xu et al., human amnionic fluid stem cells (hAFSCs), when printed with endothelial cells (ECs) and smooth muscle cells (SMCs) on a collagen scaffold, formed viable vascularized bone tissue and were implanted in mice for up to 18 weeks. We propose to test the **hypothesis** that a bioprinted device containing hepatic and capillary-forming ECs and SMCs can be successfully fabricated, and that the hepatocytes maintain their phenotype. A huge demand exists for creating replacement liver tissue, and we seek to elucidate 3D bioprinting as a means of producing functional liver tissue through the following objectives:

Aim 1: **Fabricate a viable layered hepatic tissue with sufficient vascularization using 3D bioprinting.**
Hypothesis: A 3D bioprinted tissue containing hepatic cells will remain viable if vessel-forming endothelial cells (ECs) and smooth muscle cells (SMCs) are incorporated within the tissue.

Method: An office ink-jet printer will be modified according to methods set forth by Cui et al. to print in the z-axis, and ink cartridges will be filled with hepatocytes, smooth muscle cells, and endothelial cells. It has previously been demonstrated that a fibrin gel scaffold promotes *in vitro* angiogenesis when seeded with hepatocytes and ECs. Additionally, human endothelial cells have been printed onto a fibrin scaffold and successfully formed vascular networks. First, fibrinogen and thrombin will be printed and subsequently polymerized to form a fibrin scaffold layer. All three cell types will then be printed onto the fibrin scaffold, after which a second fibrin layer will be applied and polymerized with thrombin. This process of layering with fibrin, and subsequent cell printing will be repeated, and the device will be incubated in a bioreactor. The viability of the hepatocytes and SMCs, and the proliferation of EC's will be assessed using a two-color fluorescent dye and mitochondrial metabolic activity (MTT) assay, respectively. Maintenance of hepatocyte phenotype will be determined by measuring albumin concentration.

Aim 2: **Quantify proliferation and viability of the hepatocytes, ECs, and SMCs on fibrin gel scaffolds of various stiffness.**
Hypothesis: An optimal fibirin gel matrix modulus for which hepatocyte adhesion, proliferation, and growth is maximized will exist in the range of $10 - 100$ kPa.

Method: The elastic moduli of the fibrin gel matrices will be controlled by adjusting the concentration of fibrin and thrombin. Printing of cells onto these matrices and subsequent viability and proliferation testing using the techniques in *Aim 1* will be implemented to determine a stiffness at which hepatocyte adhesion, growth, and functionality is maximized.

The long term goal is to develop a biocompatible fibrin tissue containing hepatic cells with sufficient vascularization using 3D printing. Optimizing the mechanical properties of the bioprinted tissue, attaining abundant vessel formation, and maintaining hepatic phenotype will be crucial for the success of this device. Simultaneous printing of hepatocytes and endothelial cells has not yet been explored, and this study seeks to advance the technique of 3D printing and the printing of multiple cell types. The development of a high throughput bioprinting process will enable the fabrication of more complex tissues and structures. For example, printing liver tissue with additional cell types, such as bile duct and sinusoidal cells, may become feasible following this study.

Significance

The liver is an essential organ of the body, serving a multitude of functions. These include the secretion of bile; metabolism of proteins, carbohydrates, and fats; nutrient processing, glycogen and vitamin storage; synthesis of blood-clotting factors and proteins such as albumin; removal of nitrogeneous waste and toxins from the blood; regulation of blood volume; and elimination of senescent red blood cells [1]. Associated with these functions are a variety of diseases whose progression may dictate the need for a liver replacement. Unfortunately, the number of donor livers available is far less than those needed, and thus an alternative method of treatment is necessary to generate replacements for patients in need.

1. Health Issues
Some of the most common diseases affecting the liver are hepatitis, cirrhosis, glycogen storage disease, and cancer [1]. Each year, approximately 21,000 individuals are diagnosed with liver cancer in the U.S. [2]. Once liver cancer progresses to a point where tumor removal surgery is not an option, patients will require a replacement liver. In addition, about 31,000 deaths occur each year in the U.S. from liver cirrhosis, a large portion are attributed to chronic alcohol abuse.

The progression of these diseases and other liver-associated diseases, in addition to acetaminophen overdose, can lead to acute liver failure (ALF), in which the liver ultimately shuts down and no longer performs its vital bodily functions [2,3]. The survival rate of ALF is less than 20% [4]. In 2011, 15,807 patients were in need of a donor liver, while only 5,805 patients received liver transplants [5]. Today, the waitlist exceeds 16,000 patients, and only a fraction of these patients are fortunate enough to receive a liver transplant [6].

