Tampereen yliopisto

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IPSC-DERIVED HEPATOCYTES IN 3D LIVER-ON-A-CHIP MODELS

Thesis

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TIIVISTELMÄ

Jani Jokela: iPS-soluista erilaistetut hepatosyytit kolmiulotteisissa liver-on-a-chip-malleissa
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Tiettyjen biologisten ympäristöjen, mallien tai järjestelmien ymmärtämistä sekä jäljittelyä käytetään hyväksi monilla tekniikan aloilla. Moniin ihmiskunnan ongelmiin on jo olemassa luonnosta johdettu ratkaisu evoluution ja sopeutumisen kautta. Luontoa jäljittelemällä pyritään luomaan luonnolliset kasvuolosuhteet. Lääketieteessä on elintärkeää pystyä luomaan luonnollinen vaste ja välttämään haitallisia sivuvaikutuksia, kun suunnitellaan mitä tahansa lääketieteellistä työkalua, lääkettä tai hoitoa.

Kolmiulotteisen soluviljelyn kehityksen ansiosta, elinkelpoisten soluviljelyalustojen määrä on kasvanut. Toimivan kasvualustan ylläpitäminen edellyttää rajoittamatonta solujen kasvamista kaikkiin mahdollisiin suuntiin sekä tarvittavien ravintoaineiden, kaasupitoisuuksien ja stimulaatioiden vastaanottamista. Organ-on-a-chip luo *in vivo*-ympäristön elinkohtaisille soluille laboratorio-olosuhteissa käyttämällä mikrokanavia, bioyhteensopivia hydrogeelejä ja biomateriaaleja. Yhdistämällä useampia organ-on-a-chip-systeemejä samaan järjestelmään, muodostetaan multi-organs-on-a-chip- tai jopa body-on-a-chip-järjestelmiä. Nämä systeemit jäljittelevät koko ihmiskehoa ja vuorovaikutuksia useiden elinten kesken sen sijaan, että ne jäljittelisivät vain yhtä elintä.

Maksa on monimutkainen ja välttämätön elin, jolla on kyky uusiutua itsestään. Maksasolututkimus on huomattavan tärkeää, sillä maksan kyky lääkeaineenvaihduntaan tekee siitä yhden tärkeimmistä elimistä lääkkeiden valmistuksessa. Monia lääkkeitä on poistettu markkinoilta niiden aiheuttamien maksavaurioiden vuoksi. Maksasoluviljelmät tuottavat tietoa maksan toiminnasta ja rakenteesta. Suurimmat ongelmat kaksiulotteisessa hepatosyyttien viljelyssä ovat solujen toiminnallisuuden nopea heikkeneminen sekä toiminnallisten solujen saannin väheneminen. Indusoitujen pluripotenttien kantasolujen (iPS-solut) tuottaminen ja erilaistaminen maksasoluiksi voi viedä viikkoja, korostaen tarvetta ylläpitää solujen toiminnallisuutta. Muita iPS-solujen käytön etuja ovat niiden suurempi tarjonta sekä parempi tarkkuus lääkkeiden aiheuttamiin maksavaurioihin verrattuna primaarisiin ihmisen hepatosyytteihin.

Tämä opinnäytetyö tutkii perusteellisesti kolmea liver-on-a-chip-järjestelmää kolmessa eri osassa. Opinnäytetyö painottuu järjestelmien toiminnallisuuteen, materiaaleihin sekä suunnitteluun. Opinnäytetyössä käydään läpi mitkä ovat toimivan liver-on-a-chip-järjestelmän tärkeimmät piirteet. Opinnäytetyö ei anna lukijalle vaiheittaista ohjetta toimivan liver-on-a-chip-järjestelmän rakentamiseen. Nämä chipit valittiin niiden erilaisten rakenteiden, mallien ja toimintojen perusteella. Tutkimuksessa käytiin läpi sekä hepatosyyttien että 3D-biotulostuksen perusominaisuuksia ja uusimpia tutkimustuloksia hepatosyttien 3D-tulostukseen liittyen.

Avainsanat: organ-on-a-chip, liver-on-a-chip, 3D-tulostus, hydrogeeli

ABSTRACT

Jani Jokela: iPSC-Derived hepatocytes in 3D liver-on-a-chip models
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Understanding and imitating a certain biological environment, model or system is used in numerous engineering fields. Many problems facing humankind already have a naturally derived solution through evolution and adaptation. Mimicking nature is used to create identical natural atmospheres and functions. In medicine, it is vital to be able to generate a natural response and avoid any harmful side-effects, when designing any medical tool, drug or treatment.

Due to the developments in three-dimensional cell culture, the number of viable cell culturing platforms has increased. Maintaining a functional platform requires the possibility for cells to grow freely in all directions and receive necessary nutrients, gasses concentrations and stimulations. Organ-on-a-chip creates an *in vivo* environment in a laboratory for organ specific cells using pumps, microchannels, biocompatible hydrogels and biomaterials. Applying other organ-on-a-chips into the same system, creates a multi-organs-on-a-chip or even a body-on-a-chip system. These chips mimic the whole human body and interactions between other organs, instead of mimicking just a single organ.

Liver is a complex and vital organ with the ability to regenerate itself. The demand for liver cell research is substantial because of liver's capacity for drug metabolism. Many drugs have gotten withdrawn from the market due to drug related liver injuries. Liver culture systems provide specific information on liver function and structure. The main issue with 2D hepatocyte culturing is the rapid decline in cell functionality and decrease in yield of functional cells. iPSC generation and differentiation into hepatocytes can take weeks, emphasizing the need for improvement in upkeeping cell functionality. Other benefits for using iPSCs is their greater supply and better sensitivity towards drug induced liver injuries compared to primary human hepatocytes.

This thesis studies three liver-on-a-chip systems in depth and is divided into three parts, with the main emphasis on discussing the three chip systems, hydrogel materials and designs. This thesis examines the main aspects required for a functional liver-on-a-chip but doesn't give the reader a step-by-step tutorial on how to construct a functioning chip. These chips were selected due to their different structure, design and functions, while still being verifiably functioning. Basic characteristics and 3D bioprinting production methods as well as current research done on 3D bioprinting of hepatocytes were also examined.

