Microfluidic platform incorporating bioactive PEG hydrogel for optimized hepatocyte function

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NANYANG
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**MICROFLUIDIC PLATFORM INCORPORATING
BIOACTIVE PEG HYDROGEL FOR OPTIMIZED
HEPATOCTE FUNCTION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Master of Engineering

2016

Dedicated to my Mum and Dad

*“The more that you read, the more things you will know.
The more that you learn, the more places you’ll go”
—Dr. Seuss*

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Date

.....

Student Name

Abstract

To engineer a functional *in vitro* liver tissue platform, necessary for drug testing and fundamental liver disease studies, it is important to recapitulate important parameters of the hepatic microenvironment. Recent introduction of microfluidics to cell culture has enabled a high degree of control over the specific culture parameters, analysis of cells, and development of scaffolds. In this thesis, we focus on the microfluidic scaffold and propose to develop and integrate a bioactive synthetic hydrogel in a perfusable sealed microfluidic device (μ FD) as a platform for encapsulated hepatocyte cell culture. Our overall hypothesis is that collagen type I (Col I)-functionalized poly (ethylene glycol) (PEG) hydrogel integrated in a sealed μ FD is a hepatocyte cell culture platform comparable to widespread μ FDs using Col I gel. The macromer concentration and bioactivity of the PEG hydrogel were tailored using simple chemistries and the integration of the bioactive hydrogel in the μ FD was evaluated. Huh-7.5 cells were encapsulated in the scaffolds and biological assays were employed to identify the best conditions for cell culture. Another parameter explored was the introduction of pressure-induced flow to see if the microfluidic hydrogel could be perfused. The findings of this thesis research demonstrate the potential of PEG hydrogel to be used as a tailorable integrated microfluidic hydrogel for liver tissue engineering. This thesis research will contribute to the further development of an artificial liver platform that can then be used to test the efficacy of drugs.

Biographical Sketch

Supriya Kumar received a B.S. in Biological Engineering with minors in Biomedical Engineering and Applied Economics and Management from Cornell University in May 2013. She expects to receive an M.Eng by research in Materials Science and Engineering from Nanyang Technological University in August 2016. Though her career path has yet to be defined, she has a passion for helping people and will most likely make her mark in the field of healthcare.

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
BSA	Bovine serum albumin
Calcein AM	Calcein acetomethoxy
CLSM	Confocal laser-scanning microscopy
Col I	Collagen Type I
CYP	Cytochrome P450 enzyme
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
Ethd-1	Ethidium homodimer-1
FBS	Fetal bovine serum
HA	Hyaluronic acid
HHEMP	2-hydroxy-4'-(hydroxyethoxy)-2-methylpropiophenone
HSC	Hepatic stellate cells
KC	Kupffer cells
LSEC	Liver sinusoidal endothelial cells
MMP	Matrix metalloprotein
MW	Molecular weight
NPC	Nonparenchymal cell
PAA	Poly (acrylic acid)
PDL	Poly-D-lysine hydrobromide solution
PDMS	Polydimethylsiloxane
PEG	Poly (ethylene glycol)
PEG-DA	Poly (ethylene glycol) diacrylate

PEG-Col I	Collagen Type-I conjugated poly (ethylene glycol)
PEG-NHS	Acryloyl-PEG-N- hydroxysuccinimide
PLGA	Poly (glycolic acid)
PI	Photoinitiator
PLLA	Poly (l-lactic acid)
PVA	Poly (vinyl alcohol)
RT	Room temperature
μFD	Microfluidic device
UV	Ultraviolet

Chapter 1

Introduction

The research presented in this thesis examines the design and development of a microfluidic platform incorporating bioactive PEG-DA hydrogel to improve the function of liver parenchymal cells, hepatocytes. This chapter introduces the motivation, objectives and novelty of this research, in addition to providing the framework of the thesis.

1.1 Problem Statement

In the drug development pipeline, drug induced liver injury is a leading cause of drug failure both during clinical trials and after the drug reaches the public.^[1, 2] Though some of these deleterious effects may be the result of idiosyncratic reactions, it is well known that animal models used in pre-clinical trials are not accurately predictive of a drug's hepatotoxicity in humans,^[3, 4] let alone adequately representative of human physiology and disease.^[5] Therefore, an *in vitro* model that hosts human liver parenchymal cells, hepatocytes, while maintaining the cells' metabolic activities and other functions may serve as a better model for drug testing and fundamental studies.^[6, 7]

Liver tissue engineering embodies the idea of combining synthetic scaffolds and liver cells to promote liver regeneration and native tissue development. To engineer a functional *in vitro* liver tissue platform, it is critical to understand the *in vivo* liver physiology. The liver is a highly vascularized organ with a multitude of functions and a complex 3D microstructure, comprising of multiple cell types, blood flow through sinusoids, area-specific biomatrix composition and oxygen concentration gradients. Given this elaborate structure, it has been difficult to create a complete liver model *in vitro*, but steps have been taken towards studying and optimizing the various parameters in order to maintain hepatocyte phenotype. The recent introduction of microfluidics to cell culture has permitted a high degree of control over the specific culture parameters and analysis of cells on a smaller scale. Advantages of using microfluidic devices (μ FDs) for cell culture include the ability to control critical cell micro-environmental factors, the capability of introducing multiple cell types in a single system, the establishment of biochemical gradients, the high resolution visualization of cellular events in real-time, precise control of spatial and temporal environment, the small quantities and reagents needed and the control of cell patterning and seeding.^[8, 9] With the high-throughput of μ FDs and their ability to host human hepatocytes, the development of a hepatic μ FD would also be optimal for drug hepatotoxicity testing.

From a materials perspective, the initial focus would be to design the scaffold that provides structure and biochemical cues for the residing hepatocytes within the μ FD.

Conventional *in vitro* cell culture techniques, over the last century, have observed cell growth on two-dimensional (2D) substrates. However, primary hepatocytes experience a loss of phenotype and viability over time due to lost cell-matrix and proper cell-cell interactions.^[10-12] Emerging advances in tissue engineering and biomaterials have shown that three-dimensional (3D) platforms overcome these shortcomings and recapitulate spatial aspects of the native cellular microenvironment. The 3D models often utilize hydrogels, either natural extracellular matrix (ECM) molecules or synthetic polymers, which retain a high percentage of water.^[13] Currently, a majority of research in microfluidic hepatocyte culture platforms uses a major hepatic biomatrix ECM protein, collagen type I (Col I), to encapsulate or sandwich hepatocytes, due to its familiarity, ease of use, and proven maintenance of cellular function. However, as a natural hydrogel it does not allow high control over mechanical properties that are important for cell health and from a commercialization point of view, it is more expensive than synthesized polymer hydrogels. Biocompatible synthetic hydrogels, such as poly (ethylene glycol) (PEG), have recently gained attention because their mechanical properties are tunable and they can be rendered bioactive with simple chemistries but are hardly used as integrated microfluidic scaffolds for hepatocyte culture.

It is therefore imperative to ascertain the benefits of incorporating and using the tunable synthetic polymer scaffolds in μ FDs and to understand the differences between this and more common natural ECM hydrogels used in liver tissue microfluidic technologies. A fundamental understanding of the optimal microscaffold material will allow further knowledge of the hepatocyte-biomaterial relationship and the integration parameters needed for synthetic or natural biomaterials. Once this is accomplished, the various other layers of complexity can be added to the system in a bottom-up approach. Future applications of such a platform include the study of liver morphogenesis,^[14] infection by viruses,^[15] liver fibrosis,^[16] and other liver diseases.

1.2 Objectives and Scope

1.2.1 Objective and Aims

The over-arching objective of this research is **to develop and integrate a bioactive synthetic hydrogel in a perfusable sealed μ FD as a platform for encapsulated**

hepatocyte cell culture. The polydimethylsiloxane (PDMS) μ FD design, chosen in collaboration with Professor Roger Kamm's SMART BioSyM research group, is simple and contains one gel channel that allows for basic microfluidic biomaterial testing. Biocompatible and photopolymerizable PEG hydrogel, prepared from poly (ethylene glycol) diacrylate (PEG-DA) macromers and rendered bioactive by conjugation to Col I, is chosen as the synthetic scaffold material for our model hepatocyte cell Huh-7.5.

Addressing the dearth of integrated microfluidic PEG hydrogels for liver tissue engineering, this research will extend insight into future photopolymerizable and tunable biomaterials use for hepatocyte cell culture in PDMS μ FDs. The central hypothesis of this thesis is that **Col I-functionalized PEG (PEG-Col I) hydrogel integrated in a sealed PDMS μ FD with a specific PEG concentration, controlled bioactivity and the capability to withstand perfusable flow is a comparable encapsulated Huh-7.5 cell culture platform to widespread μ FDs using Col I gel.**

The following specific aims were defined to address the hypothesis:

Aim 1: To characterize PEG hydrogels of different concentrations and select the concentration that allows highest stability and improved Huh-7.5 cell function and viability in the μ FD

Aim 2: To compare PEG-Col I microfluidic hydrogels, with controllable bioactivity, to Col I microfluidic hydrogels and select the composition that enhances encapsulated Huh-7.5 cell behavior

Aim 3: To understand the effect of passive flow on the PEG-Col I hydrogel integrity and encapsulated Huh-7.5 cell function and viability in the μ FD

1.2.2 Scope and Limitations

This study encompasses the development, characterization, and evaluation of PEG-based hydrogel as an integrated microfluidic scaffold for hepatocyte culture. There are many parameters in the development of a microfluidic hydrogel for tissue engineering, including physical properties. (mechanical characteristics, degradation, etc.), mass transport properties (permeability, porosity, etc.), and biological properties (biocompatibility, cellular adhesion, etc.). The research in the thesis focuses on

certain topics in these categories, namely integration into a PDMS μ FD, hydrogel mechanical properties and basic structure, bioactivation for cell adhesion, and ability to endure flow. Hydrogels were evaluated as a cell culture platform by *in vitro* Huh-7.5 cell culture and subsequent possible biological assays to analyze the cell behavior.

Though the current research tethers Col I to the PEG hydrogel due to its prevalence in the hepatic microenvironment, the conjugation of multiple ECM proteins will provide a more *in vivo* like hepatic biomatrix. Therefore further studies must investigate the exact composition of ECM proteins, such as fibronectin, collagen type IV and laminin, to tether to the hydrogel. Similarly, in order for this platform to be considered as a drug hepatotoxicity-testing platform, future studies must add more complexity to the results of this project, with co-culture, growth factors and other parameters to recapitulate the most accurate physiological conditions.

1.3 Thesis Outline

This thesis contains seven chapters to provide readers with a better understanding of the rationale behind the project and the methodologies used in the research, to delve into the aims defined earlier by experimental means, and to explain the outcomes and outlook, as follows:

Chapter 2 provides background information of liver tissue engineering, focusing on liver physiology, microfluidics for cell culture, and biomaterials for tissue engineering. An in-depth literature review highlights previous works in this field and the gap in knowledge that our research serves to close.

Chapter 3 describes the experimental methodologies utilized. The materials and techniques are subdivided into six sections covering the PDMS μ FD preparation, Huh-7.5 cell culture and encapsulation with the μ FD, characterization of cell-laden PEG hydrogel, the introduction of passive flow into the system, and biological assays employed to determine the success of the platform.

Chapter 4 reports the integration of Huh-7.5 encapsulated PEG hydrogel in the μ FD. By changing the PEG-DA macromer concentration and varying the hydrogel stiffness, the objective is to understand the PEG network in the PDMS μ FD and determine a suitable PEG-DA concentration for the cells, which will be used in later chapters.

Chapter 5 addresses the conjugation of Col I to the hydrogel in defined concentrations and compares these results to a Huh-7.5 encapsulated collagen platform. The objective of this chapter is to ascertain the differences between the collagen tethered synthetic matrix and the conventionally used natural hydrogel matrix and to select a collagen concentration that shows the most promising results and can be used in experiments with flow.

Chapter 6 introduces a new parameter to the platform by establishing a pressure-induced flow in the μ FD. The flows are generated at volumetric rates similar to other *in vitro* platforms and the objective is to observe the differences with and without the dynamic environment.

Chapter 7 summarizes the findings and implications of the research and presents the direction and future outlook for the field.

1.4 Findings and Originality

This research led to several novel outcomes including:

1. Establishing the integration of Huh-7.5 encapsulated PEG hydrogel in the μ FD (Chapter 4)
2. Controlling the cell-adhesive properties of the integrated microfluidic PEG hydrogel in the μ FD (Chapter 5)
3. Introducing passive flow through the bioactive PEG-Col I hydrogel to ensure perfusion is possible for the platform (Chapter 6)

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Chapter 2

Relevant Background and Literature Review

To design a hydrogel-integrated microfluidic device for the fundamental studies of the in vitro hepatic environment, this liver tissue engineering project requires the understanding of three fields of science, namely liver physiology and anatomy, biomaterials for tissue engineering, and microfluidics for cell culture. This chapter highlights the key concepts utilized in this research and concisely presents a review of the state-of-the-art research trends in liver tissue engineering, with a focus on choice of scaffold material and microfluidic technology platforms. Common applications for many of these technologies include the testing of xenobiotics' cytotoxicity on the hepatocyte cells, the study of liver diseases, and fundamental studies regarding the different environmental stimuli and their effect on the hepatocytes. For a more in depth review, Griffith et al.^[1] and Bhatia et al.^[2] have recently discussed the implications of such cell-based therapies for treating liver disease and liver failure.

