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HUMAN MICROVASCULATURE FABRICATION USING THERMAL INKJET PRINTING TECHNOLOGY

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HUMAN MICROVASCULATURE FABRICATION USING THERMAL INKJET
PRINTING TECHNOLOGY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Xiaofeng Cui
December 2008

Accepted by:
Dr. Thomas Boland, Committee Chair
Dr. Delphine Dean
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ABSTRACT

The current tissue engineering paradigm is that successfully engineered thick tissues must include vasculature. As biological approaches alone such as VEGF have fallen short of their promises, one may look for an engineering approach to build microvasculature. With the advent of cell printing, one may be able to build precise human microvasculature with suitable bio-ink. Human Microvascular Endothelial Cells (HMVEC) and fibrin were studied as bio-ink for microvasculature construction. Endothelial cells are the only cells to compose the human capillaries and also the major cells of blood vessel intima layer. Fibrin has been already widely recognized as tissue engineering scaffold for vasculature and other cells, including skeleton/smooth muscle cells and chondrocytes. In our study, we comprehensively studied changes in heat shock protein expression and cell membrane morphogenesis in printed mammalian cells with thermal inkjet printers. The heat shock protein expression of the printed cells has minor difference between the untreated cells and lower than manually heated cells. The cell membrane of printed cells developed pores which allow small molecules such as propidium iodide and dextran molecules (up to 70kD) to pass. We then precisely fabricated micron-sized fibrin channels using a drop-on-demand polymerization. When printing HMVEC cells in conjunction with the fibrin, we found the cells aligned themselves inside the channels and proliferated to form confluent linings. The 3D tubular structure was also found in the printed patterns. We conclude that cell printing technology can be used for precise cell seeding in tissue engineering fabrication with minor effect and damages to the printed mammalian cells.

DEDICATION

I dedicate this dissertation to my mom Xinggu Yao, my dad Songchuan Cui, and my grandmother Dongxian Yang, for their unconditional love and endless support.

谨以此博士文献给我的母亲姚杏姑，父亲崔松传，和祖母杨冬仙，感谢他们无条件的爱和无限的支持。

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CHAPTER ONE

LITERATURE REVIEW

Introduction of Tissue Engineering

Millions of Americans suffer lost organs and tissues or end-stage organ failure every year. Although the transplantation of organs like heart, liver, and kidney is a highly successful therapy for the incurable end-stage diseases, the need for donor organs far exceeds the supply. There are more than 25% patients for liver or heart transplantation die while they are still on the waiting list in the United States alone each year (1). Some new surgical techniques have been developed due to this organ shortage, such as transplanting whole organs (e.g. kidneys) from living, related donors and splitting adult organs for transplant (e.g. a part of a liver or a lung from a parent to a child) (2). However, the problem of donor shortage still remains in spite of the excellent results with these well developed transplant techniques. This organ donor shortage prompted several approaches that were proposed to solve this problem: artificial mechanical organs, xenotransplantation, tissue engineering, and regenerative medicine (3). However, some artificial organs which are already available to patients showed significantly reduction of life quality and may have many unwanted side effects. Xenotransplantation sources are referred as organ donor sources from other species (e.g. pig). The idea of xenotransplantation becoming suitable in the long term still remains problems (4). Besides the immunological barrier, there is still great concern about potential spreading of animal viruses (5). However, tissue engineering completely avoids the risks of

immunological responses such as rejections (acute and chronic), as well as viral infections using autologous cells. This is why tissue engineering has been attracting more and more attentions since past two decades as a promising solution for this critical organ donor shortage issue.

Principles of Tissue Engineering

In definition, tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (6).

There are three general strategies accepted for the creation of new tissues. The first approach is isolated cells or cell substitutes. This strategy avoids the surgery complications and allows replacement of only the cells which supply the needed function and permits manipulation of the cells before infusion. Its potential limitations include failure of the infused cells to maintain their function in the recipient and immunological rejection. The second approach is tissue-inducing substance. The success of this strategy depends on the purification and large-scale production of the appropriate signal molecules including hormones and growth factors. It also depends on the development of the methods of delivering these signal molecules to their targets. The third approach is cell seeding on the porous biomaterial scaffolds. In closed systems, the cells are isolated from the body by a membrane which allows the nutrients and wastes permeation but prevents large molecules like antibodies or immune cells from destroying the transplant. In open systems, cells attached to the matrix scaffolds are implanted and become

incorporated into the body. The scaffolds can be natural materials such as collagen or synthetic polymers. Immunological rejection maybe prevented by immunosuppressive drugs or by using the autologous cells (7). The third strategy becomes the basic concept and tradition approach of tissue engineering. It includes a scaffold that provides architecture for cell seeding which can organize and develop into the desired tissues and organs in vitro before implantation. The biomaterial scaffolds can provide the initial biomechanical profile for the seeded cells until they fabricate their own natural extracellular matrix. During the formation, deposition and organization of the newly generated tissue matrix, the original scaffold is either degraded or metabolized; finally leaving a vital organ or tissue that restores, maintains, or improves tissue function (2). This approach should be distinguished from the guided tissue regeneration which uses acellular matrices that are repopulated by the host after implantation (6). For vascular prosthesis, autologous reseedling of a large surface area from the adjacent native tissue is seen commonly in animals but seems very limited in humans (8). Whether the observed autologous repopulation of acellular and unseeded matrices can be transferred to humans still seems highly speculative (9).

Scaffold Materials for Tissue Engineering

The most appealing approach in tissue engineering is utilizing a combination of patient's own cells with polymer scaffolds for lost or aged tissues and organs replacement. The tissue-specific cells are isolated from the patient and harvested in vitro. By incorporating the harvested cells into the three dimensional polymer scaffolds, the

cell-matrix combinations work as the natural extracellular matrices found in the tissues. The scaffold materials must be biocompatible and are designed to meet both biological and nutritional needs for the specific cell type involved in the tissue formation. These biomaterial scaffolds will deliver the incorporated cells to the desired site in the human body. Not only provide a space for new tissue formation, the scaffolds will also potentially control the structure and function of the newly engineered tissues (10, 11). Recently, various types of tissues are being engineered using this approach including fabricated bladder, skin, cartilage, artery, bone, ligament, and tendon. Several of these engineered tissues are now at or near clinical applications (12, 17). In addition, a variety of approaches have been introduced about transferring undifferentiated cells, like stem cells, into the desired cell phenotype in tissue engineering (18). Almost all tissue engineering scaffolds are polymer. The polymer mimics many properties of extracellular matrices found in tissues. Extracellular matrices are comprised of various amino acids and sugar-based macromolecules. Extracellular matrix brings cells together and controls the tissue structure, also regulate the function of the cells and allow the diffusion of nutrients, metabolites and growth factors (19). There are various types of polymers have been studied and utilized in tissue engineering today (20). The most widely used synthetic polymers are aliphatic polyesters including poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and their copolymers (PLGA) (21, 22). PGA was the first synthetic polymer used for the successful creation of new tissue (23). Although these polymers have a long history of use in the medical applications and are also considered safe in many cases by the FDA, the use of these types of polymer scaffolds requires surgical

procedures to make large incisions to enable placement of the polymer/cell constructs. An excellent alternative approach to cell delivery for tissue engineering is the use of polymers that can be injected into the body. This enables the transplantation of the cells and polymer scaffolds into patients' bodies in a minimally invasive matter. Hydrogels represent an important type of biomaterials in biotechnology and medicine because many hydrogels have excellent biocompatibility and minimal inflammatory responses, thrombosis, and tissue damage (24, 25). Hydrogels can also swell large quantities of water without the dissolution due to the hydrophilic and cross-linked structure. This gives hydrogels physical characteristics similar to soft tissues. In addition, hydrogels have high permeability for oxygen, nutrients, and other water-soluble metabolites. Hydrogels have been found numerous applications in medicine such as contact lens, biosensors, linings for artificial implants, and drug delivery devices (26, 27). Hydrogels currently used in tissue engineering are divided into two categories, which are natural and synthetic polymers.

Hydrogels from Natural Polymers

Collagen and Gelatin

Collagen is a main component of the extracellular matrices of mammalian tissues including skin, bone, tendon, cartilage, and ligament, and it is the most widely used tissue-derived natural polymer in tissue engineering. Physically cross-linked collagen gels offer a limited range of mechanical properties and are thermally reversible. Chemical cross-linking of collagen using glutaraldehyde (28) or diphenylphosphoryl azide (29) can

greatly improve the physical/mechanical properties. But these chemically cross-linked collagen gels are still short of physical strength, potentially immunogenic, and could be quite expensive (30). There could also be big variations between different collagen production batches. However, collagen meets many biological design parameters, since it is composed of specific combinations of amino acid sequences that are recognized by cells and degraded by enzymes secreted from the cells, like collagenase. This is why collagen has been widely used as tissue culture scaffolds or artificial skins due to the ready attachment of various cell types and the cell-based degradation. Cell attachment on collagen gel can also be tuned by chemical modification, including incorporation of fibronectin, chondroitin sulfate, or low level of hyaluronic acid into the collagen matrix (31). Collagen gels have been utilized for reconstruction of liver (32), skin (33), blood vessel (34), and small intestine (35).

Gelatin is denatured collagen. It is formed by breaking the natural triple-helix structure of collagen into single stranded molecules. There are two types of gelatin, gelatin A and gelatin B. Gelatin A is produced by acidic treatment before thermal denaturation. Gelatin B is processed by alkaline treatment that causes a high carboxylic content (36). Gelatin can form gels easily by changing the temperature of the solution. It is also widely used in tissue engineering applications due to the biocompatibility and ease of gelation. Gelatin gels can also be used for growth factor delivery to promote vasculature in the engineered tissue (37). However, gelatin also faces the weakness of the physical properties and many chemical modifications have been studied to improve the mechanical properties of gelatin gels (38, 39).

Hyaluronate

Hyaluronate is one component of glycosaminoglycan in natural extracellular matrix and plays an important role in wound healing. Hyaluronate can be covalent cross-linked into hydrogel using various types of hydrazide derivatives and radical polymerization of glycidyl methacrylate (40). Hyaluronate can be degraded by hyaluronidase, which exists in serum and cells (41). Although hyaluronate has been utilized widely in tissue engineering applications such as artificial skin (42), facial intradermal implants (43), wound healing (44), and soft tissue augmentation (43), it requires thorough purification to remove impurities and endotoxins that may potentially cause diseases or immune responses (45). In addition, hyaluronate gels typically have lower mechanical properties which cause their limited applications.

Fibrin

Fibrin plays a significant role in natural wound healing. Fibrin gel has been widely used as sealant and adhesive during surgery. Fibrin can be produced from the patients' own blood and used as an autologous scaffold for tissue engineering. Fibrin can be polymerized using fibrinogen and thrombin solutions at room temperature (46). Fibrin gels might promote cell migration, proliferation, and matrix synthesis through the incorporation of the transforming growth factor β and platelet derived growth factors (47). Fibrin has also been utilized in tissue engineering to engineer tissues with skeletal muscle cells (48), smooth muscle cells (49), and chondrocytes (50). However, the

limitation in mechanical properties prevents their applications. Lots of research has been going on to increase the mechanical properties of fibrin gels.