Keywords: organ-on-a-chip, liver-on-a-chip, 3D bioprinting, hydrogel

PREFACE

I want to thank my instructor PhD Leena Viiri at BioMediTech's Heart group for giving me the opportunity to write my bachelor's thesis on the topic as well as sharing her knowledge on the subject. I received guidance on theoretical and practical parts throughout the whole project. She also provided feedback and review on my thesis. At last I would like to thank my family and friends for all of their support and assistance.

Tampere, 8.5.2020

Jani Jokela

CONTENTS

TERMS AND ABBREVIATIONS

Hepatocyte	Liver's parenchymal cell type
HepG2 cells	Human liver cancer cell line extensively used for *in vitro* research
Pluripotent stem cells	Cells that can propagate indefinitely and differentiate into many cell types excluding extra-embryonic cells.
Induced pluripotent stem cells (iPSCs)	Pluripotent stem cells generated from differentiated cells like skin fibroblasts or blood cells
Embryonic stem cells (ESCs)	Pluripotent stem cells derived from human embryo
In situ	Locally inside the body
In vivo	Inside the body
In vitro	Outside the body
Hydrogel	Absorbent polymer network storing large amounts of water
Extra cellular matrix (ECM)	Three-dimensional network supporting surrounding cells
Parenchymal cell (PC)	Cells that perform the function of the particular organ
Non-parenchymal cell (NPC)	Cells that support parenchymal cells
Polycaprolactone (PCL)	Synthetic biodegradable polyester
Polydimethylsiloxane (PDMS)	Transparent biostable silicone

1. INTRODUCTION

Organ-on-a-chip is a microphysical system aimed to assist culture living tissue *in vitro* to study its natural physiology or drug response. Tissue shows a more *in situ* like response when grown in a three-dimensional (3D) platform instead of a two-dimensional (2D). As multiple chips with different organ tissues are added into the same circulatory system, a multi-organs-on-a-chip or even body-on-a-chip systems is accomplished. With multiple organs, the system becomes more sophisticated in emulating the mechanisms of a real human body compared to single organs. [1] Present clinical trials for drugs must include a test on animal models, which can produce incorrect data and fail to predict outcomes in some cases. This can be avoided by testing drug response in an organ-on-a-chip system. [2]

Addition of 3D bioprinting introduces a more efficient way to produce cellular structure. Current 3D bioprinters create models layer-by-layer with multiple different methods. Compared to spin coating the chip and implanting the cells afterwards, 3D bioprinting uses already cell embedded bio-ink as biomaterial. As 3D bioprinting is an easier way to form microchannels, which mimic vascularization and adds a wider biomaterial selection, it is the more advanced option in chip preparation. [3]

Liver is an essential organ in drug metabolism, protein synthetization and biochemical production. This increases the interest to create a liver-on-a-chip, in order to better simulate, apprehend and replicate the liver mechanisms. In addition to hepatocytes, the main parenchymal liver cell type, nonparenchymal liver cells like liver sinusoidal endothelial cells or Kupffer cells have an effect on liver functionality, making it important to culture several organ specific cell types inside a liver-on-a-chip. [4]

This thesis reviews three modern liver-on-a-chip systems, their characteristics and differences. The thesis goes through the needed steps to put an organ-on-a-chip to use in research. This thesis also handles a certain type of hepatocyte cell culture method, liver physiology, cell culturing in hydrogels and organ-on-chips as well as 3D bioprinting hepatocytes and 3D bioprinting in general.

2. CELL CULTURE

Researching human cells and their behavior is crucial for developing any kind of new medical device or method. Gaining relevant information from a study requires the testing circumstances to be similar with the application site. Mimicking these *in situ* conditions can be achieved *in vitro*. [5]

Some major issues with *in vitro* conditions are achieving 3D growth, which can be overcome with growth supporting platforms like hydrogels, and controlling nutrients and exchanging produced waste. To be able to study the anatomy and physiology of the tissue, there must be an efficient number of living cells. Cell culture renders the possibility of cell proliferation to gain the needed amount of living tissue for the research. [5]

2.1 Differentiation of stem cells to hepatocytes

Hepatocyte differentiation is done from human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs). HiPSCs can be derived from many different somatic cell sources and they possess similar properties as hESCs like infinite proliferation *in vitro*, pluripotency and ability to differentiate into cells from all three germ layers. [6] hiPSCs are crucial in the development of disease modelling, as they supply an unlimited amount of tissue specific cells. A timeline for a hiPSC differentiation process is shown in Figure 1. [7]

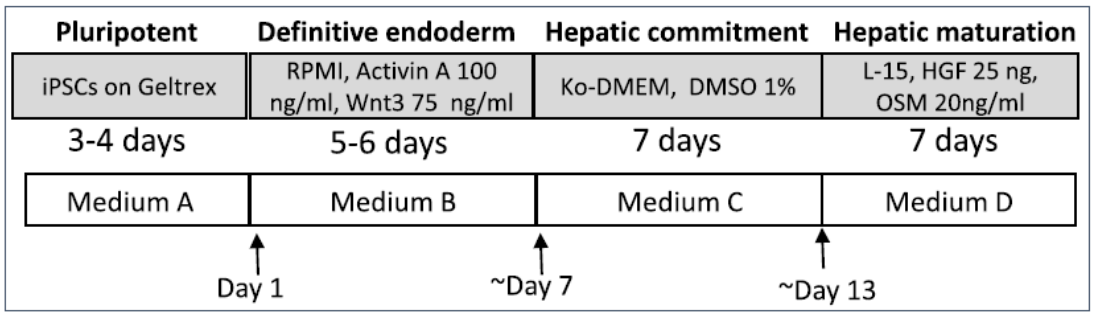

Figure 1. *Differentiation process from iPSCs to hepatocytes showcasing the three necessary stages over time. Used growth factors and mediums with their correct concentrations are presented in gray boxes. [7]*