2.1 The Liver: Microstructure and Function

2.1.1 Architecture and Blood Supply of the Liver

The liver is the second-largest organ in the human body, cleverly situated such that it receives portal blood rich in nutrients that has exited the stomach, small and large intestine, pancreas and kidney. A classic way of viewing the organization of the liver tissue is to envision each liver lobule, the functional unit of the liver, as a hexagon (**Figure 2.1A**). Each of the six vertices of the hexagon contains a portal triad that is composed of a portal vein, hepatic artery and bile duct.^[3] The nutrient-rich blood from the portal vein and the well-oxygenated blood from the hepatic artery combine and flow through the hepatic sinusoids to the central vein, located at the center of the lobule.^[4] Blood flowing through the sinusoid induces a shear stress force that affects the sinusoidal endothelial cells and also helps drive a mitigated hepatic interstitial flow that allows molecules carried in the blood to reach hepatocytes and the removal of waste (**Figure 2.1B**).^[5] Along with the network of sinusoids, there are networks of ~1 μm bile canaliculi, formed by the apical membranes of adjacent hepatocytes, which carry secreted bile from the hepatocytes to the bile duct.^[6]

2.1.2 Liver Parenchymal and Nonparenchymal Cells

There are a variety of cells that play distinct roles in the liver. Hepatocytes, the parenchymal cells, make up approximately 80% of the liver cell population.^[7] The other 20% consists of nonparenchymal cells (NPCs), namely cholangiocytes, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), liver stem cells and Kupffer cells (KCs), which support the hepatocytes via cell-cell interaction and paracrine signaling.^[6, 7]

Hepatocytes are highly differentiated epithelial cells that are approximately 20-30 μm in size and cuboidal in their native shape.^[8] They perform vital functions that are elaborated in detail in the next section. Unlike other epithelial cells, hepatocytes are not surrounded by a basement membrane, but rather by the Space of Disse, defined as the area between the hepatocytes and LSECs. LSECs line the sinusoids and with their leaky junctions and membrane fenestrations, they allow diffusion of nutrients and substances between the

Figure 2.1 The structure and microcirculation of the liver. (A) Schematic illustrations of the (i) liver, (ii) hexagonal-shaped liver lobules, and (iii) liver acinus detail the hepatic microarchitecture. (B) Hemodynamic factors, such as sinusoidal and interstitial flow, affect the hepatocyte phenotype. [Adapted from Mescher, 2013^[3] and Dash, 2013^[9]]

blood and the hepatocytes. Cholangiocytes are biliary epithelial cells that regulate immune responses, contribute to bile secretion and may regulate certain cytokine secretion. HSCs are located in the Space of Disse and primarily function by storing vitamin A and fat, secreting certain growth factors, and producing ECM proteins. The hepatic progenitor cells are normally quiescent until activated by chemical, physical, and mechanical means (i.e. when there is liver tissue damage) and can then differentiate into hepatocytes or other non-parenchymal cell types. KCs are

macrophages within the hepatic sinusoid walls and play a role in immune responses by engulfing particles from the portal blood via endocytosis.^[5, 8]

2.1.2.1 Hepatic Function

Hepatocytes perform a majority of functions commonly associated with the liver, such as glucose-homeostasis, urea formation, storage of lipids, vitamins and minerals, synthesis of carbohydrates, proteins, and metabolites, and the metabolism of endogenous compounds and exogenous substances.^[6, 10] The functions and metabolic capacities of the parenchymal cells depend on their spatial location within the liver acinus (summarized in Table 2.1). From the portal vein to the central vein, an oxygen and biochemical gradient exists, resulting in different parenchymal cell enzymes, subcellular components and ultimately functional heterogeneity.^[5, 11]

Periportal zonal hepatocytes (near the portal vein) specialize in ureagenesis, gluconeogenesis, plasma protein and bile secretion, and cholesterol synthesis. Ammonia, produced by the breakdown of amino acids and toxic in elevated concentrations, is detoxified in the hepatic cytosol during the urea cycle. The urea produced during the cycle is then excreted by the kidneys.^[6] Glucose, the major energy source for cells, is formed from non-carbohydrate precursors.^[12] Synthesized and secreted serum proteins albumin and fibrinogen respectively help maintain oncotic pressure and aid in blood clotting. Hepatocytes also produce carriage proteins, prohormones, apolipoproteins, and factors involved in hemostasis and fibrinolysis.^[6] Bile synthesis is necessary for the elimination of cholesterol, lipid transport, stimulation of bile flow, and feedback mechanisms for bile and cholesterol production.^[13]

Hepatocytes in the perivenous zone (near the central vein) are responsible for the metabolism and biotransformation of xenobiotics among other functions. In compound biotransformation, the cytochrome P-450 (CYP) superfamily of enzymes catalyze the Phase I oxidation, reduction, or hydrolytic reactions, making the substrate more polar. These heme-containing proteins are often located near the cell mitochondria. CYP3A4 is the most abundant CYP isoenzymes found in the human liver.^[14] In Phase II, the metabolic products from phase I are conjugated and made more water soluble for secretion.^[6, 15] Hepatic CYPs are sometimes involved in the pathogenesis of certain liver diseases and hepatotoxicity. Oxidation of certain drugs,

such as acetaminophen in high doses, can result in toxic byproducts that then interfere with nuclear function and lead to apoptosis.^[16]

Table 2.1 Zonation of hepatocyte functions. The heterogeneity of hepatocyte function arises from a gradient of oxygen concentration from the periportal zone (near the portal vein) to the perivenous zone (near the central vein). Cells located between the two zones tend to have mixture of functions from both periportal and perivenous zones [Sourced from Jungermann, 1996^[17]]

Parenchymal cells					
Periportal zone			Perivenous zone		
Physiologic function	Metabolic function	Enzyme or protein involved	Physiologic function	Metabolic function	Enzyme or protein involved
Oxidative energy metabolism		Succinate dehydrogenase			
Glucose output	1. Glucose from pyruvate (gluconeogenesis) 2. Glucose from glycogen 3. Glycogen from pyruvate	Glucose-6-phosphatase Phosphoenolpyruvate carboxykinase	Glucose uptake	1. Glucose to pyruvate (glycolysis) 2. Glycogen from glucose 3. Glycogen to pyruvate	Glucokinase Pyruvate kinase type L
Urea formation	Urea from amino acid nitrogen and from NH ₃	Carbamoyl phosphate synthetase	Glutamine formation	Glutamine from NH ₃	Glutamine synthetase
Protective metabolism	1. Glutathione peroxidation 2. Glutathione conjugation	Glutathione peroxidase (Glutathione level)	Xenobiotic metabolism	1. Monooxygenation 2. Glucuronidation	Cytochrome P450 UDP-glucuronosyl transferase
Plasma protein synthesis		Albumin α ₂ -Macroglobulin Fibrinogen	Plasma protein synthesis		α-Fetoprotein Angiotensinogen α ₁ -Antitrypsin
Cholesterol synthesis	Hydroxymethyl glutaryl-CoA reductase				
Bile formation	Taurocholate uptake carrier Bile acid export carrier				

2.1.2.2 Parenchymal Cell Sources

The source of cells is very important in liver tissue engineering. Primary human hepatocytes are the most preferred parenchymal cell source for cellular therapies.^[18] However, access to primary human hepatocytes is limited and conventional *in vitro* cultures of these cells have seen rapid loss of function.^[19] Tumor-derived human cell lines, such as HepG2, C3A, Huh 7 and derivatives, and immortalized cell lines have been developed to enhance the availability of human hepatocytes and overcome growth limitations.^[18, 20] Given the permissibility of Huh-7 and HepG2 cell lines to Hepatitis C virus (HCV), they are often used in studies requiring the virus. Though these cell lines are easily available and retain most parenchymal function, lower levels of certain functions do exist. Stem cells—human adult, oval, fetal or induced pluripotent—are cells that can differentiate into specialized cell types and have also

been utilized for liver tissue engineering.^[21] However, the differentiation of stem cells to fully functional hepatocytes poses difficulties and increased costs.

2.1.3 Extracellular Matrix

ECM, the region surrounding cells in a tissue, is composed of proteins, proteoglycans, and other molecules. This biomatrix provides the physical support for cells as well as biochemical and biomechanical cues necessary for regulating biological processes, such as differentiation and morphogenesis.^[22] The cell-matrix adhesion interactions are mediated through a family of cell surface receptors called integrin, which transduce the external signals to an intracellular cascading pathway. Both the matrix composition and stiffness are important factors in regulating cell behavior.^[23]

Each tissue has a unique ECM. Though only a small portion of the liver is ECM, it plays a large role in maintaining hepatocyte phenotype. Liver stiffness depends on the ECM and external and internal organ pressures.^[24] A normal healthy liver has a shear stiffness ranging from 0.75-2.5 kPa^[25, 26] and no more than 6kPa.^[24] These stiffness values are known to increase as certain liver diseases and viral infections that lead to fibrosis (i.e. hepatitis C virus^[27, 28] and ALD^[29]) progress. There is a zonal distribution of ECM components in the liver acinus, again supporting the heterogeneity of the hepatocyte function (**Figure 2.2**).^[30] Given a main research focus of liver tissue engineering is the optimal microenvironment for the hepatocyte, the Space of Disse composition is the most important for biomatrix studies.^[31]

2.1.3.1 Space of Disse ECM Proteins

Studies report that collagen I, III, IV, and VI and fibronectin and laminin are present in the Space of Disse. Col I is a major structural ECM protein, possessing the ability to form fibers and bind other matrix molecules. Collagen type III is closely related to the Col I fibers and has a similar structure. Collagen type IV, often a main component of the basement membrane, is also present in the Space of Disse and can bind growth factors. Fibronectin, in addition to Col I, is an abundant ECM protein in the Space of Disse and promotes cell adhesion to the ECM as well as growth factor binding such as hepatocyte growth factor. Laminins also interact with cell surface receptors.^[32]

Figure 2.2 Schematic of ECM distribution in the liver acinus. The circled area marks the Space of Disse. [Adapted from Wells, 2008 ^[30]]

2.2 Biomaterials for Tissue Engineering

Tissue engineering scaffolds are rapidly evolving to provide all the physical and biochemical cues necessary to regenerate, maintain, or repair tissues for the application of organ replacement, studying disease, developing drugs, and many others.^[33] These scaffolds serve as the ECM, allowing cells to self-assemble into different architectures, providing signaling cues, and directing the growth of the tissue. The choice of scaffold material depends on physical properties (i.e. stiffness, mesh size, swelling, mechanics, degradation, gel formation), mass transport properties (i.e. diffusion of nutrients and gases) and biological properties (i.e. cell adhesion sites and signaling).^[34, 35] A hydrogel is a 3D network of polymer chains that can retain a large amount of water and is not soluble in water. Such networks have been used as scaffold materials for cells *in vitro* due to their high water content and biocompatibility. Hydrogels are categorized as either natural or synthetic, based on their materials origin.

2.2.1 Natural Hydrogels

Natural hydrogels, such as collagen, gelatin, hyaluronic acid (HA), fibrin, alginate, chitosan, and matrigel have been widely used in biomedical applications. Their

biocompatibility, avoidance of immunological reactions, and ability to support cell function, owing to their derivation from natural sources, are advantageous.^[36, 37] However, there is often a batch-to-batch variability, mechanical properties and binding site presentation are not tunable, and the degradation of the matrix is unpredictable.^[36]

Gelatin, fibrin, and matrigel are examples of natural protein-based gels. Gelatin, a denatured form of collagen, is often prepared using acidic treatment or alkaline treatment and changing the temperature to form the gel. It has been used to encapsulate cells and is able to incorporate growth factors and cell adhesion proteins and peptides.^[38, 39] Fibrin gels are rapidly formed by thrombin-initiated polymerization of fibrinogen, easily remodeled by cells and their degradation rate can be controlled by inhibitors. Due to their intrinsic elevated bioactivity and ability to bind growth factors and clot components, fibrin gels have wide applications in stem cell differentiation and tissue engineering.^[38, 40, 41] Matrigel is a commercially available hydrogel derived from the basement membrane of Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells.^[42-44] However, the lacking details of its exact composition make it difficult for fundamental cell-ECM interaction studies.^[22]

HA, alginate and chitosan are a few of the natural polysaccharide-based gels utilized in tissue engineering. HA is a major macromolecular component of connective tissue intercellular matrix with enhanced viscoelastic properties and applications in skin, bone, and cartilage tissue engineering. However, it possesses low mechanical properties and retains impurities and endotoxins that need thorough purification.^[38, 39] Alginate scaffolds are prepared through gelation with divalent cations and are beneficial for long-term cell culture, chemically modifiable for biomolecule presentation, and are easily molded. Limitations of the cost-effective material include its uncontrollable degradation and intrinsic nonadhesive nature.^[38, 45] Chitosan, a deacetylated form of polysaccharide chitin, is soluble in dilute acids and gelled by increasing the pH. Though it is derived from a nonmammalian source, this positively charged material is degradable by human membranes and possesses antimicrobial properties.^[39, 46]

Collagen, the main protein in mammalian tissues, is the most common natural hydrogel in tissue engineering. Given the abundance of Col I in the Space of Disse

and its regard as one of the most useful biomaterials, the next section will detail collagen gel and its properties.

2.2.1.1 Collagen

Collagen is the main constituent of ECM and exists as a three-stranded polypeptide molecule twisted in a rope conformation. There are at least 28 different types of collagen found in humans and they can be subcategorized as fibrillar, non-fibrillar, or collagen-like proteins.^[47] Fibrillar collagens, such as collagen I, III and V, contribute to the mechanical properties of the tissue. Non-fibrillar collagens, such as IV, XIV, and XV, are more flexible than the fibril forming collagens and either exist as networks, fibril-associated collagen with interrupted triple helix, or multiplexins.

Collagens are widely used in biomedical applications because of their cell-binding sites, biodegradability, and their ability to form stable fibers through cross-linking and self-aggregation.^[48] The combined use of chemical cross-linkers, physical treatment, or formation of composite materials with other polymers enhances the mechanical properties of the hydrogel.^[34] Matrix metalloproteinases (MMPs) secreted by cells, such as collagenase, are able to degrade collagen during tissue remodeling processes.^[47]

2.2.2 Synthetic Hydrogels

Synthetic hydrogels, including PEG-based, poly (vinyl alcohol) (PVA)-based, and poly (acrylic acid) (PAA)-based hydrogels,^[38] are especially attractive materials for tissue engineering scaffolds because of their biocompatibility, easily modifiable mechanical properties, ability to incorporate molecules to become more ECM-like, and because they are more reproducible than natural scaffolds.^[36]

PVA is a hydrophilic polymer that is crosslinked through physical freezing/thawing processes, chemical molecules such as glutaraldehyde, or the addition of reactive acrylate groups and further polymerization steps.^[49] The hydrogels formed from PVA polymers and derivatives are nonbiodegradable in physiological conditions and relatively bio-inert, but can be covalently bonded to ECM proteins for improved cell attachment.^[50] PAA-based hydrogels are also not biodegradable and are able to modulate their hydrophobicity by changing the temperature. Poly (2-hydroxyethyl

methacrylate) and poly (*N*-isopropylacrylamide) are two derivatives that are widely used in cell culture. However, the cross-linking molecules used in the formation of the hydrogels are often toxic.^[34, 38]

PEG polymers have been investigated thoroughly to create hydrogels because of their advantageous properties for cell culture. The next sections will detail the properties of PEG-based hydrogels and bioactive modification techniques.