2. Three Dimensional (3D) Printing
The concept of three dimensional (3D) printing is similar to that of 2D printing on paper, which we are particularly familiar with, and follows the same general procedure as the typical home printer. The key difference is that 3D printers are able to print in the z-direction, in addition to the x and y-directions. This allows for structures to be fabricated layer by layer directly from a computer program. If regions in the structure require gap junctions, the printer ejects a powder that holds the place of a molecule, enabling a gap to form [7]. The powder can be any number of materials provided it is inert and does not react with the particles being printed and is removable once the structure is complete. This process is depicted in Figure 1.
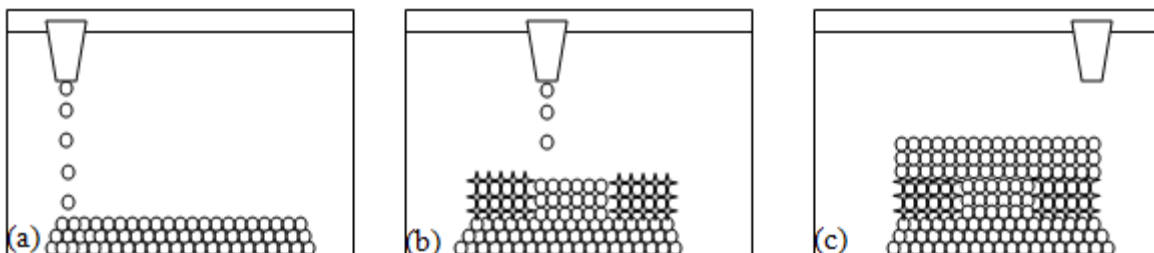
Figure 1: General 3-D Printing Process (a) Material (o) is placed layer by layer with the nozzle (b) An inert powder (✦) is used to make gaps within the structure (c) Finished product can be removed from the printer and the powder discarded

More recently, 3D printing has been extended to printing cells and biological materials, such as extracellular matrix (ECM) proteins and enzymes, in defined patterns and gradients. Inkjet printing, in which droplets of fluid are deposited without making contact with the printing substrate, has been used to print cells, layer-by-layer, onto scaffolds following a pre-programmed design [8]. Many cell types have successfully been printed, a few of which include Chinese hamster ovary (CHO) cells, rat motorneurons, human amnionic fluid stem cells, and endothelial cells [8]. By using a 3D printer to place these cells, the configuration of the cells within the tissue may be improved. One of the hurdles of engineering tissue is the inability to mimic the structure of healthy tissue found in the body when multiple cell types are incorporated [8]. With the ability to create a patterned formation of cells in 3D bioprinting, an ideal structure can be designed.

There a several advantages to 3D bioprinting. First, 3D bioprinting is a relatively inexpensive manufacturing technique because it builds onto an already existing device. A 2D office ink-jet printer can be used once modifications are applied. Second, 3D printers are adjustable; they can be readily reprogrammed with a new template, and interchangeable nozzles for injection are available to accommodate different materials or cell types. Third, 3D printing is an automated, efficient process that can minimize the waiting time of a replacement organ for a patient in need [7,8]. The further development of 3D printing could enable rapid manufacture of patient-specific tissues, and possibly production of complete organs in the future.

3. Vascularization
One of the major limitations with engineered tissue constructs is the inability to attain adequate vessel formation and angiogenesis within the tissue. In order for cells to grow and survive, they must have a source of oxygen and nutrients, as well as waste and metabolic byproduct removal method. Within most native tissue, cells are typically no more than $100 – 200$ μm away from capillaries [9]. Consequently, engineered devices and tissues are diffusion limited in size due to lack of vasculature, and formation of a necrotic core can occur when culturing cells at physiological densities without sufficient vascularization [10].

Several approaches to develop vascularized engineered constructs have been implemented, including layer by layer assembly of perfusable channels and 3D sacrificial molding, in which a rigid 3D lattice is casted into a rubber or plastic material, and the lattice material is subsequently dissolved away with an organic solvent [10]. More recently, it has been demonstrated that 3D vascular networks can be constructed using glass-like structure made of simple carbohydrates. After addition of an aqueous based extracellular matrix, sugars are dissolved to reveal a permeable device, the channels of which can be populated with vessel-forming endothelial cells [10]. In addition, incorporation of substances that promote angiogenesis, such as vascular endothelial growth factor (VEGF), has been widely practiced [9].