The hepatic differentiation process is activated with growth factors and consist of three phases: definitive endoderm (DE) formation, hepatic commitment and hepatic maturation. During DE formation, iPSCs change their shape from round and dense cells to spikey DE cells. These DE cells mature at the hepatic commitment phase and grow in size. Gradually through hepatic maturation, DE cells transform into bigger hepatocyte

morphology mimicking hepatocyte-like cells (HLCs). [7] Figure 2 displays differentiated hepatic cells at day 17. The success of the differentiation can be evaluated e.g. by measuring the expression of liver-related genes and production of liver-related proteins like albumin. [8]

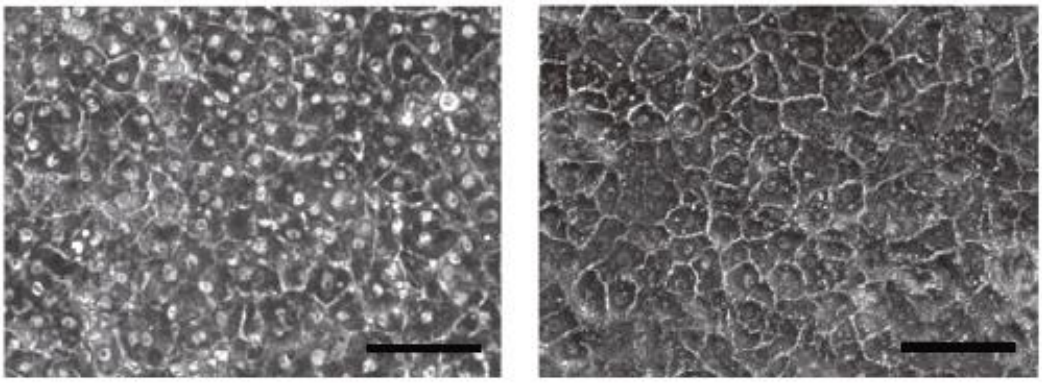

Figure 2. *Hepatocyte-like cells differentiated from hiPS and ESCs on day 17. Scale bar: 100 µm [8]*

In the study by Kajiwara et al. it was shown that the biggest impact on hepatocyte differentiation variations was the genetic background of the donor. Any differences found between two different donors with similar differentiation methods could be attributed to differences in genetics instead of differentiation methods. [8]

2.2 Cell culture in hydrogels and organ-on-a-chip structures

As mentioned in the beginning of the chapter, many experiments concerning cell adhesion and their physiology require 3D growth in order to show natural responses. Hydrogels enable 3D growth possibly mimicking the native elements of extracellular matrices (ECMs) as opposed to simple polystyrene or glass plates, where the tissue growth is two-dimensional (2D). Side-effects of 2D tissue growth can be flattening of shape, behavioral changes towards different pharmaceutical reagents, receiving less stimuli and signals and even tumorigenic growth. Other biomaterials that show similar properties with hydrogels are patterned glass substrates, elastomeric films, hydroxyapatite ceramics and fibrillar foams. [9]

Hydrogels are formed by connecting polymer chains together non-covalently by physical or covalently by chemical crosslinking. These chains reside in a precursor solution which turns into solid material after the linking procedure. Used crosslinking method depends on the polymer but the majority of peptide- or protein-based polymers connect by physical crosslinking processes. Chemical crosslinking is conducted when hydrogels need to be formed quickly, like in the case of 3D bioprinting. Chemical crosslinking can

damage cells and affect the biocompatibility of the material, which is why these methods need to be investigated carefully before being used. [9]

Organ-on-a-chip systems create a wide range of possibilities for *in vitro* experiments and tests to mimic the *in vivo* experience. This is done by creating currents that pass the tissue residing inside the hydrogel and exchange nutrients with waste products as well as perform detoxification of drugs. This flow of liquids and gasses make it a perfusion system. Organ-on-a-chip devices have some common characteristics: they are made from biocompatible and transparent materials to ease the observation of the cell culture. The devices can also be monitored with sensors and imaging systems. [4]

The most common material used in these systems is a soft and rubber-like silicone called Poly-dimethyl-siloxane (PDMS). PDMS is a synthetic and biostable polymer that due to its good hemocompatibility, high gas permeability and nontoxicity give it similar mechanical properties with soft liver tissue. Hydrophobic property of the PDMS worsens cell adhesion, which is critical for the system. New solutions have been researched and some progress has been done with laser and plasma treatment as well as material coating to increase adhesion. The high hydrophobicity of PDMS causes it to absorb small scale hydrophobic compounds in toxicity studies. This may alter the results in drug testing by decreasing the concentration of the measured drug. To avoid this problem, other polymers such as polystyrene (PS), polycarbonate (PC) and poly(methyl methacrylate) (PMMA) can be used in these occasions. [10] Some natural polymers, such as agarose and alginate, as well as mixtures of synthetic and natural polymers e.g. hyaluronan-PEG are also used as hydrogels. [11]

Current problems in academic use of liver-on-a-chip devices and their transition to industrial manufacturing is finding better fitting biomaterials, gaining the ability to use iPS-derived cells and minimizing the dead space caused by reservoirs and tubing. [12] Air bubble formation inside the small channels is an undesirable effect, as bubbles disrupt the fluid flow, influence local pressure points and can potentially damage the cells. Most commonly bubbles form through gas dissolution, especially with saturated liquids, which are heated from the room temperature to the desired research temperature of 37 ºC. [13]

Human-on-a-chip or multi-organs-on-a-chip systems are the next step in biomedicine and pharmacy to simulate an accurate *in vivo* environment of the whole human body. In researching a new drug with animal-based testing, the tissue response can alter significantly between species because of the unique metabolism and agents respond among different species. Creating an alternative to animal-based testing models with multi-organ-on-a-chips aims to achieve more reliable data in drug safety testing. [14]