2.2.2.1 PEG-based Hydrogels

PEG is a hydrophilic polymer that has been widely used in biomedical applications and is FDA-approved. Its prominent properties include biocompatibility, non-toxicity, neutral charge, injectable capacity, non-immunogenicity, solubility in water and organic solvents, resistance to protein adsorption, and its ability to be easily customized because its end hydroxyl groups can be functionalized.^[51-53] PEG acrylates, such as PEG-DA, or methacrylates are most often used to make a PEG-based hydrogel through a method called photopolymerization (**Figure 2.3A**). Though PEG hydrogels are inherently nonbiodegradable, the incorporation of degradable blocks (i.e. polyester), hydrolytically degradable blocks (i.e. poly (lactic acid) (PLLA), poly(glycolic acid) (PLGA), and poly-caprolactone) or enzyme sensitive cleavage sequences renders them degradable.^[34, 53]

2.2.2.2 Bioactivation of PEG-based Hydrogels

PEG-DA hydrogels do not exhibit any intrinsic bioactivity. In order to mimic the ECM, the PEG-DA hydrogel must allow the adherence of cells, growth factors, and other protein (**Figure 2.3B**). There are many approaches for bioactive modification of the PEG-DA hydrogel, including post-grafting, copolymerization, Michael-type addition, click chemistry, and enzymatic reaction.^[53]

Figure 2.3 Photopolymerization of PEG-DA and bioactive modification. (A) In the presence of UV light and a photoinitiator (PI), the PEG-DA monomers form a crosslinked network. (B) The introduction of cell-adhesive peptides (CAPs), enzyme sensitive peptides (ESPs) and growth factors (GFs) render the intrinsically bioinert PEG-DA hydrogel bioactive. [Sourced from Fei, 2012^[54] and Zhu, 2010^[53]]

Post-grafting is the process by which proteins or peptides are conjugated to functional groups on the hydrogel surface after the hydrogel has been formed. Lee et al.^[55] immobilized collagen on photopolymerized PAA-grafted PEG-DA hydrogel surface. Rather than cover the surface with proteins, co-polymerization of PEG-DA with monoacrylated peptides or proteins allows conjugation throughout the bulk hydrogel. Michael-type addition, evades the use of ultraviolet (UV) light, and allows incorporation of cysteine-containing peptides with functionalized PEG macromers in a stepwise growth mechanism. In another step-growth approach, click chemistry, bioactive PEG hydrogels are formed by “clicking” together azide or alkyne functionalized macromers in the presence of catalysts.^[56] Enzymes can be used to link PEG macromers functionalized with components sensitive to the specific enzyme.^[57]

2.2.3 Scaffold Materials for Liver Tissue Engineering

Scaffolds have played an important role in liver tissue engineering, as an attachment site for hepatocytes and a transplantation vehicle.

Natural hydrogels have been used in various configurations to culture hepatocytes. Alginate, with a porosity of ~90% and 100-150 μm pore sizes, has been used to culture hepatocytes as spheroids with its weakly adhesive nature.^[58-60] Yang et al. engineered the alginate scaffold with galactosylated chitosan which increased the cell adhesion from 50% to 80% and resulted in higher albumin production.^[61] Col I has been widely used in many 2D and 3D *in vitro* hepatocyte culture models and has been the most common ECM protein in the hepatocyte sandwich cultures.^[9, 19, 62-72] These culture platforms culture a monolayer of hepatocytes between two layers of ECM proteins, maintaining the *in vivo* cuboidal morphology of the hepatocytes and re-establish hepatocyte polarity. Additionally, Kim et al.^[67] showed there are also a number of transcriptional programs that are upregulated in the sandwich culture compared to the 2D monolayer on collagen. Collagen scaffolds have also maintained liver-specific function in entrapped hepatocyte spheroids.^[73] Fibrin matrices have been evaluated as hepatocyte transplantation scaffolds.^[74] Matrigel, a laminin-rich matrix with collagens and proteoglycans, has been used to culture hepatocytes even though these specialized cells are not surrounded by a basement membrane *in vivo*.^[70, 75-77] Cell-cell interaction is enhanced as hepatocytes in matrigel aggregate and display a non-polygonal morphology.^[78] The hepatocytes retained most functions and overperformed cells cultured on 2D Col I substratum.^[79] Limitations of matrigel for liver tissue applications include the potential tumorigenicity and inconsistent and unknown batch-to-batch composition. Hepatocytes have also been cultured on heparin-based hydrogels,^[80] chitosan,^[81, 82] and other scaffolds of natural origin.

There has been a rise in the use of synthetic biomaterials for liver tissue engineering, as they allow precise engineering of the *in vitro* hepatic microenvironment.^[21] Polyesters, such as PLGA and PLLA, have been widely used to support hepatocyte culture, especially in transplantation because of their biodegradability.^[83-85] Encapsulation or seeding of hepatocytes in extensively used PEG hydrogels has consistently supported urea synthesis and CYP450 enzyme activity.^[86] Recently, biodegradable PEG-diacrylamide hydrogels with incorporated MMP-sensitive peptides maintained primary rat hepatocyte phenotype stability and the cell-laden hydrogels were successfully engrafted in rodents and functioned for two weeks.^[87] Bhatia, a key researcher in hepatic tissue engineering, has worked with RGDS- (derived from fibronectin), RGES, YIGSR (derived from laminin), KQAGDV (derived

from fibrinogen), GF-(Hyp)GER(derived from collagen), and cyclic (-RGD(d-Phe)K-) peptides conjugated to photopolymerizable PEG hydrogels^[51, 88] and polyelectrolyte multilayers (PEM).^[89] She and her coworkers have taken the advantage of cell-encapsulated PEG hydrogel one step further and coupled it with photolithographic techniques to photopattern the 3D hepatocyte-laden hydrogel networks.^[90] Hepatocytes have been encapsulated in PEG-fibrinogen micropatterned scaffolds and maintained high liver-specific functions while retaining certain autocrine-controlled growth factors.^[91] Due to often small mesh sizes of the polymerized PEG hydrogel, encapsulated hepatocyte function quantification through albumin detection and imaging analysis may be limited, but are still possible.^[51] PEG hydrogel's innate resistance to protein adsorption and cell adhesion promotes hepatocyte-spheroid formation when encapsulated in the hydrogel or seeded in PEG scaffolds of specific geometries. The matrix rigidity of PEG hydrogels and ECM-conjugated PEG hydrogels can be tuned to regulate the 3D spheroid formation.^[92, 93] Another common method of spheroid culture is seeding the hepatocytes in porous bio-inert scaffolds or ordered colloidal crystal-templated hydrogel scaffolds.^[94-98] Increased cell-cell interaction and media diffusion through the ordered scaffold interconnection maintains hepatocyte albumin production and CYP450 activity, although the diameter of the spheroid is limited by oxygen penetration depth.

2.3 Microfluidic Devices for Cell Culture

Microfluidics, defined by George Whitesides as “the science and technology of systems that process or manipulate small amounts of fluids using channels with dimensions of tens to hundreds of micrometers”,^[99] has great potential for improving diagnostics and biology research.^[100] A μ FD is able to utilize the low Reynolds' number induced in the micro-channels and the micro-size lengths to achieve laminar flow.^[99] Microfluidic systems in biology have been used for DNA analysis, enzyme assays, immunoassays, cell-based assays, cellular biosensors, cytometry, and cell culturing. As a cell culture platform, μ FDs overcome the fundamental limitations of conventional cell culture techniques (summarized in Table 2.2), while also allowing the study of cell-cell interactions, cell-matrix interactions, single cell analysis, and the effect of controlled biophysical and biochemical factors in the system.^[101]

Table 2.2. Comparison of conventional cell culture and microfluidic cell culture. Utilizing microfluidic methods meets basic requirements for cell culture and shows improvement over conventional methods. [Adapted from Mehling, 2014^[101]]

Requirements	Conventional Cell Culture	Microfluidic cell culture
Control of temperature and gasses	Large fluid volumes prevent fast changes	Small volumes allow dynamic control
Addition of nutrients and removal of metabolites	Infrequent, manual exchange of large volumes	Precisely measured, continuous or transient exchange of media
Stimulation with drugs/proteins and simultaneous imaging	Mostly not feasible	Feasible
Parallelization of cellular assays	Not feasible	High capability for parallelization
Automation of cell culture tasks	Bulky, expensive fluid-handling robots must be used	High capability for automation in compact, inexpensive format
Single-cell manipulation and analysis	Manually involved, inaccurate, low throughput	Accurate and high throughput

2.3.1 Microfluidic Device Material and Design Considerations

Microfluidic devices for biological studies are often fabricated out of PDMS. PDMS is an optically transparent, soft elastomer and is hydrophobic, oxygen-permeable, biocompatible, and non-toxic. Photolithography and a photo-mask are the methods commonly utilized to create the patterned master wafer and soft lithography to create the PDMS mold.^[102] The flexibility in device design allows for numerous microfluidic applications, especially the study of multiple environmental factors on the cells.

Cell culture microfluidic platforms often have access channels that allow insertion of cells and media, gel regions to mimic the ECM, and passive or active methods for inducing a controlled flow across the gel region (**Figure 2.4**). The dimensions of the channels can mimic *in vivo* blood or lymphatic vessels. Gel region volumes and dimensions should reflect comparable ECM physiological values and allow proper permeation of nutrients and other molecules.^[103] The gel material and gelling technique must also be compatible with the housing and form a proper interface.

Figure 2.4 Schematic of a microfluidic device for cell culture. The device contains two gel regional areas and three access channels [Adapted from Shin, 2012^[104]]

2.3.2 Current Microtechnology Trends for Liver Tissue Engineering

In microtechnology, only small quantities of reagents are needed and the platforms possess the ability to control specific parameters of the microenvironment. Microfluidic devices have allowed researchers to focus on device design and patterning of the cells, the different ECM compositions of the hepatic environment, the effect of flow on the cells, co-culture of hepatocytes with non-parenchymal cells and other parameters. Many of these platforms have been developed in order to serve as a drug hepatotoxicity testing chip^[105-108] or to perform fundamental studies with the hepatocytes. With the design capabilities of modern microtechnology and the innate properties of flow on the micron scale, researchers have designed the devices in ways to allow hepatic chord or collagen sandwich culture, the patterning of cells, and networks of channels to promote hepatocyte function.^[109-112] Platforms have incorporated multiple cell types and studied the paracrine signaling between NPCs and hepatocytes.^[52, 106, 107, 112, 113] Another advantage of the microtechnology is the ability to perfuse the system. This allows proper diffusion of nutrients and removal of waste, while creating slight shear stresses that enhance hepatic function.^[9, 52, 114-122] Recently, Hegde et al. perfused a hepatocyte-sandwich culture with media at a rate of $5 \mu\text{L h}^{-1}$ and noticed an increased production of ECM. However, this effect is attributed to the replenished proline in the media and not the effect of the shear force.^[66] There is still a lack of knowledge as to the exact effect of shear on the

hepatocytes. Many hepatic microfluidic platforms have used Col I as the microscaffold material and there has been little research that explores the other ECM possibilities.^[113, 114, 123] Flaim et al.^[124] developed a microarray of different compositions of the ECM proteins (fibronectin, laminin, Col I, Collagen Type III and Collagen Type IV) in order to study the differentiation of embryonic stem cells as well as the function of primary hepatocytes. Though this study concluded certain ratios of ECM proteins that heightened the functions of fetal-like and mature hepatocytes, it is a 2D study and therefore is not exemplary of the 3D *in vivo* physiological structure. Slowly, these microtechnologies are able to couple different parameters together to create a platform hosting hepatocytes that will function more similar to the *in vivo* cells and that can be maintained for extended periods of time.

2.4 Questions Based on the Literature

Despite developments in microfluidic platforms for hepatocyte cell culture and a wealth of information accrued about biomaterials, there are still many questions that remain. To name a few:

- Can photopolymerizable PEG hydrogel, with its innate non-fouling properties but easily tunable nature, be used to encapsulate hepatocytes to produce a functional microtissue integrated within a simple μ FD?
- How does the bioactive PEG hydrogel compare to conventionally used collagen hydrogel for microfluidic cell encapsulation?
- What effect will inducing flow in the platform, to mimic the interstitial flow and pressures in the hepatic environment, have on the integrated hydrogel and encapsulated hepatocytes?

2.5 Masters in Context of the Literature

The objective and hypothesis of the thesis were designed to address the knowledge gaps in literature and to answer the questions mentioned in the section above. The main gap is the paucity of integrated PEG hydrogel as a microfluidic scaffold for liver tissue engineering, and the techniques and materials utilized in our experimental design have been chosen and modified from the literature. This thesis research will contribute to future literature, by helping to further development of

photopolymerizable materials that can then be integrated in μ FDs as a cell culture platform.

2.6 References

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Chapter 3

Materials and Methodology

Given the combination of cellular biology, materials, and microfluidic technology involved in this project, there are a multitude of techniques that can be used to test our hypotheses. We have carefully designed and tailored our experiments to focus on the fundamental studies of the biomaterial and its incorporation into the μ FD, as well as the physical effect on the cells. This chapter introduces the materials and experimental technique concepts and methods that are used as tools to develop the hepatocyte-encapsulated bioactive PEG hydrogel μ FD.