Alginate

Alginate is a well-known biomaterial from brown algae. Due to its biocompatibility, low toxicity, relatively low cost, and simple cross-linking with divalent cations such as Ca^{2+} , Mg^{2+} , Ba^{2+} , and Sr^{2+} , alginate has been widely used for drug delivery and tissue engineering scaffolds (51). Alginate can be used as an injectable cell delivery vehicle (52), wound dressing, dental impression, and immobilization matrix (53, 54). Alginate gel beads have been used for chondrocytes and hepatocytes transplantation (55).

Despite the advantages, alginate may not be an ideal biomaterial because its degradation is a process involving the divalent ions loss into the surrounding medium. This process is uncontrollable and unpredictable. Another potential disadvantage of alginate gels in tissue engineering is the lack of cellular interaction. Due to the hydrophilic character, alginate discourages protein adsorption and it is unable to specifically interact with mammalian cells (56). This is why alginate in tissue engineering is usually modified with lectin, a carbohydrate specific binding protein, to enhance ligand-specific binding properties (57). Another modification of alginate is covalently coupled with an RGD-containing cell adhesion ligand to enhance the cell adhesion. These alginate modifications provide the adhesion, proliferation, and differentiation of mammalian cells (58).

Agarose

Agarose is not like alginate and it forms thermally reversible gels (59). The physical properties and pore sizes can be mainly controlled using different agarose concentrations. Larger pore size and lower mechanical stiffness of the agarose gel at lower concentrations can enable the cell migration and proliferation (60). Cell adhesion peptides (CDPGYIGSR) have also been covalently coupled to agarose gel to enhance the interaction with cells (61).

Chitosan

Chitosan has many biomedical applications due to its biocompatibility, low toxicity, structural similarity to natural glycosaminoglycans and degradation by chitosanase and lysozyme (62). The disadvantage of chitosan is easily soluble in the acidic solution and generally insoluble in neutral conditions and most organic solvents due to the existence of amino groups and high crystallinity. Numerous derivatives have been processed to enhance the solubility and processibility of this polymer (63, 64), as well as the biological functions of chitosan, including enhancement of cellular interactions. Chitosan forms hydrogels by ionic or chemical cross-linking with glutaraldehyde (65, 66). Chitosan has also been modified with sugar residues such as fructose and galactose for hepatocytes culture (67, 68).

Hydrogels from Synthetic Polymers

Poly (acrylic acid) and its derivatives

Hydrolytically stable cross-linked poly (2-hydroxyethyl methacrylate) (HEMA) is one of the most studied synthetic hydrogels. The permeability and hydrophilicity can be controlled by the crosslinking agents (69). Although poly (HEMA) has many applications in tissue engineering, it is not degradable in physiological conditions. So dextran-modified poly (HEMA) gels have been synthesized and reported to be degradable by enzymes (70).

Poly (N-isopropylacrylamide) (PNIPAAm) exhibits phase transition behavior above the lower critical solution temperature (LCST) and is attractive for tissue engineering applications. The LCST of PNIPAAm is about 32 °C and can be adjusted to match the body temperature (e.g., 37 °C) by copolymerization (71). Therefore the application of PNIPAAm and its copolymers in tissue engineering is very promising as people can prepare a mixed cell solution and polymer at room temperature or at a lower temperature and then inject into the desired site. Then it will form a solid cell/polymer construct when the gel is warmed to body temperature. The unique temperature-responsive property of these polymers leads to various biological applications. When cells are cultured on PNIPAAm, it will be easier to recover intact cell sheets without damage by simply decreasing the temperature and modulating the hydrophilicity of the polymer (72). The phase transition property may be ideal for cell delivery since it simply depends on the temperature change after injected into the body. Limitations of these

polymers are nondegradable cross-links, and the monomers and cross-linking molecules are toxic and carcinogenic (73).

Poly (vinyl alcohol)

Poly (vinyl alcohol) (PVA) can be obtained from poly (vinyl acetate) by alcoholysis, hydrolysis, or aminolysis (74). PVA hydrogels can be formed using repeated freezing/thawing method (75) or electron beam (76) to avoid the toxicity and leaching problems of chemical cross-linking agents. Since these gels are not degradable in most physiological situations, PVA hydrogels are mostly used for long-term or permanent applications, like artificial articular cartilage (77) and bone-like apatite formation (78).

Polyphosphazene

Polyphosphazene have been used in many biomedical applications due to the biodegradable property in physiological situations. The degradation rate can be controlled by changing the side-chain structure instead of the polymer backbone (79). Polyphosphazene has alternating phosphorus and nitrogen atoms with two side groups attached to each phosphorus atom. Nonionic and ionic types of hydrogels can be prepared from polyphosphazenes. Nonionic gels are water-soluble. Ionic hydrogels have been used in controlled drug delivery due to their ability to respond to environmental changes such as pH or ionic concentration (80, 81). These polymers are useful for skeletal tissue regeneration (82).

Polypeptides

Since proteins are the major component for the natural matrices of tissues, there is wide interest in mimicking natural proteins by synthesizing polypeptides. However, to precisely control the desired amino acids sequence is very difficult and expensive. One way to bypass this problem is to synthesize genetically engineered polypeptides. People can transfer DNA templates with predetermined sequences into the genome of bacteria or yeasts to produce polypeptides with predetermined structure and controlled properties (83, 84). This method allows people to design and engineer various polypeptides with different physical/biological properties. Gly-Ala-rich sequence has been incorporated into the artificial proteins to enable reversible hydrogels according to pH or temperature change (85). However, this method is not appropriated to produce large amount of biomaterials economically. And any modification of the hydrogel will require the re-engineering of the entire genetic system.

Cells Used in Tissue Engineering

Almost all the tissue and organ types in mammals have been investigated by tissue engineering researchers. Some studies use nonspecific cell types, like fibroblast for heart valve tissue engineering (86), however, most are focusing on specific cell types for tissue engineering.

One basic approach to harvest cells is to obtain autologous organ-specific cells by biopsy. This approach applies to most organ structures, like skin, liver, blood vessels, bone, heart, bone marrow, and cartilage. But for some tissues or organs, this approach is

not applicable. Peripheral vein segments are a suitable cell source for heart valves. And for neural tissues, the approach to solve this cell-source difficulty is the isolation of stem cells. Stem cells can proliferate through multiple generations and can potentially differentiate into various cell types under proper culture conditions (87). Stem cells can be harvested from either embryonic tissue or adult tissue. Stem cells from embryonic fetal tissues may raise potential immunological and ethical issues. Recent research has been focusing on cells derived from autologous bone marrow or circulation stem cells or progenitor cells. Stem cells are totipotent and have the ability to differentiate into any cell types by definition (88). Progenitor cells are described as pluripotent since they are more differentiated than stem cells (89).

The importance to work with stem cells or progenitor cells is to know how to induce and control the cell to differentiate into the desired cell types. For any cells used in tissue engineering, it is critical to understand the mechanism of induction and control the differentiation/proliferation to obtain the functional cells. The other challenges like isolation and purification of certain cell type to obtain and maintain pure cell types. And in other hand, like tissue engineering of cardiac myoblasts requires co-culture with fibroblasts (90).

Current State of Tissue Engineering

Tissue engineering as an important interdisciplinary field in biomedical engineering has shown tremendous promises in creating biological alternatives for implants, harvested tissues, and prostheses (91). The basic concept for tissue engineering

is isolation of cells from a patient, and expanding the population in cell culture and seeding onto a biomaterial scaffold. The fabricated tissue engineering structure is then grafted back into the same patient to function as an introduced replacement tissue. The first Tissue Engineering sample was found as early as 1933 when Bisceglie implanted mouse tumor cells encapsulated in a polymer membrane into the abdominal cavity of chick embryos and showed survival of these cells (92). In 1990s, tissue engineering developed dramatically and no tissue or organ structures have been excluded from active research so far. However, only a few tissue engineered products have clinical trials, including artificial skin, cartilage, and bladders.

The major obstacle in tissue engineering of thick and complex tissue/organ is the induction of a proper nutrient and oxygen supply as well as waste removal. Although some avascular tissues, such as heart valve leaflets, can receive the nutrients by diffusion, the majority of tissues and organs depend on a complex microvascular system. The critical future challenge of creating such a system still remains unsolved.

Cardiovascular System and Microvasculature Tissue Engineering

Introduction of Cardiovascular System

The cardiovascular system is a transport system that carries blood and lymph to and from the tissues in the body. The constitutive elements of these fluids include cells, nutrients, waste products, hormones, and antibodies. Cardiovascular system includes the heart, the blood, and the blood vessels. A functional cardiovascular system is vital for survival since the tissues lack oxygen and nutrients, and waste products accumulate

without blood circulation. If cardiovascular system doesn't work well, the cells soon begin irreversible change, which quickly leads to death.

Blood

Blood is a unique connective tissue and the only fluid tissue in the body. It has both solid and liquid components. Living blood cells are the formed elements and suspended in a nonliving fluid matrix called plasma. The collagen and elastic fibers typical in other connective tissues are absent from blood, but dissolved fibrous proteins become visible as fibrin strands when blood clotting occurs.

Blood cells include erythrocytes, leukocytes and platelets. Erythrocytes normally constitute about 45% of the total volume of a blood sample. Leukocytes and platelets contribute less than 1% of blood volume and plasma makes up most of the remaining 55% of whole blood. Blood is denser than water and about five times more viscous due to its formed elements. Blood has a pH between 7.35 and 7.45. Blood accounts for approximately 8% of body weight. Its average volume in healthy adult males (5-6 L) is somewhat greater than in healthy adult females (4-5 L)

Blood cells originate in red bone marrow from hematopoietic stem cells. Platelets are from megakaryocytes. Erythrocytes are tiny and about 7.5 μm in diameter. They are biconcave discs which increase the surface area for gases diffusion. A red blood cell can readily squeeze through the narrow passages of capillaries because of its shape. Erythrocytes have hemoglobin which is responsible for the color of the blood as well as oxygen transportation.

Leukocytes protect body against disease. Some types of leukocytes have granular cytoplasm and make up a group called granulocytes. The others lack cytoplasmic granules and are called agranulocytes. A typical granulocyte is about twice the size of a red blood cell. They include neutrophils, eosinophils, and basophils. The nucleus of neutrophil is lobed and consists of two to five segments connected thin strands of chromatin. They are also called polymorphonuclear leukocytes. Neutrophils are the first white blood cells to arrive at an infection site. They phagocytize bacteria, fungi, and some viruses. Neutrophils account for 54% to 62% of the leukocytes in a typical blood sample from an adult. The nucleus of eosinophils usually has only two lobes. They moderate allergic reactions and defend against parasitic worm infestation. Eosinophils make up 1% to 3% of the total number of circulating leukocytes. Basophils migrate to damaged tissues where they release histamine to increase permeability of the capillaries and heparin to inhibit blood clotting. This increases blood flow to injured tissues. Basophils account for less than 1% of the leukocytes.