3. 3D BIOPRINTING

3D bioprinting is an additive manufacturing method in the field of tissue engineering and cell biology, where the act of bioprinting can be divided into three different stages called pre-bioprinting, bioprinting and post-bioprinting. Pre-bioprinting includes sample imaging, 3D digital design and material selection. Most familiar tissue imaging mechanisms in the medical field are computed tomography (CT) and magnetic resonance imaging (MRI). Bioprinting step comprises of using the correct hardware, cell-laden bio-ink and the previously chosen biomaterial. The post-printing phase consists of confirming the biological and mechanical functionality of the device. [15] Current 3D bioprinting methods can be divided into nozzle- and optical-based bioprinting, depending on the structure formation mechanism. In nozzle-based bioprinting the ink is passed through a nozzle and laid directly on to the template whereas in optical-based bioprinting the ink interacts with light causing polymerization or deposition of ink from a donor plate. [16] These are further divided into four techniques called inkjet, extrusion, laser-assisted and stereolithography based bioprinting. Derakhshanfar et al. assembled summary tables going through the most important properties and parameters of the biomaterials and bioprinting techniques currently used. [17] With Nozzle-based bioprinters, the most important factors to be considered are bio-ink's ability to alter viscosity, the phase transformation in extrusion from liquid to solid and finding the right process parameter window for the selected bio-ink. It is important to have the bio-ink in liquid phase before extrusion to avoid clogging the nozzle. [18]

A new method of 3D printing is called volumetric additive manufacturing, where the sample isn't formed layer-by-layer but instead every point of the device is simultaneously fabricated within a 3D geometry. The manufacturing method is called Computed Axial Lithography (CAL). CAL's advantages, compared to a normal layer-based method, are redundancy of support structures, material construction around an object to enclose it, faster manufacturing, better surface properties, scalability to larger volumes and a possibility to widen the material selection. The main principle behind CAL is to solidify a photosensitive liquid via photopolymerization. The liquid is spun around an axel as the projected light is delivered in a set of 2D images. This creates a 3D volume with enough energy to solidify the material, thanks to exposure superposition. The polymerization process operates through free radicals, which crosslink locally if the oxygen levels are low enough, as oxygen inhibits the process. If the polymerization isn't controlled with oxygen levels, it's controlled with dyes. Dyes change the light penetration of a specific

wavelength, altering the polymerization process. The fabricated sample is rinsed with a solvent to remove any uncured material. The process of fabrication is presented in Figure 3 and it also shows how the processing of about 20 mm-tall statue takes 51 seconds. The resin used in the research is gelatin methacrylate, which is a relatively common bio-ink. [19]

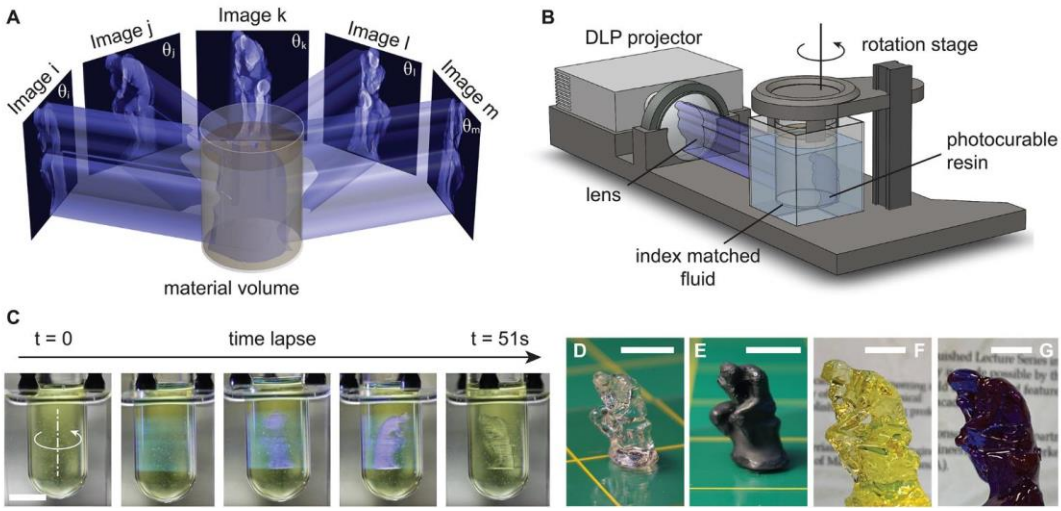

Figure 3. *CAL fabrication concept is portrayed in images A and B, where the projected light solidifies the liquid. Image C depicts a time lapse of the fabrication and images D to E showing it rinsed and colored. In images F and G, a larger (40 mm-tall) figure is manufactured and colored. Scale bar: 10 mm [19]*

According to other researches, gelatin methacrylate has good biocompatibility with cartilage cells *in vitro*. [20] This promotes using CAL in tissue engineering applications and possibly even on organ-on-chip hydrogel structures.

3.1 3D bioprinting hepatocytes

Bioprinted organs need to replicate native tissue's cell viability, functionality, physiological similarity and long-term stability [21]. Derakhshanfar et al. found some compatible bio-ink mixtures explicitly for liver tissue engineering. One bioink was a mixture of polycaprolactone (PCL), gelatin and three different types of cells. Another bio-ink consisted of hyaluronic acid and gelatin. Both of these bio-inks were printed with an extrusion printer and neither one of those printers were commercial printers. [17] Another research reports bioprinting mini-livers from hiPSCs and hESCs. After being differentiated for six days, both cell lines had matured to definitive endoderm (DE) cells before being printed inside an alginate hydrogel matrix. DE cell printing was done with a custom inkjet printer and alginate structures were dispensed as droplets and overprinted with a calcium solution to form a hydrogel matrix. Pressures applied from the nozzles in bioprinting altered

from 0.6 to 1.0 bar. These reports show how nozzle-based bioprinting is gentle enough to render cell proliferation, tissue growth and spheroid formation possible. [22]

Organ-on-a-chip devices are relatively small in size, as described more closely in the following chapter. These micro sized structures have difficulty to portrait the environment inside the organ in a real-life scale. This is because the current manufacturing methods limit the size of the fabricated structure. With 3D bioprinting, the manufacturing can be automatized, and the size of the structure is only restricted by the printing and medium chambers. [3] Fluid flow through human vascular system is an important feature to mimic in an organ-on-a-chip. Fluidic microvascular structures supply tissues with nutrients and oxygen as diffusion of molecules is not sufficient on its own. With advanced 3D bioprinting technologies, there is a possibility to create dual- or multiple-organ-on-a-chip systems in a single chip model. [23]