3.1 Materials

The SU-8 silicon wafer used for the μ FD was previously designed in the BioSyM laboratories (SMART). Sylgard 184 silicone elastomer base and curing agent were attained from Dow Corning (Singapore). Microscope cover glasses (24 x 50 mm; thickness 0.13-0.16 mm) were obtained from Deckgläser (Germany). 2,2-Dimethoxy-2-phenylacetophenone (DMPA), 1-Vinyl-2-Pyrrolidinone (NVP), Col I (from rat solution; 4 mg mL⁻¹), 2-hydroxy-4'-(hydroxyethoxy)-2-methylpropiophenone (HHEMP), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Singapore). Poly-D-lysine (PDL) hydrobromide solution was bought from Sigma-Aldrich as well and prepared by SMART member Yan Teck Ho. PEG-DA (molecular weight (MW) 4600 Da) was precipitated by acrylation of linear PEG (MW 4000 kDa; Sigma-Aldrich) and later purified by lab members Dr. Jeongeun Seo and Weibeng Ng, following a previously published method.^[1, 2] Ethanol was obtained from Merck (Singapore). Acryloyl-PEG-N- hydroxysuccinimide (PEG-NHS; MW 3400 Da) was procured from Laysan Bio (AL, USA). Huh-7.5 cell line had been obtained from RIKEN (VA, Japan). Calcium and magnesium free 150 mM PBS, 0.25% Trypsin and fetal bovine serum (FBS) were obtained from Gibco (Singapore). Penicillin-Streptomycin (Cat No. 10378016), DEPC treated water and Dulbecco's Modified Eagle Medium (DMEM; Cat No. 10569010) containing 4.00 mM L-Glutamine, 4500 mg L⁻¹ Glucose and Sodium Pyruvate were provided by Invitrogen Life Technologies (NY, USA). CCK-8 Assay was bought from Dojindo Laboratories (Singapore). Live/Dead® Cell Imaging Kit (488/570), 4', 6-diamidino-2-phenylindole (DAPI), and anti-mouse secondary antibodies conjugated with Alexa Fluor 488 were from Molecular Probes, Life Technologies Corp. (CA, USA). Anti-mouse primary antibodies against CYP3A4 and albumin were procured from Santa Cruz Biotechnology (Shanghai). Paraformaldehyde (PFA) and microscope cover slips (24 x 60 mm; thickness 1.3-1.6 mm) were acquired from VWR (Singapore). Triton X-100 was purchased from Bio-Rad Laboratories (Singapore). The equipment used and rationale for the techniques will be further described in the individual experimental technique subsections.

3.2 Microfluidic Device Preparation

3.2.1 Microfluidic Device Fabrication

Soft lithography is a microfabrication technique that generates micropatterns using physical contact between a soft polymer and a mold containing a patterned relief structure.^[3, 4] The fabrication of μ FDs for this platform employed standard soft lithography stamp methods and strictly followed the protocol^[5] used by my collaborators in the BioSyM laboratories. In brief, a mixture of the silicone elastomer base and curing agent at a ratio of 10:1 were poured on the SU-8 patterned silicon wafer that had been securely attached to a petri dish. The device pattern (**Figure 3.1**) has a long gel region flanked by two parallel media channels, and was chosen for basic biomaterial testing purposes. The width of the media channel and gel channel are 0.5 mm and 1.3 mm, respectively. The length of the channels is 15 mm and the height is 120 μ m. Trapezoidal micropillars have been designed to maintain solution stability upon introduction into the gel region. The PDMS was then allowed to degas, to remove all microbubbles present, using a DOA vacuum pump (Gast; Model No. DOA-P504-BN) and was later cured in an 80°C oven for 2 h. Following the peeling and the division of the six replicas of microchannel patterns, 1 mm and 3 mm biopsy punches (static devices) or 1 mm and 4 mm biopsy punches (flow devices) were used to punch the inlet and outlet holes for the gel and media channels, respectively. A piece of scotch tape was used to remove any small pieces of dust from the devices and the devices were autoclaved in a beaker full of de-ionized water for 20 min at 120°C. After being autoclaved, the devices were placed channel side up in empty pipette boxes under the BSC, using sterilized tweezers, and dried in a 70° oven for at least 12 h. The devices were then irreversibly bonded to clean glass cover slides using oxygen plasma treatment (conditions: 760 torr, 21% O₂ gas, 7.5 intensity and 2.5 min; Covance, Femto Science). Devices for collagen hydrogels were treated with PDL (described in section 3.2.2.1) and immediately kept with non-treated devices in the 80°C oven for at least 24 h in order to restore hydrophobicity. For cell culture purposes, the petri dishes were only opened under the BSC and non-PDL treated devices were usable for up to 1 month.

Figure 3.1 AutoCAD microfluidic device design for the silicon wafer. Each wafer makes six PDMS devices with three channels. The enlarged image shows the dimensions of the media channels and gel channel. Feature height is 120 μm . [The AutoCAD file was sourced from SMART members and designed in 2009 by Dr. Waleed Farahat, an MIT affiliate]

3.2.2 Microfluidic Device Pre-treatment

Proper attachment of the PEG-DA hydrogel and collagen hydrogel to the PDMS channels is necessary for long-term cell culture and stable dynamic conditions. DMPA PI and PDL, respectively, were utilized to coat the sides to ensure proper hydrogel adhesion.

3.2.2.1 Photoinitiator Immobilization

In free radical photopolymerization, oxygen molecules inhibit the crosslinking process by interacting with the free radicals (photopolymerization further explained in Section 3.3). During photopolymerization of macromers in a PDMS μ FD, oxygen easily diffuses through the walls and creates an un-cross-linked layer near the walls of the device (**Figure 3.2**).^[6, 7]

One method to reduce and almost eliminate this unpolymerized area, and the method utilized in this research, is to coat the channel surfaces with a less-water soluble PI prior to gel precursor solution insertion and polymerization.^[8-13] The increase in PI concentration enhances the probability that photopolymerization will be successful near the PDMS channel walls. Briefly, 1 h prior to PEG pre-polymer solution insertion, 300 mg ml⁻¹ DMPA in NVP was loaded into the devices for 5 min. Excess PI was removed by aspiration and sequential pipetting of ethanol and nuclease-free water. The devices were then kept in a vacuum and protected from light to remove any remaining moisture that would interfere with gel insertion.

Figure 3.2 Photopolymerization within an oxygen-permeable PDMS microfluidic device. The rapidly replenished oxygen diffuses through the PDMS walls and reacts with free radicals, leaving an un-polymerized layer near the walls. [Sourced from Dendukuri, 2008^[6]]

3.2.2.2 Poly-D-lysine Coating

PDL is a positively charged molecule that enhances surface adhesion to negatively charged molecules such as collagen, therefore preventing detachment of collagen from surfaces during cell culture. PDMS devices prepared for cell culture with

collagen hydrogels were treated with 60 μL of PDL immediately after plasma bonding, while the device is hydrophilic. The devices were then incubated for 4 h at 37°C and the device channels were washed thoroughly by pipetting and aspirating 200 μL of DI water 4 times.

3.3 Cell Culture

Huh-7.5 cells, the model hepatocytes used in this research, are a cell subline highly permissive for HCV replication and derived from human hepablastoma cell line Huh-7.^[14] Huh-7.5 cells were cultured in high glucose DMEM supplemented with 10% heat-inactivated FBS and 1% Penicillin-Streptomycin and incubated in 37°C in a humidified atmosphere with 5% CO_2 . Cells used in experiments were allowed to reach 80% confluency in T-75 flasks and then detached using 0.25% trypsin. Cell counting was performed automatically with the TC20TM Automated Cell Counter (Bio-Rad Laboratories, Inc., Singapore). Once cells were properly encapsulated in the μFDs (see Section 3.4), 80 μL of media was pipetted as a droplet at each media channel inlet/outlet under static conditions. Cells were cultured in the μFDs for a period of 7 days and media was changed daily.

3.4 Cell Encapsulation within Microfluidic Device

Cell encapsulation is the process by which cells are initially immobilized within the cross-linked hydrogels. Hydrogel crosslinking can occur by physical stimuli, such as UV radiation and thermal change, or chemical stimuli, such as the addition of cations or enzymes.^[15] Photopolymerization, that is the crosslinking of polymers to form a network in the presence of a PI and light, is a common physical method utilized to form PEG-DA hydrogels as 3D scaffolds for cells. Upon irradiation by UV light, the PIs undergo a cleavage reaction and release free radicals. These radicals then react with the acrylate groups and initiate the chain propagation and polymerization.^[16] PI and UV exposure can be cytotoxic to cells depending on the PI type and concentration, UV intensity, and UV exposure time. HHEMP, also found commercially as Irgacure 2959, was chosen as the PI because studies have shown that it is well tolerated amongst many cell types^[17]. Altering the MW of the polymer, the concentration of the polymer and PI, and the time and intensity of UV exposure easily control the structural and mechanical properties of the hydrogel.^[18, 19] Preliminary

studies determined the lowest concentration of HHEMP, 0.05%, and UV exposure time, 2.5 min, needed to polymerize the PEG-based hydrogels in the μ FD with minimal damage to cells. Thermally-cured biomaterials, such as collagen, use adjustments in temperature to crosslink polymers and change from solution to hydrogel.

Figure 3.3 Schematic of hydrogel polymerization process within microfluidic device. After device assembly, (A) μ FDs intended for Huh-7.5 encapsulated PEG hydrogel cell culture are first treated with DMPA (green). Cells and pre-polymer solution are then introduced and the device is exposed to UV for curing. (B) Devices for collagen hydrogel and cell culture are treated with PDL (red). After pipetting the precursor solution in the gel region, devices are kept in the 37°C incubator for thermal curing of the collagen gel. [After filling image in (A) adapted from Farahat, 2012^[20]]

3.4.1 Cell Encapsulation in PEG Hydrogel

Cell-encapsulated PEG hydrogels were prepared in the μ FD using different concentrations of PEG-DA macromers. PEG hydrogel pre-polymer solutions (composition volumes detailed in Table 4.1) consisted of Huh-7.5 cells (7.5×10^6 cell mL^{-1}), PEG-DA solution, 10X PBS with phenol red, 1X PBS, and 0.05% HHEMP PI (w/v) and were prepared in batches of 200 μL . Eight μL of the well-mixed precursor solution including cells was then injected into the gel region of the DMPA-coated

μ FD (**Figure 3.3A**) via the hydrogel inlet and outlet. The device was flipped so that the glass slide was facing up and exposed to UV light (long-wave $\lambda = 365$ nm; intensity 10.84 mW cm^{-2}) from the Bluewave 200 UV spotlight (Blaze Technology Pte. Ltd, Singapore) for 2.5 min. After polymerization, the devices were immediately rinsed with media in order to flush any excess PI or unpolymerized PEG-based macromers away and to support the cells.

Table 3.1 Composition of 5%, 7%, and 9% PEG hydrogel pre-polymer solution. Each batch prepares 200 μL , with a final cell concentration of $7.5 \times 10^6 \text{ cells mL}^{-1}$ and 0.05% HHEMP PI (w/v).

[PEG-DA] % (w/v)	Volumes (μL)				
	20% PEG-DA stock solution	7.5×10^7 Huh-7.5 cells mL^{-1}	PBS		1% HHEMP stock solution
			10X with phenol red	1X	
5	50	20	20	100	10
7	70	20	20	80	10
9	90	20	20	60	10

3.4.2 Cell Encapsulation in PEG-Col I Hydrogel

Co-polymerization of PEG-DA macromers with acrylated proteins has enabled bioactive hydrogels with active binding sites throughout the hydrogel. The amine group of peptides and proteins react with the NHS ester of PEG-NHS, which can later be polymerized with the PEG-DA macromers. Thus PEG-DA, acr-PEG-NHS and different ratios of proteins can be combined and controlled in order to produce the ECM-mimetic hydrogel. ^[18]

Keeping the PEG concentration constant, cell-encapsulated Col I-functionalized PEG (PEG-Col I) hydrogels were prepared in the μ FD using different Col I concentrations. PEG-NHS and Col I were first incubated at room temperature (RT) for 2 h to form a PEG-protein conjugate. This PEG-protein conjugate was added to a PEG hydrogel precursor solution, similar to the one described in Section 3.4.1, consisting of Huh-7.5 cells ($7.5 \times 10^6 \text{ cell mL}^{-1}$), PEG-DA solution, 10X PBS with phenol red, 1X PBS, and 0.05% HHEMP PI (w/v) (composition detailed in Table 5.1). Injection of this PEG-Col I solution into the μ FD, photopolymerization, media flushing, and media replenishment are all the same as in Section 3.4.1.

Table 3.2 Composition of PEG-Col I hydrogel pre-polymer solution with Col I concentrations of 0, 10, 100, 200, and 400 $\mu\text{g mL}^{-1}$. Each batch prepares 200 μL , with a final PEG concentration of 9% (*w/v*), cell concentration of 7.5×10^6 cells mL^{-1} and HHEMP PI concentration of 0.05% (*w/v*). The PEG-DA to PEG-NHS molar ratio is 5:1, a ratio previously established by members of TSG.

[Col I] ($\mu\text{g mL}^{-1}$)	Volumes (μL)					
	5% PEG-NHS stock solution	4 mg mL^{-1} Col I stock solution	20% PEG-DA stock solution	7.5 x 10 ⁷ Huh-7.5 cells mL^{-1}	PBS	
					10X with phenol red	1X
0	--	--	90	20	20	60
10	46.4	0.5	78.4	20	20	24.7
100	46.4	5	78.4	20	20	20.2
200	46.4	10	78.4	20	20	15.2
400	46.4	20	78.4	20	20	5.2

3.4.3 Cell Encapsulation in Col I Hydrogel

Cell-encapsulated Col I microfluidic gels were prepared in the μFD as described previously in literature.^[21] Briefly, a 2.0 mg mL^{-1} Col I solution was prepared from 10X PBS (with phenol red), 0.5 N NaOH, Col I, and cell water added and carefully pipetted in that order. All components were initially kept on ice and the solution was adjusted with NaOH to a pH of ~ 7.4 (checked by phenol red color and pH indicator strips) before addition of Huh-7.5 cells (7.5×10^6 cell mL^{-1}). A summary of the Col I gel composition is presented in Table 5.2. Eight μL of the well-mixed precursor solution was then injected into the gel region of the PDL-coated μFD and immediately placed in a humidified pipette box and kept in the 37°C incubator (**Figure 3.3B**). After 30 min, devices were taken out of the incubator and fresh media was carefully pipetted into the device. Media was changed daily.

Table 3.3 Composition of 2 mg mL^{-1} Col I hydrogel pre-polymer solution. Each batch prepares 200 μL .

[Col I] (mg mL^{-1})	Volumes (μL)				
	10 X PBS with phenol red	0.5 N NaOH	4 mg mL^{-1} Col I stock solution	7.5 x 10 ⁷ Huh-7.5 cells mL^{-1}	Cell/DI water
2.00	20	3	100	20	57

3.5 Hydrogel Characterization

In this research, the hydrogel is characterized by (1) the polymerization efficiency measured as the ability of the hydrogel to reach the PDMS micropillars post-

polymerization in the μ FD and (2) the gel mechanical and structural properties measured via oscillatory shear rheometry.