Monocytes and lymphocytes form the agranulocyte group of leukocytes. Monocytes are the largest blood cells. Their nuclei are spherical, kidney-shaped, oval, or lobed. Monocytes leave the bloodstream and become into macrophages which phagocytize bacteria, dead cells, and other debris in the tissue. They make up 3% to 9% of the leukocytes. The major types of lymphocytes are T cells and B cells, both important in immunity. T cells directly attack microorganisms, tumor cells, and transplanted cells. B cells produce antibodies, which are proteins that attack foreign molecules.

Lymphocytes account for 25% to 33% of the circulating leukocytes and may live for years.

Platelets are not complete cells. They adhere to exposed ends of injured blood vessels and any rough surface, particularly to the collagen in connective tissue underlying the endothelial lining of blood vessels. The shapes of platelets change drastically when they contact collagen. Platelets adhere to each other to form a platelet plug in the vascular break.

Heart

The cardiovascular system consists of a pump represented by the heart and blood vessels, which provide the route by which blood circulates to and from all the parts of the body. The heart contains four chambers, the right and left atria and right and left ventricles, through which blood is pumped. Heart valves guard the exits of the chambers, preventing the backflow of blood. Heart size varies with body size. But an average adult's heart is generally about 14 centimeters long and 9 centimeters wide.

The wall of the heart is composed of three distinct layers: an outer epicardium, a middle myocardium, and an inner endocardium.

Blood that is low in oxygen and high in carbon dioxide enters the right atrium through the venae cavae and the coronary sinus. As the right atrial wall contracts, the blood passes through the right atrioventricular orifice and enters the chamber of the right ventricle. When the right ventricular wall contracts, the tricuspid valve closes the right atrioventricular orifice, and the blood moves through the pulmonary valve into the

pulmonary trunk and its branches, which are pulmonary arteries. From these vessels, blood enters the capillaries associated with the alveoli (microscopic air sacs) of the lungs. Gas exchange occurs between the blood in the capillaries and the air in the alveoli. The freshly oxygenated blood, which is now relatively low in carbon dioxide, returns to the heart through the pulmonary veins that lead to the left atrium. The left atrial wall contracts, and the blood moves through the left atrioventricular orifice and into the chamber of the left ventricle. When the left ventricular wall contracts, the mitral valve closes the left atrioventricular orifice, and the blood passes through the aortic valve into the aorta and its branches.

Blood Vessels

The blood vessels are organs of the cardiovascular system, and they form a closed circuit of tubes that carries blood from the heart to cells and back again. These vessels include arteries, arterioles, capillaries, venules, and veins. The arteries and arterioles conduct blood away from the ventricles of the heart and lead to the capillaries. The capillaries are sites of change of substances between blood and the body cells, and the venules and veins return blood from the capillaries to the atria.

Arteries are strong, elastic vessels that are adapted for carrying the blood away from the heart under high pressure. These vessels subdivide into progressively thinner tubes and eventually give rise to the finer branched arterioles. The wall of an artery consists of three distinct layers. The innermost layer, tunica intima, is composed of a layer of simple squamous epithelium, called endothelium, which rests on a connective

tissue membrane that is rich in elastic and collagenous fibers. The endothelial lining of an artery provides a smooth surface that allows blood cells and platelets to flow through without being damaged. Additionally, endothelium helps prevent blood clotting by secreting biochemicals that inhibit platelet aggregation. Endothelium also may help regulate local blood flow by secreting substances that either dilate or constrict blood vessels. The middle layer, tunica media, makes up the bulk of the arterial wall. It includes smooth muscle fibers, which encircle the tube, and a thick layer of elastic connective tissue. The connective tissue gives the vessel a tough elasticity that enables it to withstand the force of blood pressure and at the same time, to stretch and accommodate the sudden increase in blood volume that accompanies ventricular contraction. The outer layer, tunica adventitia, is thin and consists of loose connective tissue with irregularly organized elastic and collagenous fibers. This layer attaches the artery to the surrounding tissues. It also contains vasa vasorum that give rise to capillaries and provide blood to the more external cells of the artery wall.

Although the walls of the larger arterioles have three layers similar to those of arteries, the middle and outer layers thin as the arterioles approach the capillaries. The wall of a very small arteriole consists only of an endothelial lining and some smooth muscle fibers, surrounded by a small amount of connective tissue. Arterioles, which are microscopic continuations of arteries, join capillaries. Capillaries are the smallest diameter blood vessels. They connect the smallest arterioles and the smallest venules. Capillaries are extensions of the inner linings of arterioles in that their walls are endothelium, which is a single layer of squamous epithelial cells. These thin walls form

the semipermeable layer through which substances in the blood are exchanged for substances in the tissue fluid surrounding body cells. The higher a tissue's rate of metabolism, the denser its capillary networks. Muscle and nerve tissues, which use abundant oxygen and nutrients, are richly supplied with capillaries. The cartilaginous tissues, the epidermis, and the cornea, where metabolism is slow, lack capillaries. The patterns of capillary arrangement also differ in various body parts. Some capillaries pass directly from arterioles to venules, but others lead to highly branched networks. Such physical arrangements make it possible for the blood to follow different pathways through a tissue that are attuned to cellular requirements. Blood flow varies among tissues. During exercise, blood is directed into the capillary networks of the skeletal muscles, where the cells require more oxygen and nutrients. At the same time, the blood bypasses some of the capillary networks in the tissues of the digestive tract, where demand for blood is less critical. Conversely, when a person is relaxing after a meal, blood can be shunted from the inactive skeletal muscles into the capillary networks of the digestive organs. The distribution of blood is mainly regulated by the smooth muscles that encircle the capillary entrances. The vital function of exchanging gases, nutrients, and metabolic by-products between the blood and the tissue fluid surrounding body cells occurs in the capillaries. The biochemicals exchanged move through the capillary walls by diffusion, filtration, and osmosis. Diffusion is the most important means of transfer. Blood entering certain capillaries carries high concentrations of oxygen and nutrients, these substances diffuse through the capillary walls and enter the tissue fluid. Conversely, the concentrations of carbon dioxide and other wastes are generally greater in the tissues,

and such wastes tend to diffuse into the capillary blood. Normally, more fluid leaves the capillaries than returns to them. Lymphatic vessels collect the excess fluid and return it to the venous circulation.

Venules are the microscopic vessels that continue from the capillaries and merge to form veins. The veins, which carry blood back to the atria, follow pathways that roughly parallel those of the arteries. The walls of veins are similar to those of arteries in that they are composed of three distinct layers. However, the middle layer of the venous wall is poorly developed. So veins have thinner walls that contain less smooth muscle and less elastic tissue than those of comparable arteries, but their lumens have a greater diameter. Many veins contain flaplike valves, which project inward from their linings. The valves are pushed closed if the blood begins to back up in a vein. These valves aid in returning blood to the heart because they are open as long as the flow is toward the heart but close if it is in the opposite direction.

The blood vessels can be divided into two major pathways. The pulmonary pathway consists of vessels that carry blood from the heart to the lungs and back to the heart. The systemic pathway carries blood from the heart to all parts of the body, except the lungs, and back to the heart again.

Human Endothelial Cells and Angiogenesis

Endothelial cells form the lining of blood vessels and have a remarkable capacity to adjust their number and arrangement to suit local requirements. Almost all tissues depend on a blood supply and the blood supply depends on endothelial cells. They create

an adaptable life-support system spreading into almost every region of the body. Endothelial cells extending and remodeling the network of blood vessels makes it possible for tissue growth and repair. The largest blood vessels are arteries and veins, which have a thick, tough wall of connective tissue and smooth muscle. The wall is lined by an exceedingly thin single layer of endothelial cells, separated from the surrounding outer layers by a basal lamina. The amounts of connective tissue and smooth muscle in the vessel wall vary according to the vessel's diameter and function, but the endothelial lining is always there. In the finest branches of the vascular tree, like capillaries, the walls consist of nothing but endothelial cells and a basal lamina. Thus endothelial cells line the entire vascular system, from the heart to the smallest capillary, and control the passage of materials and the transit of leukocytes into and out of the bloodstream (93).

Throughout the vascular system, endothelial cells have the ability for cell division and movement. If a part of the wall of the aorta is damaged and denuded of endothelial cells, neighboring endothelial cells proliferate and migrate in order to cover the exposed surface. Newly formed endothelial cells will even cover the inner surface of plastic tubing used by surgeons to replace parts of the damaged blood vessels. Endothelial cells not only repair the lining of damaged blood vessels, they also create new blood vessels. They must do this in embryonic tissues to keep pace with growth, to support recurrent cycles of remodeling and reconstruction, and to repair the damaged adult tissues (94).

New vessels always originate as capillaries, which sprout from existing small vessels. The process of angiogenesis occurs in response to specific signals. Endothelial cells will form a new capillary grow out from the side of a capillary or small venule by

extending long processes or pseudopodia. The cells at first form a solid sprout, which then hollows out to form a tube. The process continues until the sprout encounters another capillary, with which it connects and allowing blood to circulate. Experiments in culture show that endothelial cells in a medium containing suitable growth factors will spontaneously form capillary tubes even if they are isolated from all other types of cells. The first sign of tube formation in culture is the appearance of an elongated vacuole in cytoplasm. Contiguous cells develop similar vacuoles, and eventually the cells arrange their vacuoles end to end so that the vacuoles become continuous from cell to cell to form the capillary channel. Formation of capillary tubes is promoted by basal lamina components, such as laminin, which the endothelial cells themselves can secrete. The capillary tubes that develop in a pure culture of endothelial cells do not contain blood, and nothing travels through them, which indicates that blood flow and pressure are not required for the formation of a capillary network (95, 96).

Angiogenesis is controlled by growth factors released by the surrounding tissues. In living animals endothelial cells form new capillaries wherever there is a need for them. It is thought when cells in tissues are in need of oxygen, they release angiogenic factors that induce new capillary growth. Similarly, after wounding a burst of capillary growth is stimulated close to the damaged tissue. Angiogenesis is also important in tumor growth. The growth of a solid tumor is limited by its blood supply. To grow further, a tumor must induce the formation of a capillary network that invades the tumor mass. So the invading endothelial cells must respond to a signal produced by the tissue that requires a blood supply. The response of the endothelial cells includes at least four components. First, the

cells must breach the basal lamina that surrounds an existing blood vessel. Endothelial cells during angiogenesis have been shown to produce protease, which enable them to digest their way through the basal lamina of the parent capillary or venule. Second, the endothelial cells must migrate to the source of the signal. Third, they must proliferate. Fourth, they must form tubes. There are identified growth factors that can evoke all four components of the angiogenic response together. The foremost among these factors is vascular endothelial growth factor (VEGF). This acts selectively on endothelial cells to stimulate angiogenesis in many different circumstances, and it seems to be the agent by which some tumors acquire their rich blood supply. Other growth factors like some members of fibroblast growth factor family also stimulate angiogenesis but at the same time influence other cell types besides endothelial cells. These growth factors are released during tissue repair, inflammation, and tissue growth. They are made by various cell types, including macrophages, mast cells, and fat cells. A number of natural inhibitors have also been found that can block the formation of new blood vessels. Angiogenesis seems to be regulated by complex combinations of signals rather than by one signal alone (97).