There are multiple research teams that have been able to print functional hepatocyte tissue. In 2015, Xuanyi Ma et al. created a hydrogel-based 3D bioprinted model that had embedded hiPSC-derived hepatic progenitor cells, human umbilical vein endothelial cells and adipose derived stem cells. The 200 µm high structure was printed with a custom-built system. [24] Hyungseok Lee and Dong-Woo Cho 3D printed a liver-on-a-chip system in 2016. They printed with PCL as the platform material using a pneumatic pressure printer with a 200 µm nozzle. The liver-on-a-chip had a fluidic channel on top of the cell encapsulated hydrogel ECM. The goal of the research was to achieve a 3D bioprinted liver-on-a-chip and to compare its functionality against a PDMS chip. They found that their chip absorbed less proteins from the medium, which is beneficial for cell growth. Figure 4 shows how the chip is planted with desired cells. [25]

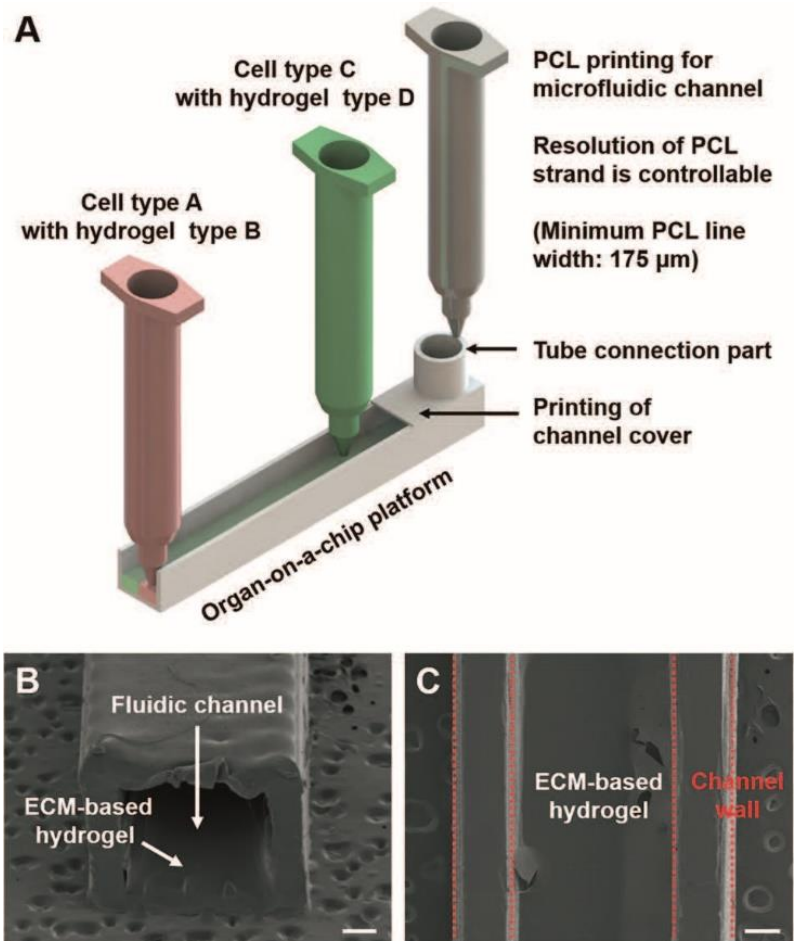

Figure 4. *A) Illustration of a 3D bioprinted organ-on-a-chip device. B) The device's vertical cross-section and C) horizontal cross-section. Scale bar: 350 μm [25]*

There is also a company named *Organovo* aiming to create several different chips by bioprinting to advance the research of drug delivery and tissue growth *in vitro*. Many university research groups use their ExVive™ Human Liver to achieve a ready liver-on-a-chip system. The process begins with designing the chip depending on the application. Their bio-ink utilizes mostly primary human cells. The bioprinter uses a layer-by-layer nozzle-based method that applies pneumatic pressure to dispense the bio-ink. [26]

4. CURRENT LIVER-ON-A-CHIP SOLUTIONS

Liver culture systems provide multiple types of reliable and viable cells including both parenchymal (PC) and non-parenchymal (NPC) cells. Adding NPCs to the system replicates *in vivo* functionality, signaling and metabolic responses to drugs more accurately. Culture systems can be divided into two types, static and perfusion systems. Static systems provide a low cost and easy to operate environment, mimicking liver structure and functions. Unfortunately, static systems can't recreate a fully realistic environment as these systems have no blood circulation declining the functionality of the hepatocytes. Static systems used for hepatocyte cultures are 2D micro-patterned and 3D spheroid culture systems.

Organ-on-a-chip devices are perfusion systems achieving crucial functions for liver-cell maintenance, such as drug detoxification as well as oxygen and chemical gradients in the tissue. Perfusion systems are divided to macroscale (5-300mL) and microscale (0.1-3mL) depending on their size. Microscale systems are used more frequently in research as they require less hepatocytes and allow higher cell-to-media volume ratios mimicking *in vivo* environment better. [4] In microfluid systems the perfusion is done by using capillary, gravity flow, micropump and micro pipetting techniques. Passive perfusion mechanisms are more difficult to recreate and modify, whereas active perfusion systems have difficulties on scaling to even smaller and precise systems. [10]

Blood flow in liver tissue is presented with a diamond-shaped functional acinus unit in Figure 5, where blood flow passes through sinusoids from portal vein and hepatic artery to central vein in three zones. The zones have different degrees of metabolism, with the first zone receiving the greatest supply of oxygen and nutrients whereas the third zone having the smallest supply.