3.5.1 Polymerization Efficiency within Microfluidic Device

Proper hydrogel polymerization in the μ FD gel region is important for embedded microfluidic hydrogels to ensure uniform flow across the hydrogel in a dynamic device system and cell density maintenance. Unpolymerized hydrogel solution will be washed away during the media insertion into the μ FD, exposing a defined hydrogel boundary in the gel region. Thus, this boundary reveals the extent of successful hydrogel polymerization in the μ FD.

Images of the cell-encapsulated hydrogel and its boundary within the μ FD were captured using Nikon Eclipse TS100 inverted routine microscope (Nikon Instruments Inc.; Melville, NY, USA) at 4X magnification one day post-polymerization. The cell density was measured following Live/Dead assay, detailed in Section 3.7.1.

3.5.2 Measuring Stiffness and Mesh Size with MCR Rheometer

Oscillatory shear rheometry enables us to quantify viscoelastic properties of a soft material, including hydrogel. By inducing sinusoidal shear deformations in a sample between two plates and measuring the stress response, a rheometer determines specific material properties which can then give information about the material structure.

Cell-laden hydrogels constructs (diameter $\sim$ 8-10 mm) were prepared similarly to the hydrogels polymerized within the μ FD, however with larger dimensions for accurate rheometer measurement. Briefly, 50 μ L of PEG-DA, PEG-Col I or Col I prepolymer solution (preparation described in Sections 3.4.1-3.4.3) was pipetted on a flat PDMS surface between two 1.3-1.6 mm microscope cover glass spacers. A microscope cover slip (0.13-0.17 mm) was placed over the drop of pre-polymer solution. Pre-polymer solutions containing PEG macromers were exposed to UV light (long-wave $\lambda = 365$ nm; intensity 10.84 mW cm^{-2}) from the Bluewave 200 UV spotlight (Blaze Technology Pte. Ltd, Singapore) for 2.5 min and Col I solutions were placed in a humidified pipette box and kept in the 37°C incubator. Once polymerized, the

hydrogels were removed from the PDMS set-up and kept in a 24 well plate in 1 mL media for 24 h.

Rheological measurements were performed using a Physica MCR 501 rheometer (Anton Paar, Germany) with an 8 mm diameter parallel plate, a 0.75-1.25 mm gap, and Peltier plate temperature-controlled base at 37°C. The testing conditions for frequency-sweep measurements were 0.5% strain amplitude at an oscillation frequency of 0.1–10 Hz within the linear viscoelastic regime. Following the device measurements, the complex shear modulus (G^*) was determined as

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad (3.1)$$

where G' is the storage modulus and G'' is the loss modulus. The mesh size (ξ), was calculated as

$$\xi = \left(\frac{G' N_A}{RT} \right)^{-\frac{1}{3}} \quad (3.2)$$

where N_A the Avogadro constant, R is the molar gas constant, and T is the temperature.^[22, 23]

3.6 Introducing Passive Flow in Microfluidic Device

Microfluidic devices can either have flow induced by a pressure gradient or use external flow machines to drive the flow. Complications and limitations arise when using external flow machines because of the extra tubes and equipment. Thus, pressure driven flow is commonly used in the μ FDs.^[24] Liquids have a natural tendency to flow from an area of higher pressure to an area of lower pressure. In each microfluidic channel, a pressure gradient can be developed by creating a greater pressure at the inlet than at the outlet, either with a syringe or by pipetting a droplet at each reservoir. To induce interstitial flow, a pressure difference is created between parallel channels (**Figure 3.4**).

Figure 3.4 Pressure gradient induced to drive interstitial flow across gel scaffold. Media flows from the channel with high pressure to the channel with lower pressure. As the fluid flows across the gel region, the pressures of the two channels come into equilibrium. [Adapted from Sudo, 2009^[25]]

For microfluidic culture under flow, a pressure gradient was induced one day after cell encapsulation in the PEG-Col I microfluidic hydrogel. Four 2 mL syringe barrels were cut at the 1 mL mark and carefully inserted into the media channel inlets and outlets without creating microbubbles. The interstitial volumetric flow rate was found experimentally by starting the height difference at 10 mm H₂O and noting the volume passing through the gel and the new height difference every 30 min. The flow rate (Q) was calculated as

$$Q = \frac{\Delta V_n - \Delta V_{n+1}}{t} \quad (3.3)$$

in which ΔV_n is the volume difference at time point n , ΔV_{n+1} is the volume difference at the next time point $n + 1$, and t is the time between the two time points. The pressure difference (ΔP) was calculated as a conversion from the height difference as

$$\Delta P = h * 9.8 \frac{\text{Pa}}{\text{mm H}_2\text{O}} \quad (3.4)$$

in which h is the height difference in mm H₂O.

To generate an initial interstitial flow of $\sim 5 \mu\text{L h}^{-1}$, a height difference of 0.8 mm H₂O between the two media channels was established. The height difference equilibrated after approximately 12 h and media was replenished every day. Cut tops of 1 mL centrifuge tubes were placed on top of the open syringes to minimize media evaporation.

3.7 Cell Biological Assays

The size of the microfluidic hydrogel, hydrogel accessibility restrictions and small mesh size of PEG hydrogels limit the assays that can be used to quantify cell function and viability in the μ FD. Media-based quantitative assays such as Cell Counting Kit-8 and Albumin ELISA were attempted, but gave unreproducible and inaccurate results. Therefore, assays that primarily use cell and protein staining have been utilized to investigate the effect of the different hydrogel parameters on cell behavior. In order to determine the viability of the Huh-7.5 cells encapsulated within the various hydrogels in the μ FD, Live/Dead® Cell Imaging Kit assay was utilized. Given the hepatocytes main functions in drug metabolism and serum protein secretion, CYP450 enzyme activity and intra-cellular albumin production were assessed and visualized using immunocytochemistry.

3.7.1 LIVE/DEAD Cell Viability Analysis

LIVE/DEAD® Cell Imaging Kit labels live and dead cells through the use of fluorescent dyes, optimized for imaging filters used with fluorescein isothiocyanate (FITC) and Texas Red. In living cells, cell-permeant non-fluorescent calcein acetomethoxy (calcein AM) is enzymatically converted, by intracellular esterases, to the intensely fluorescent calcein, which is retained within the cells and stains them green. The kit's red cell-impermeant component, Ethidium homodimer-1 (Ethd-1), enters the cells with damaged membranes and binds to nucleic acids, thereby producing fluorescence and staining the dead cells red.

Following the manufacturer's protocol, on Days 1, 3, 5 and 7 the kit vial components were mixed, 100 μ L of the solution was introduced into the media channel of the μ FD and the devices were incubated for 15 min at RT protected from light. Z-stack images of the stained cells were collected using confocal laser-scanning microscopy (CLSM) with a Zeiss LSM 780 microscope (Carl Zeiss AG, Oberkochen, Germany) with a 60X objective. For each sample, six different areas in the hydrogel were imaged.

3.7.1.1 Quantification with Imaris Software

IMARIS software (Bitplane AG, Zurich, Switzerland) was utilized to process the Z-stacks, track the cells and quantify the viability of Huh-7.5 cells and the cell density

of the cell-laden hydrogels. The spot tracking function was utilized (**Figure 3.5**), setting the live and dead cell sphere diameter to 12.5 μm and 10 μm respectively, to count the total number of live (green) and dead (red) cells in each confocal stack. Cell viability and cell density of each Z-stack were then determined as

$$\text{Cell viability} = \frac{N_L}{N_L + N_D} = \frac{N_L}{N_T} \quad (3.3)$$

and

$$\text{Cell density} = \frac{N_T}{A * h} \quad (3.4)$$

where N_L is the number of live cells, N_D is the number of dead cells, N_T is the total number of cells, A is the area of one confocal image, and h is the height measured between the top-most and bottom-most cells in the hydrogel. The Z-stack's viability and density numbers were averaged to find the sample's cell viability and cell density.

Figure 3.5 Imaris software spot tracking to quantify cell viability. By defining an estimated cell diameter for live cells and dead cells, Imaris is able to differentiate and count the number of live cells (green spots) and dead cells (red spots) that have been stained using Live/Dead Cell Imaging Kit.

3.7.2 Immunocytochemistry

Immunocytochemistry is a method used to detect target proteins using specific antibodies to those proteins. The liver cell metabolic activity and protein production inside the PEG or Col I hydrogel were investigated by immunostaining the cells with CYP3A4 and albumin antibodies. On Day 7, the Huh-7.5 cells were fixed with 4% paraformaldehyde for 30 min (PEG-DA/PEG-Col I hydrogel) or 10 min (Col I hydrogel) at RT. After the devices' channels were washed 3 times with PBS, the devices were kept at 4°C until the immunostaining process was started. To commence the cell staining, cell membranes were permeabilized by pipetting 0.1% Triton X-100

into the devices and incubating them for 30 min (PEG-DA/PEG-Col I hydrogel) or 10 min (Col I hydrogel). The samples were then washed 3 times with PBS and blocked with 5% BSA/PBS for 1 h. Primary antibodies against CYP3A4 or albumin were then added at 1:50 dilution in 0.5% PBS/BSA solution and left at 4°C overnight. After washing the devices 3 times in PBS/BSA buffer, Alexa Fluor 488 conjugated goat anti-mouse secondary antibodies were administered at 1:200 dilutions in PBS/BSA at RT for 2 h. The devices were counterstained with DAPI, washed 3 times with 0.5% PBS/BSA and twice with PBS and then imaged via CLSM with the Plan-Apochromat 63X / 1.4 Oil DIC objective lens (LSM 510; Carl Zeiss AG, Oberkochen, Germany).

3.7.2.1 Fluorescence Quantification with ImageJ Software

ImageJ software (developed at the National Institutes of Health, Bethesda, Maryland) was employed to process the Z-stacks of confocal immunocytochemistry images and quantify the fluorescence intensity of the target protein averaged per unit volume of the cell. For each image slice in the Z-stack that contained part of a cell (double-checked with the DIC image and DAPI image), the freehand selection tool was used to manually circle the perimeter of the cell part and the area and integrated density were analyzed. Once all images in the z stack were complete, the average fluorescence per cell volume was calculated as

$$\text{Fluorescence Intensity per } \mu\text{m}^3 = \frac{\sum I_n}{d \sum A_n} \quad (3.5)$$

in which I_n is the fluorescence intensity of the selected area in each image n in the z-stack, A_n is the area selected for each image n , and d is the distance between each confocal image.

3.8 Statistical Analysis

Experiments were repeated two or three times. One representative image is presented for qualitative experiments and each data point represents the mean and standard deviation for quantitative experiments. Error bars represents the standard error of the mean. A one-way ANOVA (analysis of variance) was employed to determine statistical significance and post-hoc student t-tests were also performed with $p < 0.05$.

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Chapter 4

Huh-7.5 Encapsulated PEG Hydrogel within Microfluidic Device

An important facet in the development and testing of a microfluidic hydrogel is proper integration of the basic biomaterial and cells within the μ FD. The microfluidic hydrogel should be stable in the device and properly encapsulate or host the cells, maintaining viable cell density and parenchymal cell function. In this chapter, Huh-7.5 cells are encapsulated within PEG hydrogels with different PEG-DA concentrations in a PDMS μ FD to understand the PEG network in a μ FD and to evaluate its potential as a microfluidic liver cell culture platform. Cell viability and function are measured and the PEG hydrogel is characterized to study the effect of hydrogel mechanical properties on cell behavior and to select a PEG-DA concentration suitable for Huh-7.5 cells. The experimental outcomes are presented and discussed in detail.

4.1 Introduction

Development of *in vitro* cell culture μ FDs recapitulating *in vivo*-like conditions of the hepatic microenvironment offers potential for highly-accurate liver-tissue engineering-based applications, such as xenobiotic toxicity testing and studying liver diseases. The scaffold, in which cells are embedded or seeded upon, is an integral part of the platform, providing biophysical and biochemical cues that regulate cell behavior. Proper microscaffold integration with the μ FD housing material is important, as are mechanical hydrogel properties including stiffness, mesh size, biodegradability, and swelling. Synthetic hydrogels, such as PEG-based hydrogels, have advantages to tailor their chemical and structural properties. PEG-based hydrogels have been widely developed as a scaffold material for liver tissue engineering,^[1] given the ability to tune the hydrogel's mechanical properties,^[2] bioactivity,^[1] geometry^[3-5] and degradation rate.^[6] Despite significant advances in engineered microfluidic scaffolds for hepatic cell culture, there has been a paucity of sealed μ FDs incorporating photopolymerizable synthetic PEG hydrogel materials in literature.

Herein, we integrated Huh-7.5 cell-encapsulated PEG hydrogel in a PDMS μ FD, controlling the mechanical and structural properties by using different PEG-DA concentrations. By capturing images of the PEG hydrogel-PDMS micropillar interface, the extent of polymerization and success of integration was confirmed. Rheological measurements of the PEG hydrogel examined mechanical and structural properties of the cell-laden hydrogel to compare to physiological liver conditions. Cell culture in the μ FD was observed over 7 days and hepatocellular viability, density and function were probed.

4.2 Experimental Design

PDMS μ FDs were fabricated and pre-treated with DMPA as described in Section 3.2.1 and Section 3.2.2.1, respectively. Cell culture was performed as shown in Section 3.3. Huh-7.5 cell-encapsulated PEG hydrogels with concentrations of 5%, 7% and 9% (*w/v*) were polymerized and integrated into the PDMS μ FDs as detailed in Section 3.4.1. PEG hydrogels were characterized by rheometry and qualitative light microscope image analysis as seen in Section 3.5. Live/Dead assay and

immunohistochemistry were performed on encapsulated cells as described in Section 3.7.