Microvasculature Tissue Engineering

Tissue engineering still faces many challenges, including the isolation and proliferation of appropriate cell types, the arrangement of various cell types into the correct spatial organization and creation of the optimal microenvironments for growth and differentiation. To date, the most successful tissue engineering applications are

restricted to relatively thin or avascular structures, like skin and cartilage. Those tissues have relatively low requirements for nutrients and oxygen and can be supported by diffusion from the host vasculature. Development of an artificial microvasculature is a critical obstacle to engineer thicker, metabolically demanding organs, such as heart, kidney, lung and liver. These organs have high metabolic rates and further limit the maximal thickness of engineered tissues. By building capillary networks within the engineered tissues can help to transport oxygen and nutrients also to remove the waste (98). The ability to microvascularize tissue constructs would be a significant step forward in tissue engineering and regenerative medicine. Tissue-engineered organs rely mainly on host vasculature for oxygen, nutrients, and waste removal without proper vascularization (99, 100). When the thickness of engineered tissue exceeds the limit of 100-200 μm , it must overcome the challenge of creating the functional blood vessels to supply the cells with oxygen and nutrients and also to remove the waste products from the tissue. Without an intrinsic capillary network, the maximal thickness of engineered tissue is approximately 150-200 μm because of oxygen diffusion limitations (101, 102). This is why microvascularization is so important for engineered tissue constructs before implantation. Microvascularization in vitro can maintain the cell viability during the tissue growth, induce the structural organization and promote the angiogenesis after implantation.

For biomaterial scaffolds, it is a critical challenge of how to use the polymers to promote the capillary network formation in the fabricated tissues. It is important to provide the nutrient and oxygen transport to the engineered tissues and also integrate

them with the rest of the body (103). One important approach for microvascularization is to deliver either angiogenic factors or capillary forming cells (e.g., endothelial cells) to the engineered site using hydrogel. The controlled and sustained release of angiogenic factors from hydrogels can optimize and promote the localized blood vessel formation. Various growth factors including vascular endothelial factor (VEGF) (104), epidermal growth factor (EGF) (105), basic fibroblast growth factor (bFGF) (106), and bone morphogenetic protein (BMP) (107) could be incorporated into hydrogels depending on the specific tissue types. Additionally, the delivery of DNA plasmid containing genes encoding angiogenic proteins may be another solution to increase the vascular network formation in engineered tissues (108). Co-transplantation of endothelial cells along with the primary cell type of interest may allow people to rapidly form microvasculature in the engineered tissues. This is because the endothelial cells will form tube-like structures in vitro if cultured in an appropriate condition (109).

Another approach is endothelialized microvasculature based on a biodegradable elastomer. The capillary networks were microfabricated with a biodegradable and biocompatible elastomer, poly (glycerol sebacate) (PGS). The capillary patterns were etched onto silicon wafers by standard microelectromechanical systems (MEMS) techniques. The resulted silicon wafers served as micromolds for the devices. Then the patterned PGS film was bonded to a flat film to create capillary networks that were perfused with a syringe pump at a physiological flow rate. The devices were endothelialized under flow conditions, and part of the lumens reached confluence within 14 days of culture (110).

The best studied growth factor for therapeutic angiogenesis is vascular endothelial growth factor (VEGF). However, VEGF alone is not sufficient to form mature and stable vasculature (111). In the study of using injection of adenoviral vectors to express VEGF in normal tissue, the newly formed blood vessels were disorganized, leaky and hemorrhagic. VEGF may also initiate inflammation by increasing the expression of adhesion molecules on endothelial cells or the release of chemokines. The newly formed blood vessels may not be adequate to the tissues' metabolic need.

Cell and Organ Printing

The traditional tissue engineering approach is using porous biomaterial scaffolds seeded with isolated autologous cells from the patient, culturing the constructs in bioreactor, and implanting the resulting cell/biomaterial complex back to the patient. However, it is a critical challenge to rapidly fabricate tissue and organs with well defined structures and functions in tissue engineering (112). Engineered tissue constructs require spatial control over cells in three dimensional spaces to assemble and organize them into a functional structure (113). The formation of 3D cellular structures requires control of both cells and scaffold components (114). All the methods currently being investigated for 3D cellular patterning have the advantages as well as large limitations.

Introduction of Inkjet Printing

Because of the large limitations for the old 3D cell matrix fabrication methods, researchers have been recently looking at the modified off-the-shelf inkjet printers.

Inkjet printing is a non-contact printing technique. By receiving the digital image or character data from computer, inkjet printers have the ability to reproduce the data onto substrate (e.g., paper) with tiny ink drops (115). Inkjet printers can drop ink continuously or drop-on-demand. Drop-on-demand means the ink drops are ejected only where and when they are required to create the images on the substrate. The ink stream is broken into equal-sized droplets by applying a piezoelectrically modulated pressure wave behind the nozzle (116). There are thermal, piezoelectric, and electromagnetic methods for creating drops. Most inkjet printers are thermal and piezoelectrical printers and the operating frequencies are about 10 KHz. For thermal inkjet printers, little air bubbles are created by heating and then collapse to provide the pressure pulse to eject a tiny drop of ink out of the nozzle. As for piezoelectric inkjet printers, the actuator of polycrystalline piezoelectric ceramic in each nozzle provides the transient pressure to eject the ink drops onto the substrate (115).

Similar printing mechanisms are used in both standard Hewlett-Packard and Canon commercial inkjet printers. Inkjet printing technique was invented in 1979 at HP laboratories. At the same time, Canon invented their “BubbleJet”. The first inkjet product appeared on the market in 1984. The inkjet printer has high operating frequency, high orifice density, integrated power, and interconnect electronics. In thermal inkjet printers, when a small heating element heats, the expanded air bubble will eject a very small drop of ink out of the nozzle. The current pulse lasts a few microseconds and raises the plate temperature as high as 300 °C (117).

Inkjet printing technology has also been widely used in electronics and micro-engineering industries for printing electronic materials and complex integrated circuits (118). Recently, inkjet technology has been successfully applied into biomedical field, such as drug screening, genomics, and biosensors (119-121). Although biological molecules and structures are usually thought to be fragile, DNA molecules have been directly printed onto glass slides using commercial inkjet printers for fabrication of high-density DNA microarrays without degradation (122).

Recently, a novel application of inkjet printing is using the commercial available inkjet printers to print cells and biomaterials in order to fabricate 3D cellular scaffolds (123). It showed that the standard HP and Canon desktop inkjet printers can be modified to perform cell printing. Organ printing, defined as computer-aided inkjet based tissue engineering, has the advantages to construct 3D structures with living biological elements. An important advantage of this process is the ability to simultaneously deposit living cells, nutrients, growth factors, therapeutic drugs along with the biomaterial scaffolds at the right time and location (124). Based on the properties of high-throughput efficiency, cost-effective, and fully automated, the cell and organ printing has become a promising approach for tissue engineering.

Cell Printing Using Thermal Inkjet Technology

The Hewlett-Packard Deskjet 500 inkjet printer has the droplet volume of 130 pL. There are 50 firing nozzles on the printer head and the actual heating occurs during 10- μ s pulse. The energy supplied to the ink during the printing process is transferred into

kinetic energy and heating of the ink drop. Mathematical modeling studies indicated that the bulk drop temperature in the ink rises between 4 and 10 degrees above ambient during printing. This makes it possible for printing living systems (125). It has been proved successful to print cell suspensions.

These commercial available inkjet printers can be modified for cell printing (126). During cell printing, the paper feed sensor mechanism of the printer is adjusted and the printer is UV sterilized and placed inside a sterile hood. A sterile hydrogel sample was placed on a stage below the printer head. The ink cartridges are modified and cleaned for cell suspension. They are rinsed thoroughly using 70% ethanol multiple times for sterilization. This procedure was proved sufficient sterilization for all the experiments (125). Quail mesoderm QCE-6 stem cells were printed successfully into specific patterns on 2 mg/mL collagen hydrogels using modified HP Deskjet 500 with clean and sterilized HP 51626A ink cartridge. The stem cells were found to proliferate on the collagen gel surface and to emit green fluorescence because of the GFP expression. This indicated the live cells. Fewer cells were observed individually at the early stage. After 6 days of culture, the number of cells increased. And after 12 days, most cells were found. These evidences indicated that the printed QCE-6 stem cells could maintain their viability and proliferate on the collagen gel after being printed (127).

By replacing the regular printing ink with the cell suspensions, this can be considered as bio-ink which could be printed using a commercial thermal inkjet printer. Because the mammalian cells are very sensitive to heat and mechanical stress, there was a major concern that the cells could be damaged or killed by the printing process.

However, the studies proved that many viable cell types can be delivered using modified inkjet printers (125).

3-D Printing Using Modified Thermal Inkjet Printers

In order to build 3D biomaterial scaffold, an automatically controlled elevator stage with z-axis controlled moving platform was designed (127). A round glass cover-slip with 2.5 cm diameter was fixed onto the top of a metallic rod and controlled by a stepper motor. The motor was powered by a 4 V signal and operated through a series of four toggle switches. A modified sterile 50 mL or 15 mL sized conical tube was used as chamber to house the elevator stage. Sterile silicone gel was used for sealant.

For 3D alginate scaffold formation, alginate solution with certain concentration was filled into the chamber. The crosslink solution was filled into the modified ink cartridge (128). In this case, 0.25 M CaCl_2 was filled into the ink cartridge. It can promote the cross-linking of the individual alginate chains to form the 3D structure. This cross-linker was printed layer-by-layer to form the 3D alginate structure. After the cross-linking occurred on the first layer, the platform was submerged into the uncross-linked alginate solution for a next layer formation on the previous layer. The procedure was repeated until the desired structure was achieved. This printing method may apply to smaller structures since larger one may cause deformation or collapse. Many biomaterial scaffold structures can be fabricated using this method, such as tubes or branched tubes (127).

Simultaneous Deposition of Biomaterials and Cells

People can use modified thermal inkjet printers to print cells and biomaterials individually. Simultaneous deposition of endothelial cells and biomaterial scaffold fabrication can also be achieved using the modified inkjet printers. The idea is to mix the cells along with the cross-linker in the ink cartridge. When printing the patterns, the mixed cells can be printed and aligned into the cross-linked biomaterial patterns. The embedded cells can then attach onto the printed biomaterial pattern and proliferate along the pattern. Eventually a 2D or 3D cell/biomaterial construct can be fabricated (127).