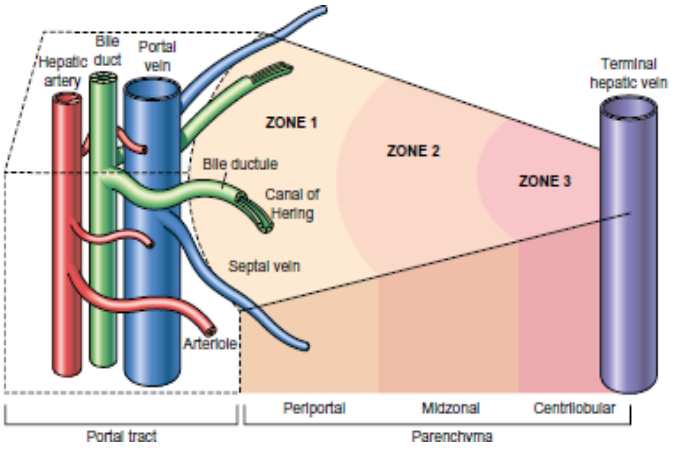

Figure 5. *Acinus unit showing the three zones of functionality in liver [27]*

This affects the size of the lobule, creates differences in anatomic results and changes the ratio of endoplasmic reticulum and cytoplasmic lipid. [27] Microfluidic flow is vital for creating a viable environment for the cells but it also creates average shear stresses of 0.1 - 0.5 Pa in hepatic sinusoids. Stresses exceeding 0.5 pascals cause changing cell morphology, hindering cell adhesion as well as dramatically decreasing hepatic cell functionality within just 1-3 days. [28] Achieving a functional liver-on-a-chip system also requires parallel scaling laws between the organ and the micro-sized chip, similar geometry as liver tissue (Figure 6), shear stress protection for hepatocytes, universal cell culture media and dynamic real-time monitoring and measuring of the system. [29]

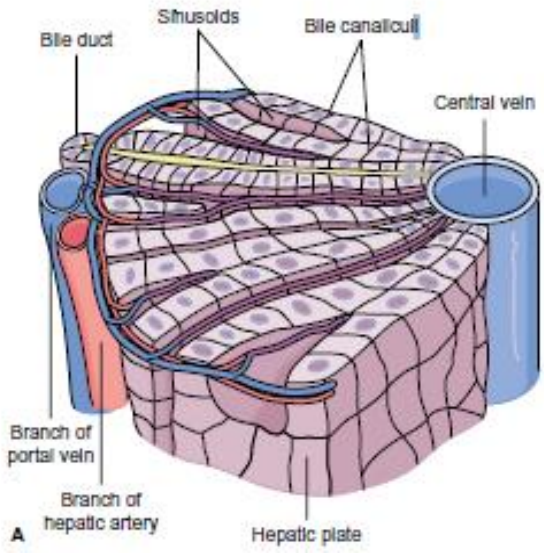

Figure 6. *Microstructure of the liver tissue [27]*

Modern time liver-on-a-chips can focus on different points of interest. System can focus on applying both parenchymal and non-parenchymal cells to showcase the importance of their interactions. Some chips are designed to mimic *in vivo* liver whereas other systems center on having large volumes, accessibility to the tissue after the experiment or improving adhesion of the cells after implantation. Chips examined in this thesis must be compatible with hiPSCs and be able to integrate into a multi-organ-on-a-chip system. [30]

4.1 Very large-scale liver-lobule (VLSLL)-on-a-chip device

Banaeiyan et al. designed and constructed a very large-scale liver-lobule (VLSLL)-on-a-chip device, which has the possibility to customize its size, usage of cell mass, surface area and the volume of the device. This system can also be integrated in multi-organ

human-on-a-chip system experiments and its main characteristics are shown in a summary of all the chips in Table 1 on page 20. [29]

The chip design imitates liver tissue by having a hexagonal structure in the chambers and an integrated multi-chamber layout in the bottom layer (Figure 7 b). The nutrient channels are on the top layer (Figure 7 a), and the structure of a bottom layer single chamber is depicted in Figure 7 (e) and (f). [29]

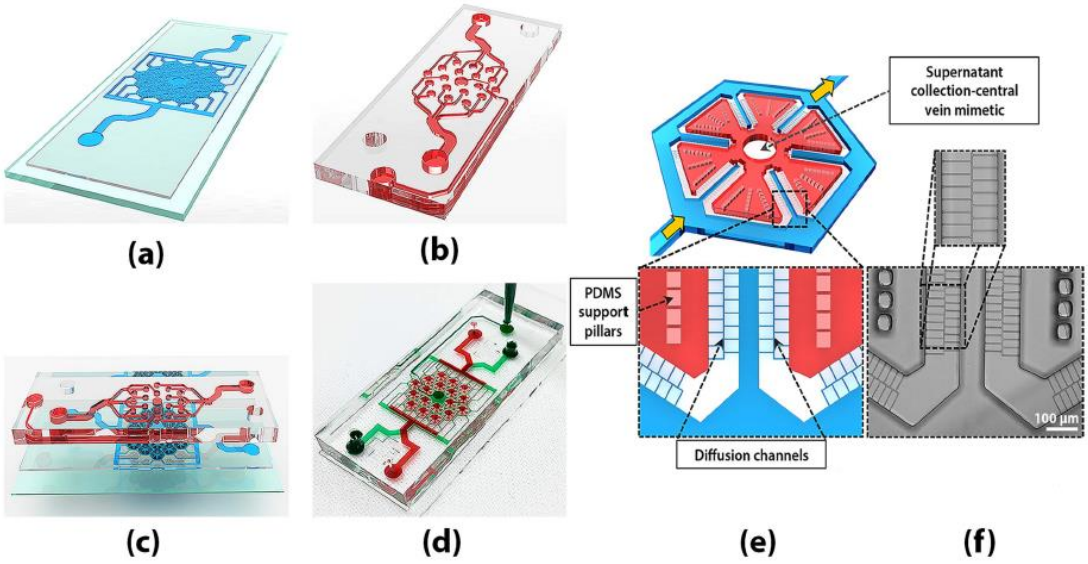

Figure 7. *VLSLL-on-a-chip system separated in bottom (a) and top layer (b). The whole system put together (c) and (d). A magnified photo of a single chamber also depicting the diffusion channels (e) and (f). [29]*