Innovation

1. Heterogeneous Cell Printing

The main obstacle in construction of liver tissue is the ability to create tissue that can readily form vascular networks. We plan to overcome this obstacle by engineering tissue that consists of a specific combination of cells to enhance the vascularization properties. Research proves that incorporating endothelial cells (ECs) and smooth muscle cells (SMCs) with a desired cell type can benefit vascular diffusion within the constructed tissue [16]. Therefore, we believe that printing and co-culturing hepatocytes with ECs and SMCs will produce a suitable liver tissue with enhanced vascular development. This may be a difficult task, as many of the previous bioprinting studies have involved printing only a single cell type. Additionally, no previous researchers have attempted to print hepatocytes with ECs and SMCs. To enhance vascularization within the tissue, which in turn will lead to a healthy, more productive tissue, we believe it is necessary to bioprint all three cell types simultaneously.

2. Scaffold Printing

In the experiments performed by Xu et al., only the cells themselves were printed, where as the scaffold was layered by repeatedly immersing the printing platform in an unpolymerized collagen solution [16]. We intend to print the hepatic, endothelial and smooth muscle cells *and* our fibrin scaffold consecutively. This would involve the incorporation of two more cartridges within our printer: one housing the fibrinogen solution and the other the enzyme thrombin, which catalyzes the polymerization of fibrinogen to fibrin. A two-template program will be used to guide the printer. The first program contains instructions for the scaffold design, in which fibrinogen and thrombin droplets will be deposited and mixed so that a fibrin scaffold is created. Multiple versions of this first program will be created so that the scaffold can be tuned to a desired stiffnes. Once this layer has sufficiently polymerized, the second program will be initiated, which guides the deposition of cells onto this scaffold. This cycle of scaffold deposition, scaffold polymerization, and cell deposition will be repeated. Ultimately, coupling cell and scaffold printing will completely automate the bioprinting process.

Approach

We seek to develop a viable, vascularized hepatic tissue supported on a fibrin scaffold using three dimensional printing. Aim 1 will be performed using a layered fibrin scaffold with a stiffness of 50 kPa. Once proven that the three cell types remain viable after printing and proliferating within the device, we will then seek to test different fibrin elastic moduli ranging from 10 – 100 kPa in order to identify a stiffness that promotes optimal hepatocyte viability. .

Aim 1: **Fabricate a viable layered hepatic tissue with sufficient vascularization using 3D bioprinting.**

Hypothesis: A 3D bioprinted tissue containing hepatic cells will remain viable if vessel-forming endothelial cells (ECs) and smooth muscle cells (SMCs) are incorporated within the tissue.

1. Using a modified HP deskjet printer, a fibrin scaffold will be deposited along with the three aforementioned cell types in a layer-by-layer fashion. Ten layers will be printed.
2. Cell viability of the hepatocytes and smooth muscle cells will be assessed using a two-color fluorescent dye, and proliferation of endothelial cells will be assessed using a mitochondrial metabolic activity (MTT) assay. Maintenance of hepatocyte phenotype will be determined by measuring albumin concentration.

1. We will use an HP deskjet printer that has been modified to print in the z-direction, as previously described by Xu et al. [11]. Ink cartridges will be thoroughly washed with ethanol and rinsed with water prior to loading with viable porcine hepatocytes, human ECs, and human SMCs at a concentration of 10^7 cells/mL. These cells will subsequently be printed onto a 50 kPa fibrin scaffold, prepared with fibrinogen and thrombin, the latter of which polymerizes the fibrinogen glycoproteins. Cells will be printed according to the pattern in Figure 2. After printing of the first cell layer, a second fibrin layer will be printed and polymerized, and cell printing will once again be executed. This pattern of cell deposition followed by fibrin layering and polymerization will be repeated until a construct of approximately 10 layers has been fabricated. Once completed, each bioprinted tissue will be incubated in a perfusion bioreactor as described by Lovett et al. [12].
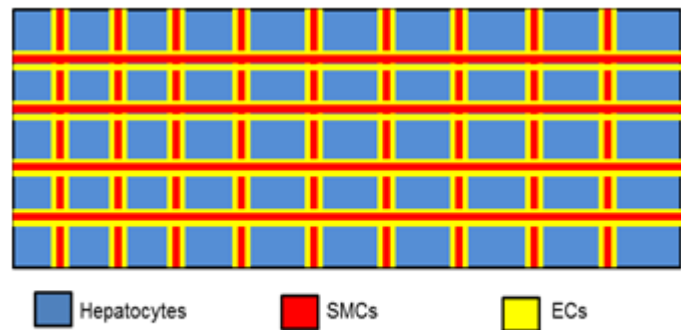