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CHAPTER TWO

PROJECT RATIONALE

The traditional approaches in tissue engineering have limitations in precise cell seeding and penetration to the fabricated biomaterial scaffolds (1). It is also difficult to fabricate vascularization into thick engineered constructs (2) and assemble multi-cellular heterogeneous tissue structures (3). The current rapid prototyping techniques, such as stereolithography (4-7), fused deposition modeling (8-9), and 3D printing[®] (10-11) are difficult to apply to tissue engineering because most of them require high temperature and harsh organic chemical (12), which are unfriendly factors to cells of interest and may result into apoptosis (13). To address these issues, we have developed thermal inkjet printing technology for printing cells and biomaterial scaffolds (14-15). It is hypothesized the modified commercial inkjet printers can simultaneously deliver cells and biomaterials. The printed endothelial cells will be printed and aligned into the fabricated biomaterial scaffold channel for human microvasculature fabrication. The printed endothelial cells will finally proliferate and grow into confluent linings which are the capillaries. By layer-by-layer inkjet printing technology we can finally fabricate a capillary tree into the thick engineered tissues, like heart, lung, and livers.

First we are going to explore the feasibility of using the modified thermal inkjet printers to print living cells and biomaterial scaffolds. This includes the study of the printing capability of printing mammalian cells using modified thermal inkjet printers; the possible genetic and morphogenetic changes and damages for the printed cells

developed during the thermal printing process. If the cells survive after the printing, how fast it takes the cells to repair the developed pores. After assuring the safety of printing process for cell and organ printing, we will continue to use the modified thermal inkjet printers for human microvasculature fabrication using human microvascular endothelial cells and appropriate biomaterial scaffolds, like fibrin.

Objective 1

Printing Capability Study and Evaluation of the Possible Damages to the Printed Cells Using Modified Thermal Inkjet Printers

Hypothesis

It was hypothesized commercial thermal inkjet printers could be modified for precise deliveries of living cells and cell-friendly biomaterial scaffolds into predefined patterns. The printed cells have high viable rate however there may be cell membrane pores developed during the printing process. These developed pores may be repaired shortly after the printing. Cell printing using modified thermal inkjet printers can be applied not only for tissue engineering, it can also be used for plasmids transfer and targeted drug delivery to the printed mammalian cells.

Approach

HP Deskjet 500 printers were modified specially for cell and biomaterials printing. Chinese hamster ovary cells (CHO) were printed for printing capacity study of

the modified HP 51626A ink cartridges and the printer. Furthermore, the heat shock protein 70 expression of the printed CHO cells were examined comparing the heat shock protein expressions from the manually heated CHO cells and the untreated cells. Possible developed pores in cell membrane were also studied using fluorescent labeled dextran molecules with different molecule weight. The cell membrane healing time of the printed CHO cells were also studied using the same procedure.

Objective 2

Construction and Confocal Evaluation of Human Microvasculature by Thermal Inkjet Printing Technology

Hypothesis

It was hypothesized that human endothelial cells and biomaterial scaffold can be simultaneously deposited into specific microvasculature patterns using modified thermal inkjet printers. The printed endothelial cells would be printed and aligned into the printed biomaterial scaffold channel and proliferate to align a confluent lining after culturing for certain time period. The printed endothelial cells were able to show tubulogenesis or angiogenesis inside the printed biomaterial scaffold which could be confirmed by laser scanning confocal microscope.

Approach

Human microvascular endothelial cells and fibrin biomaterial scaffold were printed by the modified thermal inkjet printers. The printed microvasculature was cultured using MCDB-131 media for 21 days before evaluated by immunostaining and live/dead assay. The endothelial cells were entrapped in fibrin channel to construct the designed microvasculature. The fluorescent staining and confocal microscope were helped to show the tubular structure of the microvasculature. Scanning electron microscope was also used to study the channel structure of the printed fibrin scaffold and the surface for cells to attach. The mechanical properties of the printed microvasculature were also tested for future handling and suturing.

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CHAPTER THREE

HEAT SHOCK PROTEIN EXPRESSION AND CELL MEMBRANE DAMAGE INDUCED BY THERMAL INKJET PRINTING OF CHINESE HAMSTER OVARY CELLS

Introduction

The traditional approach of tissue engineering is to manually seed cells into fabricated biomaterial scaffolds for artificial tissue or organ fabrication (1). There has been growing interest in looking for other approaches for more rapid and precise cell seeding and biomaterial scaffolds fabrication technologies (2), including cell and organ printing which was introduced in 1999 showed great promise for improved cell seeding in tissue engineering applications (3). Drop-on demand cell printing has the advantage to combine high through-put, digital control and high precision placement (4). There are thermal, piezoelectric, and electromagnetic methods for creating drops on demand (5); most inkjet printers employing heat or a mechanical compression. In thermal inkjet printers, small air bubbles are created by heating that then collapse to provide the pressure pulse to eject a 10-150 picoliter drop of ink out of the nozzle (6-8). The sizes of droplets are various according to the applied temperature gradient, frequency of the current pulse, and the sample viscosity (6-8). As for piezoelectric inkjet printers, the actuator of polycrystalline piezoelectric ceramic in each nozzle provides the transient pressure to eject the ink drops onto the substrate (9). Because of the well documented damages to the cell wall and cell lysis after sonication at 15-25 kHz (10), which is in the

range of frequencies employed by piezoelectric inkjets, we focused on the use of thermal inkjet printers to eject mammalian cells (11-14).

In thermal inkjet printers, the heat used to generate a bubble is maybe of a concern as many biological molecules denature at temperatures above 37 °C. When cells are undergoing sudden increased temperature or other kinds of stresses, cells will express elevated amount of heat shock proteins to protect themselves from the damages (15). This self-defense mechanism for cells was first reported for drosophila cells in 1974 (16). Heat shock proteins are a group of proteins which exist in cells under normal conditions but whose expression will be elevated when the environment changes suddenly (17). Those protein expressions are triggered by heat shock factor which is a key element during heat shock response (18). Heat shock proteins, which are named according to their molecular weight, protect cells by stabilizing other proteins, preventing their denaturation (19) and can bind to denatured proteins preventing their aggregation (20). In order to quantify the intensity of heat and stress to the printed mammalian cells during the printing process, we examined the expression of Heat shock protein 70 (Hsp70), one of the most widely studied heat shock proteins with a molecular weight of 70 kD (17).

In addition to the Hsp70 expression, we also observed the appropriate cell concentration for the best printing results, and changes or damages to cell membranes of the printed cells.

Materials and Methods

Materials

Dulbecco's modified eagle medium (DMEM), fetal bovine serum, penicillin and streptomycin, sodium bicarbonate, Dulbecco's phosphate buffered saline solution (DPBS), trypsin-EDTA, fibronectin were from Sigma Chemicals (St. Louis, MO, USA). Rat tail Collagen I was from BD Biosciences (San Jose, CA, USA). Live/Dead Viability/Cytotoxicity Kits for mammalian cells were purchased from Invitrogen (Carlsbad, CA, USA). Mouse monoclonal antibody to heat shock protein 70 (Hsp70) were obtained from Abcam Inc (Cambridge, MA, USA). 10X cell lysis buffer, WesternBreeze chromogenic western blot immunodetection kit, propidium iodide, dextran Texas Red 3000 MW, dextran Texas Red 10,000 MW, dextran Texas Red 40,000 MW and dextran Texas Red 70,000 MW were from Invitrogen (Carlsbad, CA, USA). 30% Acrylamide/Bis solution, PVDF (polyvinylidene difluoride) membrane, the Mini-Protean 3 cell, and Trans-Blot electrophoretic transfer cell were acquired from BioRad (Hercules, CA, USA). The Centra-8R centrifuge was bought from International Equipment Company (Needham, MA, USA). The microprocessor controlled 280 series water bath was purchased from Precision Scientific (Winchester, VA, USA) and the Z2 Coulter Counter was obtained from Beckman Coulter, Inc (Fullerton, CA, USA). The HP DeskJet 500 inkjet printer and 51626A black ink cartridges were manufactured by Hewlett-Packard Company (Palo Alto, CA, USA), and the LSM 510 confocal laser scanning microscope was built by Carl Zeiss MicroImaging (Thornwood, NY, USA).

Cell Culture and Cell Solutions

A green fluorescent protein (GFP) expressing Chinese hamster ovary (CHO) cell line kindly provided by Dr. Gabor Forgacs was used for heat shock protein study of printed mammalian cells(21). CHO cells were cultured with DMEM media and incubated at 37°C with 5% CO₂ for 14 days before passaging. Media was changed every two days. A cell pellet was collected using centrifugation at 1000rpm for 5 minutes. The supernatant was aspirated and the cell pellet was resuspended using 1 ml DPBS. The cell concentration was determined using a Coulter counter and DPBS added to achieve the desired final concentration between 2×10^6 and 20×10^6 cells/ml.

Ink Cartridge and Printing

A new HP 51626A black ink cartridge was used for each cell concentration. 2mg/ml collagen gel was prepared from rat tail collagen 1 using ice cold DPBS and neutralized with sterile 1N NaOH. Collagen solution was held on ice before use. The collagen solution was pipetted onto a microscope cover slip using a cold pipette tip which was then placed in an incubator at 37°C for 30 minutes to allow gelation. Cell suspensions were pipetted into a cartridge as described elsewhere (22) and the collagen coated cover slip placed below the cartridge inside a sterile hood. Each of the 50 inkjet nozzles of the cartridge were fired twice while the printer head was moving across the cover slip causing 100 drops to be ejected; this was repeated a total of three times for each cell concentration. The samples were then incubated for 20 minutes before DMEM media was carefully added onto the samples. Samples were incubated for 12 hours before

analysis, to avoid cell proliferation to influence the cell count. The cells were then imaged by fluorescence microscopy and counted.

Cell Printing and Protein Sample Preparation

In order to study Hsp70 expression of printed CHO cells, 5 samples were prepared. As positive controls, we used cells that were heated to 43°C for two hours; cells that were pipetted instead of printed were negative controls. Cells were printed into petri dishes as described above continuously until a pattern of drop of several mm in diameter was visible. Cells were diluted with 1 ml DPBS then collected into an Eppendorff tube. The samples were centrifuged at 1000rpm for 5 minutes and the supernatant aspirated. A volume of 220 µl DPBS was used to resuspend the cell pellet. Of this suspension, 20 µl was used for cell counting to determine the cell concentration in the suspension. The remainder was equally divided into two Eppendorff tubes. Two control samples were prepared by diluting cell concentrations in DPBS to the identical numbers as the printed samples. One printed cell sample and one control cell sample were heated to 43 °C for 2 hours in a water bath. One sample containing no cells but DPBS was also prepared. Following, 10 µl 10X cell lysis buffer was added into all five samples; the samples were incubated at 37°C for 45 minutes, and then centrifuged at 1000rpm for 5 minutes. Finally, 50 µl protein supernatant of each tube was collected and transferred to a new Eppendorff tube for Western blot analysis.