4.3 Results and Discussion

A preliminary step taken towards integration of the cell-encapsulated PEG hydrogel in the PDMS μ FD was to determine design parameters of the PEG photopolymerization process that would allow gel stability and minimize cell damage. Given the ability to support materials with low stiffness in μ FDs and a desire to achieve higher viability for the cells,^[7] PEG hydrogels of 4.6 kDa MW were tested at low concentrations of 5%, 7%, and 9% (w/v), henceforth denoted as 5% PEG-DA, 7% PEG-DA and 9% PEG-DA, respectively. The photopolymerization parameters were optimized for cell health,^[8-10] which required minimizing HHEMP PI concentration to 0.05%, lowering UV exposure time to 2.5 min and setting the UV intensity to 10.84 mW cm⁻². These conditions coupled with the initial PDMS housing pre-treatment with DMPA^[11-13] resulted in proper polymerization of all hydrogel concentrations in the μ FD (**Figure 4.1A**), as revealed by the hydrogel boundaries reaching the PDMS micropillars. Higher concentrations of PEG-DA macromers showed increased cross-linking efficiencies with boundaries closer to the media channel.

After verifying the intact PEG hydrogel-PDMS interface, we next investigated Huh-7.5 cell density in the microfluidic hydrogel to understand the encapsulation of cells after hydrogel polymerization and subsequent media replenishments (**Figure 4.1B**). Huh-7.5 cells were initially introduced into the μ FD at a concentration of 7.5×10^6 cell mL⁻¹ (7.5×10^3 cell mm⁻³). All hydrogels showed average post-encapsulation efficiencies less than 65%. A possible explanation for the microfluidic hydrogels' low cell encapsulation is cell sedimentation.^[14] Though pre-polymer solutions were mixed well before insertion into the μ FD and the time before polymerization with UV was minimized, the difference in densities of cells and other components in the pre-polymer solution in such small dimensions sometimes led to cells settling. This has primarily been found true for cells photo-encapsulated in hydrogel microbeads.^[15, 16] The degree of cell sedimentation was inversely proportional to the macromer concentration (see **Figure 4.3B**). A significantly lower post-encapsulation cell density, 37%, was found in 5% PEG-DA hydrogel and can possibly be explained by the enhanced cell sedimentation^[17] and concomitant polymerization inefficiencies.

Figure 4.1 Characterization of cell-encapsulated PEG-DA hydrogel polymerization and cell density within the PDMS microfluidic device. Following hydrogel polymerization in the μ FD, (A) optical images were taken near the end of the gel region (closer to the gel inlet) and the middle of the gel region. The white arrows in the enlarged image point to the hydrogel boundary between two micropillar posts. Scale bars are 200 μ m. (B) Cell density was quantified using a combination of Live/Dead assay, CLSM and IMARIS spot tracking software to count the total cells on days 1, 3, 5 and 7 ($n = 3$, mean $\pm$ SD; $p < 0.05$, one way ANOVA). The difference in cell densities for 7% and 9% PEG-DA hydrogels were not statistically significant ($P > 0.05$).

To understand the mechanical and structural properties of the microfluidic PEG-network, shear moduli and mesh sizes of the hydrogels were calculated from rheological measurements (**Figure 4.2**). Consistent with other hydrogel studies,^[18] the shear moduli of PEG hydrogels were dictated by macromer concentration, significantly increasing with higher PEG-DA percentages. The moduli ranged from 349.84 Pa to 775.46 Pa, and calculations based on the rubber-elastic theory^[19] revealed mesh sizes in the range of 17.32 nm to 23.10 nm. The shear modulus for 9% PEGDA fits in the range of healthy physiological liver stiffness.^[20]

[PEG-DA]	G' (Pa)	ξ (nm)
5%	349.84 ± 45.95	23.10 ± 1.02
7%	670.27 ± 29.75	18.57 ± 0.27
9%	775.46 ± 24.79	17.32 ± 0.18

Figure 4.2 Mechanical and structural characterization of cell-laden microfluidic hydrogels with different concentrations of PEG-DA. The complex shear moduli (G^*) of swollen Huh 7.5-encapsulated hydrogels (~8-10 mm diameter, 1.1 mm thickness) were obtained by oscillatory shear rheological measurements (top; $n=3$, mean $\pm$ SD; *: $P < 0.05$, **: $P < 0.01$). Mesh size (ξ) was calculated from the storage modulus (G') of the hydrogels (bottom). There are no significant differences in the mesh sizes of the hydrogels ($p > 0.05$, one way ANOVA).

Cell culture in the PEG hydrogel-integrated μ FD promoted high Huh-7.5 cell viability. After cells were treated with the Live/Dead assay, representative confocal images revealed viable green cells dispersed uniformly throughout the xy -plane (**Figure 4.3A**) but a non-uniform distribution of cells in the z direction due to cell sedimentation and its dependence on macromer concentration (**Figure 4.3B**). The increased cell settling can be explained by a lower viscosity of the precursor solution^[16] and increases in gelation time^[21] with lower concentrations of PEG-DA. Smaller hydrogel dimensions may account for proper nutrient and oxygen transport throughout the width of the hydrogel, in contrast to cell-laden hydrogels of larger dimensions which often find less viable cells in the center of the hydrogel.^[4] There tended to be a higher concentration of dead cells close to the outer edge of the hydrogel, near the PDMS micropillars. Yeh et al.^[10] hypothesized that dead cells observed near the periphery of microgels may not be properly embedded in the hydrogel, leading to a loss of viability. Given the higher concentration of DMPA PI by immobilization on the PDMS housing, we hypothesize that the cells closer to the PDMS posts were exposed to higher free radical concentrations during polymerization and thus lost viability. Another surmise is that cells near the media channels experience high shear stresses during the daily media changes, manually pipetted, which can have detrimental effects on the cells.^[22] Nevertheless, cell viability of the encapsulated cells was greater than 80% for all hydrogel conditions over the cell culture period of 7 days (**Figure 4.3C**). Increases in PEG-DA concentration from 5% and 7% to 9% on Day 1 had a significant effect on cell viability in the μ FD, with 9% PEG-DA hydrogel supporting 93% and 98% viability, respectively. The lower cell viabilities in 5% and 7% PEG-DA hydrogels can be attributed to enhanced cell sedimentation leading to exposure of settled cells to high concentrations of free radicals from the DMPA PI near the top PDMS microfluidic hydrogel region. There was no significant recovery of cell viability as time passed.

Further characterization of cell behavior by immunohistochemistry revealed the encapsulated Huh-7.5 cells produced albumin and CYP3A4 (**Figure 4.4A**), but there were no significant differences in fluorescence intensities between hydrogels of different PEG-DA concentrations (**Figure 4.4B**). Huh-7.5 cells in all hydrogels started to cells embedded in the microfluidic PEG hydrogel. Phalloidin staining of the cytoskeleton was problematic (ultimately not shown) and permeabilization and other

Figure 4.3 Evaluation of Huh-7.5 cell viability and cell spatial arrangement in microfluidic hydrogels with different PEG-DA concentrations. Confocal images of cells labeled by Live/Dead were taken on days 1, 3, 5, and 7. Representative images show an (A) xy -plane maximum projection of a Z-stack for all days and an (B) xz -plane view of the 3D hydrogels on Day 1. Calcein AM (green) indicates live cells and Ethd-1 (red) indicates dead cells. The axes show the orientation of the hydrogel; x (green) parallel to the media channel, y (blue) perpendicular to the media channel, z (red) the depth of the hydrogel. Scale bars are 150 μ m (A) and 100 μ m (B). Image analysis of the confocal images by IMARIS allowed quantification and (C) histogram construction of cell viability, defined as the number of live cells divided by the total number of cells ($n = 3$, mean $\pm$ SD; **: $P < 0.01$).

Figure 4.4 Evaluation of liver specific functions of Huh-7.5 cells encapsulated in PEG-DA microfluidic hydrogel by immunocytochemistry. On Day 7 (A) confocal images were taken of Huh-7.5 cells stained for either albumin (top; green) or CYP3A4 (bottom; green) and counterstained by DAPI for DNA (blue). Representative images show an xy-plane maximum projection of a Z-stack. Scale bars are 25 μ m. (B) The average target protein fluorescence intensity per unit cell volume (mm^3) was quantified by analyzing the confocal images with ImageJ software. There are no significant differences in the target protein fluorescence intensity for hydrogels with different PEG-DA concentrations ($p > 0.05$, one way ANOVA).

staining aggregate into small spheroids by Day 7, which is consistent with studies of hepatocytes encapsulated in bioinert materials.^[2, 23] There was noticeable difficulty in staining the processes were performed multiple times before accurate representative images could be captured. Underhill et al.^[1] attributed the challenge of staining bulk PEG hydrogels to diffusive limitations that arise from the small mesh size and

sectioned the hydrogels to achieve proper antibody labeling. Unfortunately, given the inaccessible nature of the integrated microfluidic hydrogel, sectioning is impossible without taking the device apart and removing the scaffold. Other qualitative biological assays for cell function, such as albumin or urea ELISA and transcriptional assays, were also difficult to employ accurately due to the evaporation of media from the channel inlets/outlets, the entrapment of molecules in the hydrogel network, and difficult cell recovery.

4.4 Conclusion

This work shows the successful integration of Huh-7.5 cell-encapsulated PEG hydrogel in a PDMS μ FD for hepatocyte culture, maintaining high cell viability and prominent liver-specific function. The shear modulus and mesh size of PEG hydrogel were tailored by varying the macromer concentration from 5-9%. Pre-treatment of the μ FD with DMPA solved the problem of oxygen-inhibited photopolymerization, allowing an acceptable PEG hydrogel-PDMS interface. Though varying the PEG-DA concentration from 5-9% (w/v) had little effect on Huh-7.5 viability and function, the higher PEG-DA concentration of 9% promoted a high post-encapsulation cell density and uniform cell distribution, exhibited the best crosslinking efficiency within our DMPA-coated PDMS casing, and had a shear modulus comparable to lower values in *in vivo* healthy livers. Given the physiological cell behavior were comparable, secondary important hydrogel criterion such as stability and uniformity were evaluated to pick the best PEG-DA concentration. Hence, 9% was chosen as the PEG-DA macromer concentration for future experiments developing a bioactive PEG microfluidic hydrogel. Taken together, we demonstrate that photopolymerizable materials can be employed to culture liver cells in a μ FD and provide a platform in which scaffold mechanical properties can be easily controlled to develop integrated microfluidic scaffolds for future liver tissue engineering applications.

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Chapter 5

Varying ECM Composition within the Microfluidic Device

The formation of composite hydrogels grafting bioactive natural constituents with more mechanically stable synthetic polymers often resolves drawbacks arising from either type alone. In this chapter, PEG-Col I hydrogels with different Col I concentrations encapsulate Huh-7.5 cells in a μ FD and are compared to both bare PEG hydrogels and Col I gels as microfluidic scaffolds for hepatocyte cell culture. Cell behavior in the hydrogel is examined using biological cell assays and mechanical and structural properties of the different PEG-Col I hydrogels are investigated. The results are presented and discussed in detail.

5.1 Introduction

Many μ FDs for liver tissue engineering use Col I gel to encapsulate or sandwich the hepatocytes with verified maintenance of parenchymal function. Col I is the main ECM protein found in the hepatic microenvironment and regulates cell behavior through integrin transmembrane proteins, which link the ECM to the cytoskeleton. Though Col I provides the proper cell-binding sites needed by hepatocytes, its mechanical properties are weak and non-adjustable alone.^[1] The natural hydrogel does not recapitulate key biophysical properties of the physiological liver ECM that also maintains parenchymal function.

In Chapter 4, we suggested the successful integration of hepatocyte-laden PEG hydrogel in a PDMS μ FD and selected 9% PEG-DA as the optimal macromer concentration for mechanical and stability reasons. However, a major drawback to using bare PEG hydrogel as a scaffold is its non-adhesive nature. PEG-based hydrogels can be easily rendered bioactive by conjugating proteins or peptides to the scaffold via grafting, co-polymerization, click chemistry, and Michael-type addition.^[2] These methods, in addition to others, allow precise control over the amount of protein conjugated to the scaffold which is important for fundamental studies of the optimal hepatic ECM composition. The blend of synthetic PEG hydrogel and a natural hydrogel will ultimately create a novel material with desired tunable bio-functional and mechanical properties that has the potential for specific tissue engineering applications.^[3]

Herein, we co-polymerized PEG-DA macromers with different acrylated Col I concentrations to manipulate the cell adhesive and bioactive properties of the microfluidic cell-encapsulated PEG hydrogel. Given the addition of Col I protein and the loss of an acrylic group affects the polymerization efficiency, light microscope images were again captured to understand the proper integration of the bioactive hydrogel in the μ FD. Mechanical and structural PEG-Col I properties were examined utilizing oscillatory shear rheometry. Huh-7.5 cells were cultured in the μ FDs for 7 days with Live/Dead assays and immunocytochemistry evaluating cell viability and function. The results were compared to those of Huh-7.5 cell-encapsulated Col I hydrogel in a μ FD of the same design.

5.2 Experimental Design

PDMS μ FDs were prepared as described in Section 3.2.1. Microfluidic devices for PEG-based hydrogels were pretreated with DMPA and μ FDs for Col I hydrogel were pretreated with PDL as detailed in Section 3.2.2.1 and 3.2.2.2, respectively. Huh-7.5 cells were cultured as shown in Section 3.3 and encapsulated in PEG-Col I hydrogels, with initial Col I concentrations of 0, 10, 100, 200, and 400 $\mu\text{g mL}^{-1}$, and 2.0 mg mL^{-1} Col I hydrogels in the μ FDs as detailed in Section 3.4.2 and 3.4.3. The PEG-Col I hydrogel with 0 $\mu\text{g mL}^{-1}$ Col I is the same as the 9% PEG-DA hydrogel. All hydrogels were characterized by rheometry and qualitative light microscope image analysis as seen in Section 3.5. Biological assays were performed on encapsulated cells as described in Section 3.7.

5.3 Results and Discussion

Microfluidic PEG hydrogel was rendered bioactive in a controlled manner by copolymerizing different concentrations of acrylated Col I with PEG-DA macromers, using the same photopolymerization parameters mentioned in Chapter 4. The resulting PEG-Col I hydrogels had a 9% PEG mesh backbone, encapsulated Huh-7.5 cells, and theoretical conjugated Col I concentrations of 10, 100, 200, or 400 $\mu\text{g mL}^{-1}$, hereafter denoted as PEG-Col 10, PEG-Col 100, PEG-Col 200, and PEG-Col 400. Cell-encapsulated 9% PEG-DA hydrogels and 2.0 mg mL^{-1} Col I gels were prepared as control samples.