This liver-on-a-chip system has diffusion channels guarding the cells from excessive shear stress and direct contact with air bubbles, as depicted in Figure 8 (b). This system tries to mimic the space of Disse, which exchanges substances between hepatocytes and sinusoids. The diffusion channels also create a similar metabolic zonated acinus unit as real liver tissue. This was experimented with glucose diffusion from media to the chambers. Glucose concentration was 5.5 mol/m^3 in the media and just 360 seconds after fluid flow, the middle of the chamber had half of the glucose concentration compared to feed network. Glucose concentration increased for 1260 seconds (21 minutes) after which it reached a maximum concentration of 4.5 - 5 mol/m^3. Top layer works as a feeding and seeding network with nutrient rich liquid flowing through a single outlet to the center of the bottom layer at the velocity of 0.6 mm/s. When the liquid reaches a chambers feed network, its velocity decreases to 0.3 mm/s and as it passes through diffusion channels it drops to around 8 x 10^{-4} mm/s in the tissue chamber as shown in

Figure 8 (a). This highly controlled flow needs to maintain a volume accuracy of only µl's. [29]

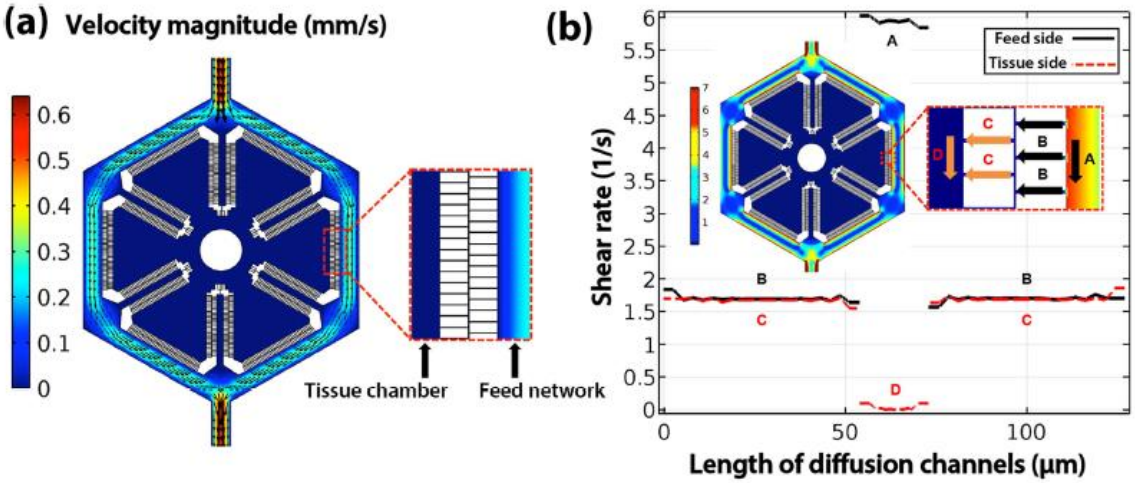

Figure 8. *The velocity of the liquid (a) and shear stress (b) within the structures decrease dramatically due to the diffusion channels [29]*

Banaeiyan et al. employed both pump- and gravity-driven Newtonian incompressible laminar flow in two different experiments. Constant media flow conditions, with the flow rate of 1 µl/min, were created with a syringe pump. Pumping was done with nutrient and oxygen full cell media in intervals of 15 minutes flow and 15 minutes static for a 24-hour period. [29] Nutrient diffusion occurs more frequently as the velocity of the liquid declines, like in the situation of static phase in the pump-driven flow. This was experimented on yeast cells uptake off Arsenite (As(III)) with similar hydro-dynamic geometry and same matrix material. It was found that the number of aggregates found per cell was about 1.7-4.7 times higher with 25 nl/min than 100 nl/min flow rates. The results could be explained with a closer contact between cells and the substance and a longer time that cells were exposed to Arsenite. [31] Gravity-driven experiment was done in a short-term culture of HepG2 cells by connecting a 2 ml media vessel to the inlet port and two smaller 1 ml vessels collecting the circulated media to the outlet ports. This mode of flow has a declining flow rate as the inlet and outlet vessels balance their media pillars at a declining speed. [29]

VLSLL -on-a-chip microfabrication is done on a 4-inch (10.16 cm) silicon wafers for its semiconductive properties. The wafer is cleaned after which a layer of photoresist is spin-coated on top, exposed to UV radiation and developed to form the feed network. After that the chip is covered with a 60 µm layer of another photoresist, exposed to UV, coated again and soft-baked in determined temperature to solidify the matter. This is done with every layer to form a 400 µm thick stencil layer. This stencil layer is developed

and hard baked in a higher temperature to enhance mechanical and chemical properties. [29]

PDMS is used to fill the microchannels of the bottom and top layers but with different PDMS:crosslink ratios. In the bottom layer a ratio of 5:1 was used whereas 15:1 was used for top layer to gain a stronger structure. PDMS was spin-coated directly on top of the silicon master on the bottom layer, creating a 400 µm-thick polymer layer. This procedure was redone for the top layer and both layers were baked, aligned together and bonded together. The whole process is depicted in Figure 9. [29]