Heat Shock Protein 70 Expression Analysis

WesternBreeze chromogenic western blot immunodetection kit and mouse monoclonal antibodies to Hsp70 were used for western blots. First, 18 μ l protein solution from each tube was mixed with 6 μ l 4X reducing sample buffer. The mixed protein samples along with marker were boiled for 5 minutes, added into the wells of polyacrylamide gel (SDS-PAGE) and assembled in the Mini-Protean 3 cell. A voltage of 100V was used to run the electrophoresis for 150 minutes in ice water. After the electrophoresis, the SDS-PAGE was taken out and soaked in transfer buffer for 15 minutes before transferred to a sandwich assembly with PVDF membrane. This sandwich assembly was then inserted into Trans-Blot electrophoretic transfer cell and 30V voltage was used to transfer protein band from SDS-PAGE to PVDF membrane in a 4°C cold room overnight. After transfer, the membrane was washed using distilled water for 5 minutes twice to remove gel, transfer buffer components and some weakly bound proteins. Then the membrane was blocked for 30 minutes on rotary shaker. The primary antibody solution was made using mouse monoclonal antibody to Hsp70 and blocking solution at a ratio of 1:5000 following the antibody as per manufacturer's protocol. The PVDF membrane was incubated in the primary antibody solution for 1 hour and in the secondary antibody solution for 30 minutes. The membrane was then incubated with the chromogenic substrate until purple bands developed on the membrane, before drying on a clean piece of filter paper in open air.

Membrane Permeability Study

We evaluated the effect of printing on the integrity of cell membranes as well. Texas Red dextran molecules with different molecular weights (3000 D, 10,000 D, 40,000 D, and 70,000 D) were used to stain printed CHO cells in order to assess whether pores had developed in the cell membrane. Cell suspension for printing was prepared as mentioned above. Texas red dextran molecules were first dissolved in DPBS at 1mg/ml stock concentrations. The working solutions to stain the printed cells were diluted 1:1000 from the stock solutions. Microscope cover slips were coated using 10 µg/ml fibronectin for 2 hours at 37°C. The cells were printed onto the fibronectin coated microscope cover slips using HP Deskjet 500 thermal inkjet printer as described above. Negative controls were prepared using cells manually pipetted onto the microscope cover slips. After incubation at 37 °C for various times ranging from 15 min to 2 hours, dextran solutions were carefully added to the cover slips. The samples were incubated at darkness for 45 minutes and then carefully washed twice using DPBS. A LSM510 confocal microscope was used to examine the fluorescent stained cells using appropriate channels for Texas red and GFP (FITC (cy2) and RHOD (cy3, Texas Red)).

Results

Quantitative Cell Printing Study

This study was conducted to relate cell concentrations in the cartridge to the number of cells actually printed. As seen in Figure 3.1, as the cell concentration changed from 2.1×10^6 to 16.5×10^6 cells/ml, the percentage of drops containing cells varied

linearly, peaking at a concentration of 8.2×10^6 cells/ml. At this concentration, we found an average of 116 cells on the cover slip after firing 100 times, indicating that most the drops contained one cell, and 15% of the drops contained more than one cell. For concentrations below 8.2×10^6 cells/ml we observed a linear relationship between the concentration of cells in the ink and ejected cells. For example at concentration of 4.1×10^6 only 54% of the drops contained cells. At concentrations above 8.2×10^6 cells/ml we observed a decrease of cells per drop to 67% at 16.5×10^6 cells/ml.

Figure 3.1: Thermal inkjet printer printing ability study of various cell concentrations

Specification of the Printer	Firing frequency	3.6 kHz (23)
	Resolution	85 μm (300 dpi) (27)
	Drop volume	130 pl (23)
Printing Parameters	Conc. of cell suspension, 10^6 cells/ml	2.1, 4.1, 8.2, 16.5
	Number of printed dots	100
Expected Value	Printed cells ^a	27, 53, 107, 215
Experimental Values	Total # of cells printed (n=3)	17, 54, 116, 76

Table 3.1: Quantitative CHO cell printing study of HP DeskJet 500 and HP 51626A ink cartridges (^aPrinted cells = Cell concentration x drop volume x number of printed dots).

Heat Shock Protein Expression

The cell concentration of printed CHO suspension was 400,000cells/ml. Thus, there were 80,000 cells total in the 200 μ l of collected cell suspension. After the 200 μ l cell suspension was equally divided into two eppendorff tubes, each tube contained 40,000 cells. Figure 3.2 shows the Hsp70 bands of the printed CHO cells and controls. All samples with cells showed bands at 70 kD indicating heat shock protein was present for all cells including the untreated CHO cell controls. Manually heated CHO cells had the strongest Hsp70 protein expression, whether printed or not. Printed CHO cells showed a somewhat weaker Hsp70 protein expression compared to the manually heated cells. The intensity of the band was that of untreated cells, indicating marginal over expression if any of Hsp70 in printed cells.

Figure 3.2: Heat shock protein 70 expression of printed CHO cells (HC: Manually heated cells; PC: Printed cells; HPC: Manually heated printed cells; UC: Untreated cells; DPBS: DPBS solution without cells)

Cell Membrane Permeability of Printed CHO Cells

Figure 3.3 shows cells incubated and stained with dextran molecules 15 min after printing, we could clearly see the red fluorescence from the Texas red-labeled dyes with molecular weights up to 40,000. For the printed cells incubated with 70,000 MW dextran molecules (Figure 3.3D), we did not see significant fluorescence, indicating the limited penetration of the dye. Control samples showed no significant fluorescence for any dextran dye employed as shown in Figure 3.3E for the lowest molecular weight dextran dye (3,000 D). Figure 3.4 showed series images taken at z-axis with 1.5 μ m interval for printed cells incubated with Texas Red conjugated dextran molecules of 3000MW. The transient nature of these pores is shown in Figure 3.5 which shows the evolution of the approximate pore size with time. We observed, that only 3,000 MW dextran molecules penetrated the cell if we incubated the cells one hour, thus we estimate the pore size after 60 min to have shrunk to somewhere between the Stokes diameter of 3,000 and 10,000 kD dextran molecules, or approximately 37Å. One hour and a half after the printing, the size of pores reduced to approximately 22Å. After 2 hours of printing, even PI couldn't enter the printed CHO cells. The pores appeared closed or repaired as none of the dyes penetrated.

Figure 3.3: Confocal microscopy of printed CHO cells cell membrane pore sizes study using Texas-Red fluorescent dextran molecular dyes (RHOD cy3 Texas Red channel). (A) Dextran 3,000; (B) Dextran 10,000; (C) Dextran 40,000 (D) Dextran 70,000 (E) Pipetted control sample incubated with Dextran 3,000

Time after Printing	PI (16Å)	Dextran 3000 (28Å)	Dextran 10000 (46Å)	Dextran 40000 (90Å)	Dextran 70000 (120Å)
0.25 hour	O	O	O	O	X
1 hour	O	O	X	X	X
1.5 hour	O	X	X	X	X
2 hour	X	X	X	X	X

Table 3.2: Fluorescent molecules penetrated through damaged cell membrane after cell printing (O: molecules entered the cells; X: molecules didn't enter the cells).

Figure 3.4: Series confocal microscopy images at z-axis with 1.5 μ m interval showed the fluorescent dextran molecules were inside the cells.

Figure 3.5: Rate of repairing pores developed during printing process by CHO cells.

Discussion

Quantification

In previous studies, we showed CHO cells can survive the thermal inkjet printing process with more than 90% viability (13). The focus of this study was to determine the exact cell numbers printed from different cell concentrations and, thus, optimize the cell concentration for the bio-ink. Figure 3.1 shows that the cell concentration of 8.2×10^6 cells/ml had the highest amount of cells printed through the printer head. In Table 3.1 some important printing parameters are summarized. The cell concentration that should result in an average of one cell per drop depends on the drop volume following $c = 1000/V_{\text{drop}}$, where c is the concentration in 10^6 cells/ml and V_{drop} the drop volume in pL. Thus, a concentration of 7.7×10^6 cells/ml of ink should assure that each drop contains one cell. In fact, this is close to our experimental results where a concentration of 8.2×10^6 cells/ml was used and we observed that most drops contained cells with some drops containing more than one cell. The highest cell concentration which was 16.5×10^6 cells/ml also had apparently lower number of cells printed than the 8.2×10^6 cells/ml. This may be the cells sticking together during the printing. The diameter of the printer head nozzle is $48 \mu\text{m}$ and the average diameter of the printed CHO cells were 28 to $30 \mu\text{m}$. When two or more cells sticking together, sometimes they could not pass through the nozzle channel. It also had higher possibility for the cells to be sticking together at high cell concentration.

Heat Shock Protein Expression

When cells are undergoing sudden changes of environment, such as extreme change of temperature, they express elevated amount of heat shock proteins to protect other proteins from denaturing (19). The expressed heat shock proteins bind to the other proteins to protect from disruption of secondary bonds. We wanted to look at the heat shock protein expression in the printed CHO cells in order to determine if the heat generated during firing caused an elevated level of HSP which may be an indication of further potential damage to printed cells. As shown in Figure 3.2, all samples including the negative controls showed some HSP expression, which has been described in the literature (17). In fact, every cell expresses HSP, but the amount will be elevated when the cells are undergoing extreme environment changes. The printed CHO cells had lower heat shock protein expression than the manually heated CHO cells, and a somewhat comparable expression to the unprinted controls. Although the modified thermal inkjet printer had a print frequency of 3.6 kHz, the heating time during the drop ejection process was as short as 2 microseconds during which 1.3×10^{-5} Joules of energy is delivered (23). If all the energy was used to heat the printed drop, in fact it was not, the temperature of the drop would increase 24°C^1 . As the cell printing was executed at room temperature, the printed cells would be heated to at most 49°C . According to Wang's work on the kinetics of Hsp70 expression, the cells wouldn't respond until at least 1.5 hours at 43°C and would be expected to have the highest expression when heated continuously for 16 hours at 43°C (24). The drops, however, will cool down to room temperature within

¹ Estimated from $\Delta T = E / (cp * V_{\text{drop}})$; cp – heat capacity of the ink ($4.18 \text{ JK}^{-1}\text{g}^{-1}$); E – energy supplied by the heating element ($1.3 \times 10^{-5} \text{ J}$); V_{drop} – volume of the drop ($1.3 \times 10^{-7} \text{ ml}$)

minutes, thus no elevated HSP levels are observed and we conclude that the printing process is safe for the cells and the heating time too short for cells to respond via the heat shock protein mechanism.

Membrane Permeability

The printed CHO cells were studied using dextran molecules with different molecular weights. A molecule which has been used extensively to estimate the pore sizes in the cell membranes (25). All the printed CHO cells incubated with dextran molecules of 3 kD, 10 kD, and 40 kD MW had solid orange fluorescence under microscope, when the assay was performed shortly after printing. Weak red fluorescence was also found from the cells incubated with 70 kD dextran molecules (Figure 3.3D). This may be from the dextran molecules which were attached to the cell membrane. Another possibility is that the molecules may have been contaminated with lower MW dextran molecules, as they were not purified after receiving. Still, the fluorescence is weak, which leads us to conclude that the cutoff molecular weight of the pores in the cell membrane of the printed CHO cells had a range from 40 kD to 70 kD or an average 105 Å pore diameter (26). The series images taken at z-axis of the printed cells stained by Texas Red conjugated dextran molecules with 3000MW clearly showed the dextran molecules were inside the cells instead of sticking only outside onto the cell membrane.