Figure 2 – Cell deposition pattern that will be utilized to construct our liver tissue

2. Constructs will be sampled 1day, 3days, and 1, 3, and 6 weeks following incubation. Hepatocyte viability will be measured using a non-invasive two-color fluorescent dye. Alamar blue measures cell viability through metabolic activity, while 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) measures cell membrane integrity [13]. Fluorescent microscopy will be used to image the constructs. EC proliferation will be quantitatively measured via the MTT assay, in which MTT reagent will be added to the device, and after a 4h incubation period, an absorbance at 540 nm will be obtained. In addition, albumin concentration will be measured to monitor maintenance of hepatocyte phenotype. Albumin is a blood-clotting factor synthesized by hepatocyte-committed cells and fully developed hepatocytes [14].

Aim 2: **Quantify proliferation and viability of the hepatocytes, ECs, and SMCs on fibrin gel scaffolds of various stiffness.**

Hypothesis: An optimal fibirin gel matrix modulus for which hepatocyte adhesion, proliferation, and growth is maximized will exist in the range of 10 – 100 kPa.
1. Fibrin stiffness will be controlled by adjusting the fibrin concentration and droplet density used to make the gels, and cells will be printed onto these matrices of varying stiffness.
2. Cell viability and proliferation studies will be conducted to determine an optimal stiffness that represents a balance between hepatocyte viability and vascularization.

1. In *Aim 1*, the hepatic tissue will be constructed initially with an intermediate 50 kPa stiffness. After demonstrating that hepatocytes remain viable within this printed construct, programs for printing scaffolds with elastic moduli ranging from 10 -100 kPa, the stiffness of *in vivo* liver tissue, will be created [15]. This will be accomplished by adjusting the fibrinogen and thrombin concentrations, as well as the volume of fibrinogen deposited per area, and measuring the corresponding stiffness of the printed scaffold via Instron testing. One these programs have been developed, simultaneous printing of the cells and scaffold will be undertaken using the same procedure in *Aim 1*. The mechanical properties of a scaffold have been shown to have a profound impact on cell migration, adhesion, and survival [15]. Fibrin was selected as the extracellular scaffold because it has previously been shown to promote co-culture of hepatocytes and vessel-forming ECs [16]. Additionally, fibrin is thought to promote cell migration and proliferation due to the incorporation of platelet-derived growth factors [17]. These constructed fibrin scaffolds should ultimately mimic the stiffness of the liver extracellular matrix, promoting both sufficient cell adhesion and cell growth.

2. The same viability and proliferation tests will be performed as was done in *Aim 1*. These assays will seek to identify the optimal fibrin scaffold stiffness. There may be two optima for stiffness, one stiffness ideal for hepatocyte adhesion and proliferation, while the other ideal for EC and SMC viability and proliferation. However, it could be reasoned that increased proliferation of ECs and SMCs should lead to more efficient nutrient and waste exchange between hepatocytes and inter-vessel media, and therefore improved hepatocyte health. Thus, studies should reveal a particular stiffness at which a balance between hepatocyte viability and vascularization is attained.

Timeline

The first two months will be set aside to calibrate the printer, program the printing sequence as needed for the different layers, and prepare the cartridges for hepatocytes, ECs, SMCs, fibrinogen and thrombin. Once the printer is prepared, two months will then be devoted to printing the fibrin scaffold alone. During this period, we will adjust the fibrinogen and thrombin concentrations, as well as the volume of fibrinogen deposited, in order to tune the scaffold stiffness. As previously mentioned, we will later need to print cells onto scaffolds with elastic moduli of 10 – 100 kPa, in 10 kPa increments. Following scaffold fabrication, hepatocytes, ECs, and SMCs will be printed onto scaffolds of intermediate thickness, and these will be incubated and assayed. This phase of the project should require at least 6 months. The remainder of the project time will be used to rerun the experiments done for the first set of tissue-scaffold constructs but with the stiffness of the scaffold being altered by a 10 kPa increment. By the end of the research period we will have significant findings about the viability of the three types of cells as printed tissue as well as a well-defined method for printing tissue-scaffold constructs with stiffness that induces higher viability.