Altering the Col I concentration in PEG-Col I hydrogels markedly affected the extent of polymerization in the μ FD. Images of the microfluidic gel region area (**Figure 5.1A**) revealed that an increase in conjugated Col I concentration past 100 $\mu\text{g mL}^{-1}$ resulted in a hydrogel boundary at least 100 μm from the PDMS posts. This decrease in crosslinking efficiency was expected,^[4, 5] given PEG-NHS, which reacted with Col I to form an acrylated Col I conjugate, is known to terminate the growing PEG chain. The 5:1 PEG-DA to PEG-NHS ratio, used in this research, means that PEG-Col I hydrogels have 92% the number of reactive acrylate groups as pure PEG hydrogels. Almany et al.^[5] described the combination of fewer functional groups available for crosslinking and possible steric hindrances by bulky PEGylated protein molecules as limiting for hydrogel crosslinking. All other microfluidic hydrogels, including the

thermally cured collagen gel and 9% PEG-DA hydrogel controls displayed proper hydrogel-PDMS integration. Thermal curing process of natural polymers is not inhibited by oxygen molecules diffusing through the PDMS housing, unlike free radical polymerization,^[6] and thus the collagen gel formed a neat boundary connecting the micropillars at the edge of the gel region.

The hydrogels crosslinking differences are reflected in the initial cell densities observed in the hydrogel integrated μ FDs (**Figure 5.1B**). Collagen gel and 9% PEG-DA had the highest average cell encapsulation numbers but were both less than 60%. PEG-Col I hydrogels had significantly smaller initial cell densities in comparison to the collagen gel (post hoc t-test Day 1, $p < 0.01$ for PEG-Col 100 and $p < 0.001$ for PEG-Col 10, 200, and 400). These encapsulation differences can be explained by crosslinking limitations in the composite hydrogels leading to unpolymerized hydrogel and cells exiting the gel region and far fewer cell-binding sites because Col I concentration is an order of magnitude lower in the PEG-Col hydrogels. Interestingly, though the cell density in PEG hydrogel decreased significantly in the first 5 days (t-test, $p < 0.01$), there are no significant changes in cell density in any of the hydrogels with Col I over the culture period. Anderson et al.^[7] explained a similar phenomenon in PEG-peptide hydrogels, suggesting the reduction in cell density is due to the increase in swelling of the hydrogel matrix over time. However, PEG hydrogel theoretically should have lower swelling ratios than the PEG-Col I hydrogels because of its enhanced crosslinking density and therefore lower mesh size.^[8, 9] Another hypothesis, supported by our data, is that the non-adhesive properties of PEG hydrogel allow decreased cell density, whereas the conjugation of proteins enhances cell adhesion despite compromising the crosslinking efficiency.^[10]

Figure 5.1 Characterization of cell-laden PEG-Col I and Col I hydrogel polymerization and cell density within microfluidic device. (A) Optical images of the hydrogel boundary (indicated by white arrows in the enlarged image) near the end and middle of the gel region were taken after hydrogel polymerization. Scale bars are 200 μm . Following the use of a Live/Dead assay on days 1, 3, 5, and 7, (B) cell density was quantified using confocal images of the stained cells and IMARIS software ($n = 3$, mean $\pm$ SD; $p < 0.001$, one way ANOVA).

Quantification of the PEG-Col I hydrogels' stiffness and structure was performed using oscillatory shear rheometry to investigate changes in the mechanical nature caused by the conjugated protein (**Figure 5.2**). Our experimental shear modulus value of control collagen gel was not presented due to instrument measurement problems. Previous studies reported modulus values in the range of 15.5-17 Pa for $\sim 2.0 \text{ mg mL}^{-1}$ Col I gels,^[11, 12] All PEG-Col I hydrogels exhibited shear moduli between that of the 2.0 mg mL^{-1} Col I gels and 9% PEG hydrogel, and the values were an order of magnitude greater than Col I gels. The conjugated Col I concentration was inversely proportional to shear modulus and positively correlated with mesh size. Only conjugation of $400 \text{ } \mu\text{g mL}^{-1}$ to the PEG hydrogel resulted in a significant shear modulus decrease to 525.19 Pa, and the modulus value was comparable to that of PEG hydrogel with lower PEG-DA macromer concentrations (see Figure 4.2). As aforementioned, both the presence of monofunctional macromers and protein in the PEG-Col I hydrogel disrupt crosslinking, subsequently resulting in lower mechanical properties. Scott et al.^[13] decoupled the effects of PEG-NHS and collagen on hydrogel mechanical stiffness and found that the presence of PEG-NHS contributed more to the reduced matrix stiffness. However, in our research, the PEG-NHS concentration was kept constant and NHS: Col I ratios were changed, indicating that it was the sole presence of Col I concentration that negatively affected the mechanical properties by interacting with or blocking functional groups. The μ FD can support low microfluidic hydrogel stiffness, as indicated by the collagen gel boundary and cell density. However, lower mechanical properties of PEG-Col I hydrogels with higher Col I concentrations negatively affects integration into the PDMS μ FD and hence photopolymerization parameters or choice of PEG concentration needs to be revisited to improve cross linking density, successively enhancing shear modulus.

To examine the viability of encapsulated Huh-7.5 in inert PEG-hydrogels, bioactive PEG-Col I hydrogels, and natural Col I gels in a μ FD, Live/Dead assay was employed. Viable cells were embedded throughout the hydrogels, as revealed by confocal z-stack maximum projections (**Figure 5.3A**). Bio-inert 9% PEG-DA hydrogels promoted the highest number of cell aggregation by Day 7, indicated by the larger green spheroids. Huh-7.5 cells encapsulated in PEG-Col 10 and 100 hydrogels were evenly spread in the microfluidic hydrogels and dead cells were found towards the edges of the hydrogel, similar to PEG hydrogels. A previous study shows collagen

Figure 5.2 Mechanical and structural characterization of cell-laden PEG-Col I hydrogels with different conjugated Col I concentrations. Viscoelastic properties of swollen Huh-7.5-encapsulated PEG-Col hydrogels were measured by oscillatory shear rheometry and the complex shear modulus (G^*) was calculated from the obtained storage (G') and loss modulus measurements (top; $n = 3$, mean $\pm$ SD). Relating mechanical properties to hydrogel structure, the mesh size (ξ) was calculated from G' of the hydrogels (bottom; $n = 3$, mean $\pm$ SD). PEG-Col 400 ($400 \mu\text{g mL}^{-1}$ Col I conjugated) showed significant differences in shear modulus and mesh size in comparison to 9% PEG-DA hydrogel ($0 \mu\text{g mL}^{-1}$ Col I conjugated) (*: $P < 0.05$).

molecules are homogeneously positioned in PEG-DA hydrogels copolymerized with monoacrylated Col I.^[13] The maintenance of a relatively uniform dispersion of cells in the bioactive microfluidic hydrogels for all culture days is potentially due to the spatial distribution of Col I in the copolymerized PEG-Col hydrogels and cell adhesion. Huh-7.5 cells in the collagen gel control seemed to be partially encapsulated in the Col I gel, as indicated by the spread morphology of some cells. A noticeable and unexpected phenomenon was the perceived increase in dead cells throughout the hydrogel in the higher percentages of conjugated Col I. However, confocal image analysis by IMARIS software revealed that there were no significant differences or trends in Huh-7.5 cell viability in all microfluidic hydrogels (**Figure 5.3B**). PEG-Col I hydrogels supported at least 80% viability throughout the culture period.

Figure 5.3 Evaluation of Huh-7.5 cell viability in microfluidic PEG-Col I hydrogels with different Col I concentrations. (A) On days 1, 3, 5, and 7, cells were labeled with calcein AM (green; live cells) or Ethd-1 (red; dead cells) from the Live/Dead assay and imaged using CLSM. Representative images show an xy-plane maximum projection of a Z-stack. Scale bars are 150 μ m. (B) Cell viability was quantified by employing IMARIS software to analyze the confocal Z-stacks and count the total number of live cells and dead cells ($n = 3$, mean $\pm$ SD). There are no significant differences in the cell viability for all hydrogel types ($p > 0.05$, one way ANOVA).

Evaluation of cell behavior by immunohistochemistry and target protein fluorescence intensity quantification revealed few differences in Huh-7.5's liver-specific functions in the different hydrogels. Confocal images (**Figure 5.4A**) revealed that on day 7, cells in all hydrogels expressed albumin and CYP3A4, and cell aggregates started to form. Though Live/Dead staining had confirmed a higher density of hepatocyte spheroid aggregates in the 9% PEG-DA hydrogel, immunostaining revealed a positive correlation between Col I concentration and aggregate size. This can be explained by the change in matrix stiffness, as detailed in other studies.^[14-16] Although qualitative images uncovered little information to compare Huh-7.5 albumin and CYP3A4 production in the different hydrogels, quantitative ImageJ analysis (**Figure 5.4B**) showed significantly higher protein and enzyme expression (fluorescence intensity) in the collagen gel compared to the "blank sheet" 9% PEG-DA hydrogel. Albumin production in PEG-Col 10 and 100 hydrogels were also significantly lower than the collagen gel control, confirming the importance of Col I in regulating liver-specific functions.

Granted the differences in both cell-adhesive nature and network mechanical properties between PEG-Col I gels and our controls, further experiments need to be performed to decouple the effects of one from the other on Huh-7.5 cell behavior. However, this is outside the scope of our work and experimental design.

Figure 5.4 Evaluation of liver specific functions of Huh-7.5 cells encapsulated in PEG-Col I microfluidic hydrogel by immunocytochemistry. (A) Huh-7.5 cells were stained for either albumin (green) or CYP3A4 (green) and counterstained by DAPI for DNA (blue) on Day 7 and imaged by CLSM. Representative images show an xy-plane maximum projection of a Z-stack. Scale bars are 25 μ m. The confocal images were analyzed by ImageJ software to quantify the cell function, allowing a (B) histogram construction of the average target protein fluorescence per unit cell volume ($n = 3$, mean $\pm$ SD; *: $P < 0.05$)

5.4 Conclusion

This study demonstrates the successful bioactivation of PEG hydrogel with Col I and investigates the potential of PEG-Col I hydrogel to culture Huh-7.5 cells in a PDMS μ FD, comparing it to natural Col I and non-adhesive PEG-DA microfluidic hydrogels. Copolymerization of PEG-DA macromers with different concentrations of acrylated Col I proteins varied the degree of bioactivity, but also reduced the amount of crosslinking which in turn weakened the hydrogel's mechanical properties and lowered cell encapsulation efficiency. Only PEG-Col I hydrogels with conjugated Col I concentrations up to $100 \mu\text{g mL}^{-1}$ produced stable microfluidic gels with proper hydrogel-PDMS micropillar interfaces. PEG-Col I hydrogels promoted high cell viability and the production of two liver-specific proteins, but did not yield results at the same level as conventional Col I microfluidic gels. Furthermore, Col I hydrogels were much easier to integrate in the gel region of the μ FD in comparison to the PEG-Col I hydrogels and there was no need to expose cells to UV or free radicals. At this point, our original hypothesis has been partially refuted as no PEG-Col I composition showed cell physiological results comparable to the Col I hydrogels, although the higher concentrations of PEG-Col 200 and PEG-Col 400 came the closest. However for future studies that may add complexity to the hydrogel to enhance the cells' response, it is important to ascertain whether the integrated hydrogel can withstand perfusable flow in the μ FD. For further studies with a low passive perfusion, neither PEG-Col 200 nor PEG-Col 400 were stable enough to withstand a uniform flow in preliminary studies (unpublished observation). PEG Col 100 had the highest theoretical amount of Col I while showing stability as a microfluidic scaffold and the ability to span the entire length of the gel region. PEG-Col 100 was chosen for future experiments understanding the effect of flow on microfluidic PEG hydrogel integrity and Huh-7.5 cell function. Taken together, we highlight a materials approach to tune both mechanical and cell-adhesive properties of an embedded photopolymerizable microfluidic hydrogel for cell culture, thereby enabling nuanced control over cell encapsulation, viability and function.

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Chapter 6

Introducing Flow to the Microfluidic Platform

Perfusion of in vitro cell culture can improve maintenance of cell phenotype by creating a more physiological environment for the cells, enhancing the transport of nutrients and oxygen to cells, and removing waste metabolites from the cells. Though hepatocytes are not in direct contact with the liver sinusoids and blood flow in vivo, there is an interstitial flow that reaches the cells and affects their behavior. In this chapter, pressure-induced passive flow is established in the μ FD with integrated Huh-7.5 encapsulated PEG-Col I hydrogel to ensure the microfluidic hydrogel retains mechanical integrity and to observe differences in cell behavior under static and flow conditions. The findings of this study are presented and discussed in detail.

6.1 Introduction

Flow-based μ FDs for testing drug toxicity^[1-4] allow dynamic evaluation of metabolite production.^[5] Though a dynamic environment is not completely necessary for screening xenobiotics,^[6] the presence of flow also creates a more *in vivo*-like environment, simulating blood or interstitial flow and enhancing efficient nutrient and waste transfer. Thus, it is important to be able to perfuse a cell culture system, taking advantage of the microfluidic design and laminar flow properties.

In Chapter 5, we suggested that the bioactivity of PEG hydrogel can be easily controlled by varying the Col I concentrations and PEG-Col I can be properly integrated in the PDMS μ FD depending on the crosslinking efficiency. Other acrylated proteins can also be co-polymerized with the PEG-DA macromers in specific proportions and the PEG concentration should be increased to maintain a proper degree of polymerization. However, given the importance of a dynamic system for cell culture, the next parameter explored is the ability to perfuse the device uniformly, while maintaining hydrogel integrity.

Herein, we induced pressure-driven flow through the bioactive microfluidic PEG-Col I hydrogel with encapsulated Huh-7.5 cells at a volumetric flow rate comparable to literature.^[7, 8] Passive flow, though unable to maintain a constant volumetric flow rate as the height difference equilibrates, is useful for basic cell culture perfusion tests. In these studies, a pressure-based system was utilized rather than using a pump, due to the number of devices that required perfusion and incubator related culture limitations. Microfluidic scaffolds undergoing flow need to span the entire length of the gel region to allow uniform flow.^[9] Therefore microfluidic PEG-Col 100 hydrogel was selected for the dynamic experiment, as it maintained the best stability at the highest conjugated Col I concentration. Utilizing light microscopy, the gel stability was examined on Day 7. The duration of cell culture in the μ FD was 7 days, Huh-7.5 cell behavior was investigated with biological assays and the static and dynamic conditions were compared.