The transient nature of the pores has been assumed; given the fact that long term survival of printed cells has been shown (14). However, we wanted to have a better understanding of pore sizes dynamics. As Figure 3.4 shows, as soon as 2 hours after

printing, virtually no fluorescent molecules were observed penetrating the cells. This was confirmed by propidium iodide (diameter 16Å) staining the cells, which did not show significant levels inside the cells.

Conclusions

From the comprehensive study of the heat shock protein expression and cell membrane morphogenesis, we concluded that the cell printing technology using modified thermal inkjet printers can be applied for quantitative cell seeding. The low expression of Hsp70 showed that the cells are not stressed beyond the normal handling, such as pipetting and centrifuging. During printing, temporary pores opened in the cell membrane allowing molecules with molecular weight up to 70,000 to pass. This may have promising applications for plasmid transfer for foreign protein expression and drug delivery purpose.

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CHAPTER FOUR

SIMULTANEOUS DEPOSITION OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS AND BIOMATERIALS FOR HUMAN MICROVASCULATURE FABRICATION USING THERMAL INKJET PRINTING TECHNOLOGY

Introduction

From previous study, we confirmed thermal inkjet printing was safe to print mammalian cells. Now we could continue our human microvasculature fabrication using human microvascular endothelial cells (HMEC) and appropriate biomaterials using this technology. The goal of tissue engineering is to solve the organ donor shortage by fabricating the replacement for the lost or damaged tissues and organs (1). Recently, there are many successes achieved in tissue engineering. However, these successes are limited in relatively thin tissue structures, like skin and bladder (2, 3). These engineered tissues can be supported by the diffusion of nutrients from the host vasculature. However, when the thickness of the engineered tissue exceeds to 150 to 200 μm , it will surpass the oxygen diffusion limitation. Then tissue engineers must create functional vasculatures into the engineered tissues to supply the cells with oxygen and nutrients, also to remove the waste product from the cells (4). This is an unsolved issue in traditional tissue engineering so far (5). However, this critical issue could be solved by cell printing technology which is based on inkjet printing.

Inkjet printing is a non-contact printing technique. Inkjet printers have the ability to reproduce the data onto substrate with tiny ink drops by receiving data from computers

(6). Drop-on-demand means the ink drops are ejected only where and when they are required to create the images on the substrate. The inkjet printer has high operating frequency, high orifice density, integrated power, and interconnect electronics. For thermal inkjet printers, little air bubbles are created by heating and then collapse to provide the pressure pulse to eject a very small drop of ink out of the nozzle (6). The current pulse lasts a few microseconds and raises the plate temperature as high as 300 °C (7). Inkjet printing technology has also been widely used in electronics and micro-engineering industries for printing electronic materials and complex integrated circuits (8). Recently, inkjet technology has been successfully applied into biomedical field, such as drug screening, genomics, and biosensors (9-11). Although biological molecules and structures are usually thought to be fragile and sensitive, DNA molecules have been directly printed onto glass slides using commercial inkjet printers for fabrication of high-density DNA microarrays without degradation (12).

Our lab has successfully developed a novel inkjet printing application using the commercial available inkjet printers to print cells and biomaterials for 3D cellular scaffolds (13). We showed that the standard HP and Canon desktop inkjet printers can be modified to perform cell printing. Organ printing, defined as computer-aided inkjet based tissue engineering, has the advantages to construct 3D structures with living biological elements. An important advantage of this process is the ability to simultaneously deposit living cells, nutrients, growth factors, therapeutic drugs along with biomaterial scaffolds at the right time and location (14). This technology can also be used for the microvasculature fabrication using appropriate biomaterials and cells.

Fibrin plays a significant role in natural wound healing. Fibrin gel has been widely used as sealant and adhesive during surgery. Fibrin Glue is used as skin grafts and tissue engineered skin replacements (15). Fibrin can be produced from the patients' own blood and used as an autologous scaffold for tissue engineering (16). Fibrin can be polymerized using fibrinogen and thrombin solutions at room temperature (17). Fibrin gels might promote cell migration, proliferation, and matrix synthesis through the incorporation of the transforming growth factor β and platelet derived growth factors (18). Fibrin has also been utilized in tissue engineering to engineer tissues with skeletal muscle cells (19), smooth muscle cells (20), and chondrocytes (21).

Endothelial cells form the whole inner lining of cardiovascular system and have a remarkable capacity to adjust their number and arrangement to suit local requirements. Almost all tissues depend on a blood supply and the blood supply depends on endothelial cells. Endothelial cells are the only cells to form capillaries. They create an adaptable life-support system spreading into almost every region of the body. Endothelial cells extending and remodeling the network of blood vessels makes it possible for tissue growth and repair (angiogenesis) (22).

In our study, a modified Hewlett-Packard Deskjet 500 inkjet printer was used to simultaneously deposit human microvascular endothelial cells and fibrin to form the microvasculature. HP Deskjet 500 inkjet printer has the droplet volume of 130 pL for each drop of ink. There are 50 firing nozzles on the printer head and the actual heating occurs at 10- μ s pulse. The energy supplied during the printing process is transferred into kinetic energy and heating of the ink drop. Mathematical modeling studies indicated that

the bulk drop temperature in the ink rises between 4 and 10 degrees above ambient during printing. This makes it possible for printing living systems (23). It has been proved successful to print cell suspensions (24).

Materials and methods

Materials

Human microvascular endothelial cells (HMVEC) were provided by Professor Peter I. Leikes at Drexel University. MCDB 131 medium, fetal bovine serum, penicillin and streptomycin, sodium bicarbonate, L-glutamine, hydrocortisone, human recombinant epidermal growth factor, heparin, Dulbecco's phosphate buffered saline solution (DPBS), trypsin-EDTA, fibrinogen from bovine plasma, thrombin from bovine plasma, bovine serum albumin (BSA), lectin-fluorescein isothiocyanate conjugate were from Sigma Chemicals (St. Louis, MO, USA). Live/Dead Viability/Cytotoxicity Kit for mammalian cells was from Invitrogen (Carlsbad, CA, USA). IEC Centra-8R centrifuge was from International Equipment Company (Needham, MA, USA). Microprocessor controlled 280 series water bath was from Precision Scientific (Winchester, VA, USA). Glass microscope cover slips and 35mm tissue culture Petri-dishes were from Fisher Scientific (Pittsburgh, PA, USA). HP DeskJet 500 inkjet printer and HP 51626A black ink cartridges were from Hewlett-Packard Company (Palo Alto, CA, USA). Advanced Micro Osmometer (Model 3300) was from Advanced Instruments, Inc (Norwood, MA, USA). Zeiss Axiovert S100 UV microscope and Zeiss LSM 510 laser scanning microscope were from Carl Zeiss (Minneapolis, MN, USA). Electromechanical testing system was from

MTS System Corporation (Eden Prairie, MN, USA). Hummer 6.2 sputter coater was from Anatech Ltd (Hayward, CA, USA). Hitachi S4700N field emission scanning electron microscope was from Hitachi (Tokyo, Japan).

Thrombin and Fibrinogen Solution Preparation

In order to assure prompt and best polymerization of fibrin gel after printing, appropriate concentrations of thrombin and fibrinogen solutions were prepared. The thrombin solution was made using 50 Unit/ml thrombin and 80mM Ca^{2+} in DPBS. The concentration of fibrinogen is 60 mg/ml. Thrombin solution was used as ink and fibrinogen was substrate on a microscope cover slip (bio-paper). After printing, the printed samples were incubated at 37°C for 5 to 10 minutes until white printed scaffolds were observed.

Osmolality Study

To assure the minimum deformation (swelling) of the printed fibrin gel in the culture media, matched osmolality is required for endothelial cells culture media (MCDB 131), thrombin solution, and fibrinogen solution. Serum free MCDB 131 media, 1x PBS, and distilled water were used to make thrombin and fibrinogen solutions for osmolality study. The osmolality of different solutions was tested using Advanced Micro osmometer.

Fabrication of Fibrin Channels

HP Deskjet 500 printer and HP 51626A cartridges were modified to print/fabricate fibrin channels. A grid pattern was used to print fibrin. 50 Unit/ml thrombin and 80mM Ca^{2+} in 1x DPBS was used as ink to print onto 60 mg/ml fibrinogen solution. The printed samples were incubated for 10 to 15 minutes after the printing for fibrin cross-linking. The printed fibrin fibers were prepared for tensile testing and SEM.

SEM of Printed Fibrin Scaffold

The microstructure of printed fibrin fibers were examined by a scanned electron microscope. The printed fibers were first dried in 100% ethanol overnight. Then were critically dried under vacuum and CO_2 . After that, the dried fibers were cut through in the middle for the cross section study. The fixed fibers were sputter-coated with a thin layer of Chromium using the Hummer 6.2 sputter coater in 100 mTorr vacuum argon environment for a 5 minutes period and 10 mA of current. SEM images were taken using the Hitachi SEM mentioned above at a 5 kV accelerating voltage.

Mechanical Properties Study of Printed Fibrin

Mechanical properties study of the printed fibrin fibers were tested by stretching the sample at a constant deformation rate of 5 mm/min at room temperature. The fibers were gripped on an MTS electromechanical testing system for uni-axial tensile testing. The original data were achieved and analyzed using the software coming with the MTS system (TestWorks, MTS System Corporation). The elastic modulus and ultimate tensile

strength (UTS) were from the acquired stress-strain data. The elastic modulus was defined as the slope of the linear part of the stress-strain curve and the highest stress which caused the fiber to break is the UTS.

Cell culture and Bio-ink Preparation

Human microvascular endothelial cells (HMVEC) were cultured in MCDB 131 media supplemented with FBS, L-glutamine, hydrocortisone, HEGF and heparin. The culture condition is standard at 37°C with 5% CO₂ for 10 days before passaging. Media was changed every two days. During passaging, cell suspension was centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and the cell pellet was resuspended using 1 ml 1X PBS to get the cell suspension. The bio-ink was made by mixing the same volume of cell suspension and 100 units/ml thrombin with 160 mM Ca²⁺ in 1x DPBS. The final condition of bio-ink has 1 million cells/ml HMVEC, 50 Unit/ml thrombin, and 80mM Ca²⁺ in 1x DPBS.

Fabrication of Microvasculature within Fibrin Channels

The bio-ink mentioned above was added into modified HP 51626A cartridge. 60 mg/ml fibrinogen solution was used to make the bio-paper on a microscope cover slip. A modified HP Deskjet 500 printer was used to print a 2D grid pattern using the bio-ink (HMVEC with thrombin/Ca²⁺) onto the fibrinogen bio-paper. This made the cells simultaneously deposited along with fibrin channel formation. The printed pattern was incubated for 20 minutes for the printed cells attaching onto the fibrin channels. This will

also allow the printed fibrin channel to crosslink. After that, a few drops of MCDB-131 media was added onto the printed pattern for nutrient and moisture. The patterns were then cultured at 37°C with 5% CO₂ for 21 days. Media was exchanged for every two days during the culture.