Future Directions

1. If bioreactor culture of the printed tissue is successful, these tissues could potentially be implanted within mice or another animal. Vascularization of the devices, and hepatocyte viability, could subsequently be observed.
2. A more complex tissue could be bioprinted, in which additional liver cell types, such as bile duct and sinusoidal cells, are printed along with vessel-forming cells. Additionally, liver extracellular matrix is a complex mixture consisting of collagens, non-collagenous glycoproteins, glycoaminoglycans, proteoglycans, matrix-bound growth factors, and matricellular proteins [16]. Printing of multiple liver ECM proteins could be used to fabricate a tissue that more closely mimics that of a natural liver.
3. Stem cells exhibit superior proliferative capabilities in comparison to other liver cell lines such as porcine, genetically modified, or adult hepatic cells. As of now, an efficient, reliable, and well-characterized means of consistently differentiating stem cells to functional hepatocytes has yet to be developed. Should such a technique be developed, printing of stem cells onto a scaffold with appropriate mechanical properties and growth factors for hepatic differentiation may be feasible.

References

1. "liver." *Encyclopedia Britannica. Encyclopedia Britannica Online Academic Edition.* Encyclopedia Britannica Inc., 2013. Web 13 Mar. 2013.

2. "Liver Cancer." *Liver Cancer, Cirrhosis, Tumors, Treatment*. The American Liver Foundation, 10 Oct. 2011. Web. 19 Apr. 2013. <http://www.liverfoundation.org/abouttheliver/info/livercancer/>.

3. "Liver Transplantation" *National Digestive Diseases Information Clearinghouse (NDDIC)*. U.S. Department of Health and Human Services, NIDDK, NIH, 23 Apr. 2012. Web. 19 Apr. 2013. <http://digestive.niddk.nih.gov/ddiseases/pubs/livertransplant/>.

4. Lai, Wai K., and Nick Murphy. "Management of Acute Liver Failure." *Continuing Education in Anaesthesia, Critical Care & Pai* 4.2 (2004): 40-43. Web.

5. OPTN/SRTR 2011 Annual Report. Available at: http://optn.transplant.hrsa.gov (Accessed 18 April 2013).

6. "Liver Disease Statistics." *Johns Hopkins Medicine*. Johns Hopkins University, Johns Hopkins Hospital, John Hopkins Health System, n.d. Web. 20 Apr. 2013. <http://www.hopkinsmedicine.org/healthlibrary/conditions/liver_biliary_and_pancreatic_disorders/liver_disease_statistics_85,P00686/>.

7. Sachs, Emanuel. *Three Dimensional Printing*. *Defense Technical Information Center*. Massachusetts Institute of Technology, Cambridge Department of Mechanical Engineering, 30 May 2001. Web. 19 Apr. 2013. <http://www.dtic.mil/cgi-bin/GetTRDoc?Location=U2&doc=GetTRDoc.pdf&AD=ADA400235>.

8. Xu T, Zhao W, Zhu JM, Albanna M, Yoo J, Atala A. Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology. *Biomaterials*, 2013, 34: 130-139.

9. Chung S, King MW. Design concepts and strategies for tissue engineering scaffolds. *Biotechnology and Applied Biochemistry*, 2013, 58(6): 423 – 38.

10. Miller J, Stevens K, Yang M, Baker B, Nguyen DH, Cohen D, Toro E, Chen A, Galie P, Yu X, Chaturvedi R, Bhatia S, Chen C. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials*, 2012, 11: 768 – 774.

11. Xu T, Jin J, Gregory C, Hickman J, Boland T. Inkjet printing of viable mammalian cells. *Biomaterials,* 2005, 26: 93 – 99.

12. Lovett M, Lee K, Edwards A, Kaplan D. Vascularization strategies for tissue engineering. *Tissue Engineering: Part B*, 2009, 15(3): 353 – 370.

13. Schreer A, Tinson C, Sherry J, Schrimer K. Application of alamar blue/5-carboxyfluroescein diacetate acetoxymethyl ester as a noninvasive cell viability assay in primary hepatocytes from rainbow trout

14. Behbahan I, Duan Y, Lam A, Khoobyari S, Ma X, Ahuja T, Zern M. New approaches in the differentiation of human embryonic stem cells and induce pluripotent stem cells toward hepatocytes. *Stem Cell Rev and Rep*, 2011, 7:748-759.

15. Peyton S. Lectures 12-14, ChE 590B, University of Massachusetts Amherst, 2012.

16. Xiong A, Austin TW, Lagasse E, Uchida N, Tamaki S, Bordier BB, et al. Isolation of human fetal liver progenitors and their enhanced proliferation by three-dimensional coculture with endothelial cells. *Tissue Eng Part A*, 2008; 14:995-1006

17. Cui, Xiaofeng and Boland, T. Human microvasculatature fabrication using thermal inkjet printing technology. *Biomataerials*, 2009, 30:6221-6227