6.2 Experimental Design

PDMS μ FDs were prepared and pre-treated with DMPA as detailed in Section 3.2.1 and Section 3.2.2.1, respectively. Huh-7.5 cells were cultured and encapsulated in

PEG-Col 100 hydrogels as shown in Section 3.3 and 3.4.2, respectively. Passive flow was introduced into μ FDs with an initial interstitial flow of $\sim 5 \mu\text{L h}^{-1}$ as described in Section 3.6 and static cultures followed normal culture protocols. Qualitative light microscope images were taken as detailed in Section 3.5.1 to analyze hydrogel integrity on Day 7. Cell viability and function assays were performed on encapsulated cells as shown in Section 3.7.

6.3 Results and Discussion

The interstitial flow through a microfluidic hydrogel is mediated by a pressure difference and affected by the material's permeability.^[10] A preliminary study determined that the height difference needed to generate a volumetric flow rate of $\sim 5 \mu\text{L h}^{-1}$ through the cell-encapsulated microfluidic PEG-Col 100 hydrogel was 0.8 mm H_2O and the pressure difference needed is 7.84 Pa (work unpublished). If conditions for the hydrogel were to change (i.e. cell concentration, PEG-DA macromer MW or concentration, photopolymerization parameters, etc.), this number would have to be optimized again.

The PEG-Col 100 hydrogel endured the pressure difference and low interstitial flow, indicated by images of the μ FD gel region (**Figure 6.1A**). By Day 7, the hydrogel maintained a PDMS-PEG Col I interface, but slight separations between the boundary of the hydrogel and the PDMS micropillars were observed near the flow entry points into the gel region. Although the hydrogel is predominantly stable, this reveals that our approach to integrating the PEG-DA hydrogel into the μ FD, supported by the Cucchiara et al.,^[11-13] has flaws as it does not graft the PEG-DA to the PDMS. More recent studies have used surface silanization solutions, such as 3-(trichlorosilyl)propyl methacrylate to treat the PDMS surfaces and direct covalent bonding with PEG hydrogel.^[14] Cell density under the static and flow conditions were similar throughout the culture period, except for Day 5, and the encapsulation efficiency did not surpass 65% (**Figure 6.1B**). This was expected from Chapter 5 results, as the hydrogel and photopolymerization conditions did not change. The low interstitial flow was too small to damage the hydrogel integrity and cells were properly encapsulated.

Figure 6.1 Characterization of cell-laden PEG-Col 100 hydrogel polymerization and cell density in a static or dynamic microfluidic system. On day 7, (A) optical images of the hydrogel boundary were taken closer to the gel inlet and the middle of the gel region to observe the hydrogel boundary (shown by white arrows in the magnified image) that formed near the PDMS micropillars. Blue arrow indicates the direction of passive flow through the gel region. Scale bars are 200 μ m. (B) The cell density was measured on days 1, 3, 5, and 7 using Live/Dead assay, CLSM and IMARIS software ($n = 3$, mean $\pm$ SD; *: $P < 0.05$).

Viability and spatial distribution in the μ FD were qualitatively analyzed using Live/Dead staining and concomitant CLSM. There were no differences in the observed in the confocal images (**Figure 6.2A**), as viable cells in both conditions were uniformly found throughout the PEG-Col 100 gel. Image analysis with IMARIS revealed that Huh-7.5 cells in both perfused and static culture devices exhibited

viability above 91% (**Figure 6.2B**). An improved viability was observed for Huh-7.5 cells exposed to flow on days 5 and 7. This is consistent with studies comparing static and flow conditions with primary human hepatocytes encapsulated in collagen gel.^[15] Goral et al.^[8] used the same perfusion rate we used, and attributed the difference in primary human hepatocyte viability to the enhanced delivery of nutrients and waste removal in the flow culture.

Figure 6.2 Evaluation of Huh-7.5 viability in microfluidic PEG-Col 100 hydrogel under static and flow conditions. (A) Confocal z-stack images of Huh-7.5 cells labeled with Live/Dead assay were taken on days 1, 3, 5 and 7. Representative images show an xy-plane maximum projection of a Z-stack. Green indicates live cells and red indicates dead cells. The blue arrow shows the direction of flow in the perfused devices. Scale bars are 150 μm . The confocal images were analyzed by IMARIS software and (B) the cell viability was calculated as ratio of live cells to the total number of cells ($n = 3$, mean $\pm$ SD; *: $P < 0.05$).

The improved cell viability led to further investigation of Huh-7.5 cell behavior in the perfused PEG-Col I hydrogel by immunostaining cells for albumin and CYP3A4 production (**Figure 6.3A**) and quantifying the results (**Figure 6.3B**). There were no significant differences between the static and flow conditions with cells in each setup producing both proteins by day 7. Confocal images show Huh-7.5 cells that start to form larger aggregates under flow conditions. Unfortunately given the limitations of

the hydrogel and device, other function assays could not be used to investigate this more thoroughly. Previous studies have shown hepatocytes exposed to flow should exhibit restored polarized morphology^[16] and enhanced CYP activity^[7] among other improved cell phenotype.

Figure 6.3 Evaluation of liver specific functions of Huh-7.5 cells encapsulated in a static or flow PEG-Col 100 microfluidic hydrogel platform by immunochemistry. (A) Huh-7.5 cells encapsulated in the PEG-Col 100 hydrogel were either stained for albumin (top; green) or CYP3A4 (bottom; green) and counterstained by DAPI for the nucleus (blue). Representative images show an xy-plane maximum projection of a Z-stack. Scale bars are 25 μm . (B) The fluorescence intensity of target proteins per unit volume of cell was quantified using ImageJ software. There are no significant differences in the target protein fluorescence intensity for Huh-7.5 cells under static and flow conditions ($p > 0.05$).

6.4 Conclusion

This chapter presents the successful establishment of pressure-driven flow through the microfluidic PEG-Col I hydrogel and evaluates the effect of perfusion on Huh-7.5 cell behavior. A volumetric flow rate of $5 \mu\text{L h}^{-1}$, used in literature for hepatic μFDs was found by varying the height of media in both of the media channels and measuring the change in volume. The PEG-Col 100 hydrogel was stable but the flow condition revealed slight separations between the hydrogel and PDMS micropillars, highlighting the need for better PEG-PDMS graft approaches. Huh-7.5 cells in flow culture displayed heightened viability in comparison to static conditions, however there were no significant differences in hepatocyte production of albumin or CYP3A4. Taken together, we validate the use of bioactive photopolymerizable hydrogel as a microfluidic scaffold for cell culture under dynamic conditions, establishing a more physiological environment to use for future tissue engineering applications.

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Chapter 7

Conclusions and Recommendations for Future Work

Our thesis work serves to aid in the development of a microfluidic platform for liver tissue engineering applications. This chapter summarizes the outcomes of the research, ties the findings to the initial hypothesis presented in the first chapter, details the implications of the results, and suggests areas for future growth in the field of developing and researching integrated microfluidic hydrogels for liver tissue engineering.

7.1 Summary of Contributions and Implications

In this thesis, we proposed the integration and development of cell-encapsulated bioactive PEG hydrogel in a perfusable sealed PDMS μ FD as a platform for liver tissue engineering. The overall hypothesis was that PEG-Col I hydrogel integrated in a sealed PDMS μ FD with a specific PEG concentration, tailored bioactivity and the capability to withstand perfusable flow can be used as a hepatocyte culture platform comparable to widespread μ FDs using Col I gel. This hypothesis was investigated using carefully designed experiments and our results led to insight in developing photopolymerizable tunable materials as microfluidic hydrogels.

First, we verified that photopolymerizable PEG hydrogel could be integrated in the μ FD with different stiffness, by varying the PEG-DA macromer concentration. Proper polymerization of PEG-DA near the oxygen-permeable PDMS housing was enabled by immobilizing DMPA PI on the surface before polymerization. Encapsulating and culturing Huh-7.5 cells in the platform showed low encapsulation efficiency within the PEG hydrogels, but cells retained liver-specific function and maintained high viability. Higher PEG-DA concentrations allowed better integration and encapsulation efficiencies, important parameters for microfluidic platforms, thereby implying that future platforms can increase the macromer concentration to ensure hydrogel stability in the μ FD while staying in the range of physiological liver stiffness. However, a balance between hydrogel stability and cell behavior must be met, as previous studies have shown that increasing the concentration beyond a point compromises cell function and ability to detect protein secretions.^[1, 2]

Second, we demonstrated that tailoring PEG microfluidic hydrogel bioactivity is easily accomplished using copolymerization with acrylated Col I proteins, and compared PEG-Col I hydrogel's integration and potential as a hepatocyte culture platform to Col I gels. Conjugating higher Col I concentrations to the hydrogel negatively affected the integration into the μ FD by lowering the crosslinking efficiency. Huh-7.5 cells cultured in the PEG-Col I hydrogels with varied bioactivity displayed high viability and those in more bioactive PEG-Col I hydrogels displayed slightly enhanced albumin production, emphasizing the importance of bioactivity for liver tissue engineering microfluidic scaffolds. However, because of the lack of

stability in the μ FD, devices with 200 and 400 $\mu\text{g mL}^{-1}$ Col I were not suitable for further experiments with flow, thereby implying future platforms have to conjugate proteins in ways that do not affect hydrogel polymerization, stiffness and integration.^[3] No composition of PEG-Col I used allowed comparable Huh-7.5 function as that found in Col I gels and overall the integration and implementation of Col I gels was far easier.

Third, we confirmed that the PEG-Col I hydrogel allows perfusion at low pressure-driven flow rates in the μ FD. A pressure difference between the media channels, specific to the PEG-Col 100 composition and therefore permeability, was found to drive a flow rate of 5 $\mu\text{L h}^{-1}$ through the hydrogel. Huh-7.5 cells exposed to flow exhibited higher viability but no differences were detected in cell function. Due to limitations of assays that could be used to evaluate cell function, we could not make a definite conclusion on the effect of dynamic condition on cells encapsulated in PEG-Col I hydrogel. Perfusion of the device also revealed flaws in our approach to integrating the PEG hydrogel, thereby implying future platforms should graft the microfluidic hydrogel to the PDMS housing rather than simply enhancing polymerization near the interface.

In summary, PEG hydrogel has shown potential to be used as a tailorable integrated microfluidic hydrogel for liver tissue engineering as it is able to support hepatocyte culture, adjust stiffness in a controlled fashion, retain stability while rendered bioactive with ECM proteins, and endure low perfusion, all within the μ FD. However, contrary to our original hypothesis, we did not find a PEG-Col I composition that supported Huh-7.5 function as well as conventional collagen microfluidic platforms. As briefly aforementioned, suggested improvements to the platform include researching ways to conjugate proteins with minimal change to the polymer crosslinking and utilizing methods to graft the PEG hydrogel to the PDMS housing.

7.2 Recommendations for Future Work

Our project is just the first stepping stone to developing a more complicated platform that can be used for the fundamental studies of liver disease and for drug testing. Thus, there are many opportunities to build upon the results found in this thesis to develop a microfluidic hydrogel and platform that recapitulates more aspects of the

liver microenvironment. One recommendation is to conjugate different proteins (fibronectin, laminin, collagen IV) to the hydrogel scaffold in a similar method that we used for tethering Col I proteins. Once again, each protein concentration must be optimized for cell function and hydrogel stability, and the ratio of proteins can be controlled in our 3D microfluidic platform as an adaptation to what Bhatia et al. did with a 2D microarray platform.^[4] Another recommendation is to investigate different PEG hydrogel geometries in the μ FD, utilizing microfabrication technologies to create more porous hydrogels^[5] or sinusoid-like configurations. With improved methods to graft PEG hydrogel to the PDMS μ FD, the integration of such scaffolds should be possible. Though we used a hepatocarcinoma cell line as a model cell to evaluate the potential of our microfluidic hydrogel, primary hepatocytes need to be used in future experiments to ultimately facilitate accurate assessments of the hydrogel as a culture platform. Once this is accomplished, a future step would be to combine parenchymal and NPCs as a co-culture in the platform in optimal ratios. The last recommendation related to the microenvironment is to design the experimental platform such that an oxygen gradient is formed through the hydrogel. The perfect *in vitro* model will encompass all the main liver parameters, namely the configuration of the ECM, various co-cultures, flow dynamics, biochemical composition and the zonal microenvironments.

Our last recommendation is related to the assessment of hepatocyte function and viability in the μ FD. Throughout our research, we found immense difficulty in evaluating our microfluidic hydrogel as a cell culture platform, partly because of the restrictions of the μ FD and partly because of the PEG hydrogel mesh size. Thus, our recommendation is to develop ways to assess the microfluidic hydrogel's potential for cell culture more easily, either inbuilt in the platform or introduced through the media inlets. Given we were only able to use staining assays to evaluate the cell viability and function, better computational models must be designed to accurately quantify the fluorescence in 3D models, an area that is currently growing.

7.3 References

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Appendix I: Publications Outside of Thesis Work

Kim, M. H., Kumar, S. K., Shirahama, H., Seo, J., Lee, J.-H., Zhdanov, V. P. and Cho, N.-J. (2015), Biofunctionalized Hydrogel Microscaffolds Promote 3D Hepatic Sheet Morphology. **Macromolecular Bioscience**. doi: 10.1002/mabi.201500338

Kim, M. H., Kumar, S. K., Shirahama, H., Seo, J., Lee, J.-H. and Cho, N.-J. (2016) Phenotypic Regulation of Liver Cells in a Biofunctionalized Three-Dimensional Hydrogel Platform. **Integrative Biology**. doi: 10.1039/C5IB00269A

Shirahama, H., Kumar, S. K., Jeon W.Y., Kim, M. H., Lee, J.-H., Ng, S. S. and Cho, N.-J. Fabrication of Inverted Colloidal Crystal Poly(ethylene glycol) Scaffold: A Three-Dimensional Cell Culture Platform for Liver Tissue Engineering. **Journal of Visualized Experiments**. *Accepted*.

“If science is to progress, what we need is the ability to experiment, honesty in reporting results—the results must be reported without somebody saying what they would like the results to have been—and finally—an important thing—the intelligence to interpret the results.”
—Richard Feynman

“Our responsibility is to do what we can, learn what we can, improve the solutions, and pass them on.”
—Richard Feynman