Fluorescent Staining of Printed Microvasculature

Lectin-fluorescein isothiocyanate conjugate (FITC-coupled lectin stain) and Live/Dead Viability/Cytotoxicity Kit were used to stain the printed fibrin channels and HMVEC. For FITC-coupled lectin stain, the samples were first blocked using 2% BSA (blocking solution) for one hour at room temperature. Then the samples were stained using FITC-coupled lectin staining (20µg/ml in blocking solution) overnight at 37°C with 5% CO₂. Samples were then washed using blocking solution twice following 1X PBS for another two times. Zeiss LSM 510 confocal microscope was used to look at the cells. For live/dead assay, the staining solution was made by adding 5µl 4mM calcein AM and 20µl 2mM ethidium homodimer-1 into 1x PBS solution. The samples were incubated in the staining solution for 30 to 45 minutes at room temperature. Zeiss Axiovert S100 UV microscope was used to look at the samples.

Integrity of Printed Microvasculature

Texas Red conjugated dextran molecules were applied to the printed microvasculature cultured for 14 days. The samples were incubated at 37°C for 40

minutes at darkness. LSM confocal microscope was used to look at the possible dextran molecules invasion to the cultured microvasculature.

Fluorescent Imaging of Printed Microvasculature

Printed human microvasculature in fibrin channels were cultured for 21 days before analyzed using Live/Dead viability/cytotoxicity assay. Z-series images with frame size of 512x512 were taken using Zeiss LSM 510 confocal microscope with the interval of 5 μm . The series Z-axis fluorescent images were rendered into 3D images using ImageJ software.

Results

Osmolality of Thrombin and Fibrinogen Solution

The osmolality of thrombin solution of 50 Unit/ml thrombin and 80mM Ca^{2+} in 1x DPBS is 359 mosM. Fibrinogen solution was made using distilled water and the osmolality of 60 mg/ml fibrinogen is 341 mosM. After fibrin printed, there were minor deformations of the fibrin in the HMVEC culture media.

Printed fibrin channels

Figure 4.1 shows the fibrin grid printed using a modified HP Deskjet 500 printer. The printed fibrin gel kept good shape after the print. Only minor changes of the pattern were found. The higher magnified images showed channel structures for cell seeding and microvasculature fabrication.

Figure 4.1 Printed fibrin fibers with grid pattern. Light microscopy images of the printed fibrin gel after 20 minutes of print.

Critical dried fibrin gels were studied using Toshiba scanning electron microscope for the channel structure study. Figure 4.2 (A) showed the cross section of printed fibrin fiber structure. The higher resolution image (Figure 4.2 (B)) of the printed fibrin surface showed many nano-sized fibers.

Figure 4.2 Cross section of printed fibrin fiber (A). Nano-sized fiber structure of printed fibrin surface shows friendly for cell to attach (B).

Mechanical properties of printed fibrin fibers

Using the MTS electromechanical testing system and the TestWorks software coming with the system, we had the elastic modulus of 2.9 ± 0.8 MPa and the UTS of 1.7 ± 0.5 MPa (n=4). The average diameter of the tested fibrin fibers was $93 \mu\text{m}$ (n=4).

Microvasculature fabrication of fibrin and HMVEC

Figure 4.3 shows the live/dead assay of the printed HMVEC after 24 hours. The printed cells aligned a line in the printed fibrin channel. After 7 days, the printed HMVEC started to proliferate in fibrin grid structure which is shown in Figure 4.4. After 14 days of culture, the printed HMVEC formed a line from the FITC-coupled lectin staining (Figure 4.5). When the printed HMVEC was cultured for 21 days, the printed cells aligned themselves and proliferated to form a confluent lining in the fibrin channel (Figure 4.6).

Figure 4.3 Printed HMVEC in fibrin channel after 24 hours (live/dead assay)

Figure 4.4 Printed HMVEC in fibrin channel after 7 days (live/dead assay). A, B shows different positions in the printed grid pattern.

Figure 4.5 Printed HMVEC in fibrin channel after 14 days (FITC-lectin assay)

Figure 4.6 Printed HMVEC in fibrin channel after 21 days (live/dead assay)

Channel Structure of Printed Human Microvasculature

Figure 4.7 shows a tube image of the printed human microvasculature. Figure 4.8 shows the Z-series images of the channel. Using free 3D image render software ImageJ, a channel structure of printed human endothelial cells was found (Figure 4.9).

Figure 4.7 Printed microvasculature tube. Left: Fluorescent image of cells. Right: DIC image of fibrin channel.

Figure 4.8 Z-series images of printed microvasculature tube.

Figure 4.9 Orthogonal sections display mode of printed microvasculature tube.

Integrity of Printed Human Microvasculature

Figure 4.10 (A) shows a printed microvasculature cultured for 14 days. Figure 4.10 (B) shows the fluorescence from Texas Red conjugated dextran molecules with 3000MW which stained the sample.

Figure 4.10 (A) Printed microvasculature cultured for 14 days. (B) Fluorescence image of Texas Red conjugated dextran molecules of 3000MW.

Discussion

Osmolality of Thrombin and Fibrinogen Solution

The osmolality of MCDB-131 culture media was 348 mosM. Osmolality of thrombin solution of 50 Unit/ml thrombin and 80mM Ca^{2+} in 1x DPBS is 359 mosM. Fibrinogen solution was made using distilled water and the osmolality of 60 mg/ml fibrinogen is 341 mosM. So the thrombin solution of 50 Unit/ml thrombin and 80mM Ca^{2+} in 1x DPBS and 60 mg/ml fibrinogen in distilled water have the similar osmolality which is very close to MCDB-131 media. After the fibrin fibers were printed, there were also minor changes of the fibrin in the HMVEC culture media. So this can avoid the distortion and swelling of the printed fibrin fibers.

Printed fibrin channel structure

Fibrin was printed into a grid pattern. After 20 minutes of printing, the printed fibrin patterns were checked using a light microscopy. Only minor deformations of the pattern were found. The hollow channel structure showed in SEM image of printed fibrin fiber was promising for cell seeding and microvasculature fabrication. The nano-sized fibers found in channel surface showed good attachment for cells to attach.

Mechanical properties of printed fibrin fibers

The printed fibrin fiber had elastic modulus of 2.9 ± 0.8 MPa and UTS of 1.7 ± 0.5 MPa (n=4). The average diameter of the tested fibrin fibers is 93 μm (n=4). Using methods mentioned to estimate the up limit of burst pressure (24), we estimated the burst pressure of the printed fibrin fiber of 2955mmHg. Comparing with the average burst pressure of tissue-engineered blood vessels which is 3340 mmHg (25), and the extruded fibrin fibers with 55-65 μm diameters of UTS of 4.5 MPa by Cornwell and Pins (26), the mechanical properties of our printed fibrin fibers are very good.

Microvasculature fabrication of fibrin and HMVEC

From figure 4.3, the printed cells aligned very well by the printer and were ready to proliferate after 24 hours. After 7 days, the printed HMVEC proliferated in the fabricated fibrin grid channels and were going to connect each other. Two weeks later of printing, the cells connected together and formed a line. When the printed HMVEC was cultured for 21 days, the printed cells aligned themselves and proliferated to form a

confluent lining in the fibrin channel (Figure 4.6). This shows the successes and possibility to use the modified thermal inkjet printers and ink cartridge to simultaneously deposit fibrin and HMVEC for human microvasculature (capillaries) fabrication.

Tubulogenesis Study of Printed Human Microvasculature

During angiogenesis, the proliferated endothelial cells form tubular structure or capillaries (27). The series images of Z-axis (Figure 4.8) clearly showed the channel structure of the fabricated microvasculature tube. A 3-D channel was rendered using ImageJ software. This showed the functionality of the printed human endothelial in the fabricated fibrin channel. The proliferated endothelial cells aligned inside the fibrin channel and intended to form a tubular structure. This showed simultaneous deposition of endothelial cells and fibrin gel using thermal inkjet printing technology can be used for human microvasculature fabrication.

Integrity Study of Printed Human Microvasculature

It is not necessary for the printed and cultured human microvasculature to be leaking-proof. However, somewhat integrity is needed for this fabricated structure. Texas Red conjugated dextran molecules with 3000MW were applied to the printed samples. In a microvasculature cultured for 14 days, the cells were not confluent yet. We can see some defects in the structure (Figure 10A). However, the dextran molecules still had difficulties to get inside the microvasculature. This showed from the obvious red

fluorescence difference between the inside and the outside the microvasculature. This study showed the integrity of the printed microvasculature.

Conclusions

Through the cell printing study of the fibrin gel and HMVEC for microvasculature fabrication, we concluded that human endothelial cells can be simultaneously deposited along with the biomaterials (fibrin) for microvasculature fabrication using the modified thermal inkjet printers. The growth factor (VEGF) enhanced medium can be used to culture printed cell and biomaterial construct for promoted microvascularization in vitro. The rendered 3D channel structure through the Z-series images showed the functionality of the printed human microvasculature. The endothelial cells tended to form a tube inside the fibrin channel. The fabricated microvasculature also showed integrity after being cultured for 14 days. The cell/organ printing technology can be a promising solution for branched 2D/3D human capillary tree fabrication and organs with complex microvasculature, like lung, liver, and heart.

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CHAPTER FIVE

CONCLUSIONS

Through the comprehensive study of the heat shock protein expression and cell membrane morphogenesis of the printed mammalian cells, we found minor difference of the expressed heat shock proteins from the printed cells comparing that from the untreated cells. The heating time during the printing is too short for the printed cells to response and initiated the heat shock expression and protection mechanism. The shearing and stress during the printing delivered to the printed cells did develop pores in the cell membrane. However, the printed cells still have a survive rate up to 90%. This showed the pores developed in the cell membrane are not big enough to kill the printed cells. From the dextran molecules pore size study, the average pore size in the cell membrane of the just printed cells was 105Å. The pores shrank to 37Å after one hour and were repaired by cells after just 2 hours of printing. The newly developed pores allowed molecules with molecular weight up to 70000 to pass. This study showed the cell printing technology can not only for tissue engineering fabrication, it also has promising possibilities for plasmid transfer of foreign protein expression and targeted drug delivery purpose.

After confirmed the cell printing technology was safe for the printed cells, in the continued study of the fibrin gel and HMVEC for microvasculature fabrication, we concluded that human endothelial cells can be simultaneously deposited along with the biomaterials (fibrin) for microvasculature fabrication using the modified thermal inkjet

printers. The 3D channel structure rendered from the series Z-axis images showed the functionality of the printed human microvasculature. The growth factor (VEGF) enhanced medium can be used to culture printed cell and biomaterial construct for promoted microvascularization in vitro. The endothelial cells tended to form a tube inside the fibrin channel. The fabricated microvasculature also showed integrity after being cultured for 14 days. The cell/organ printing technology can be a promising solution for engineering tissues with complex microvasculature, such like liver, lung, and heart.