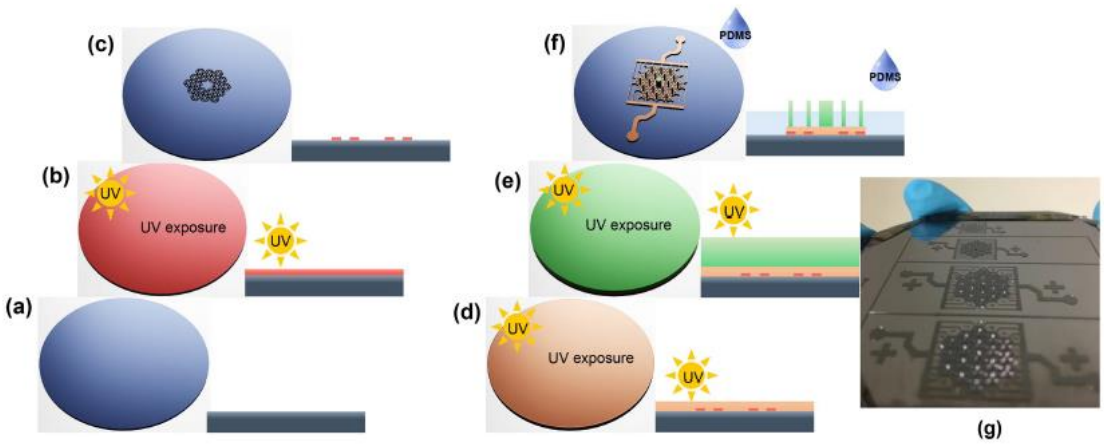

Figure 9. *Steps of producing a bottom layer for the VLSLL device from a silicon wafer. Multiple devices can be created on a single wafer with different chamber and channel sizes (g) [29]*

hiPSC-derived hepatocytes were plated in the chip and cell culture was observed daily to monitor the cell morphology of the hiPSC-derived hepatocytes. Cells started to create a 3D cluster within 48 hours, whereas in 2D culture the cells started to create a two-dimensional single layer cell formation. 2D cultured cells remained in the same morphology through the experiment unlike in the VLSLL device. The hepatocyte structure became more visible after 7 days and formation of bile-canaliculi was noticed on day 8 and bile network was finally confirmed on day 16. Hepatocyte functionality was measured by urea synthesis and albumin secretion. Albumin secretion increased significantly on day 5 as the cells had maturated and started to differentiate. Peak albumin levels were detected on day 12 at around 400 ng/h/10^6 cell reaching 2-5 times higher albumin secretion compared to 2D culture. Urea synthesis in the chip increased on day 2 and stayed between 1.3-1.8 µg/h/10^6 cells, which is considerably higher than urea synthesis in static 2D culture. Between pump- and gravity-driven flows, pumps create higher flow rates and higher nutrient flows creating higher urea synthesis levels within just 5 days after cell seeding. [29]

In Figure 10 HepG2 cells are dyed with a fluorescent to show the ratio of living cells to dead cells. Even though the test is done with HepG2 type cells, it shows features that could be universal with liver cells of different origin. Varying with dye coloring happens most likely because of physical traits and properties such as cell density. The authors concluded that lower cell density caused dye penetration to increase and vice versa with higher density. They also observed that areas around diffusion channels sent higher signals than average because longer dye incubation duration resulted in increased penetration of the dye inside the tissue. This study suggests that 3D culturing induces more healthy and viable cells than 2D culturing. [29]

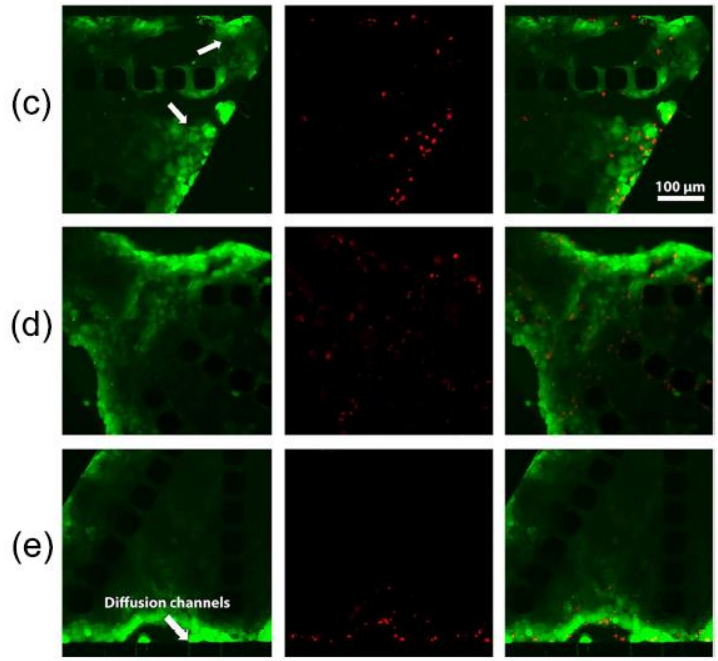

Figure 10. *HepG2 cells dyed to indicate how many live (green) and dead (red) cells there were on day 4. Figure (c) shows how dyes penetrated areas with smaller cell density better and the other way around with areas of higher cell density (d). This makes the edges of the structure and diffusion channel entrances (e) send higher signals. [29]*

4.2 Commercial chips for liver applications

Commercial alternatives for liver-on-a-chip are made to be universal and have the possibility to grow multiple tissue types. These chips can be used to replicate the whole body with multiple organs as body-on-a-chip systems. Another focus with commercial organ-on-a-chip systems is on parenchymal cells and the functionality of the organ. Commercial chips do not represent the most refined technology, they are versatile and adaptable to be used in several occasions. When commercial chips are not specified to a single cell type these chips do not mimic the liver-specific structure, perfusion method or liquid flow as precisely as the VLSLL-chip. These chips focus on tissue interface and how mechanical forces and stimulation contribute to cell growth. Often protective endothelial cells need to withstand shear stress generated by blood mimicking media, whereas functional cells receive nutrients from cell media. [32] An example for a commercial tissue interface-on-a-chip is Liver-Chip manufactured by Emulate Inc. [33] This Liver-Chip is configurated from their Chip-S1™ Stretchable Chip, which emulates the stretching forces caused by the flow of fluids within its elastic membrane. [34] The chip contains two channels that are separated by a porous membrane. The membrane is allowing a connection between the hepatocytes on the top channel and the liver specific endothelial cells on the bottom channel. Even though the channels are independent and separated by the elastic membrane, tissues create cell-cell interactions between each other. To further emulate liver's *in vivo* atmosphere, Kupffer cells can be implanted below and hepatic stellate cells above the endothelial cells on the bottom channel. This recreates the cellular layers that are arranged inside the liver. [33]

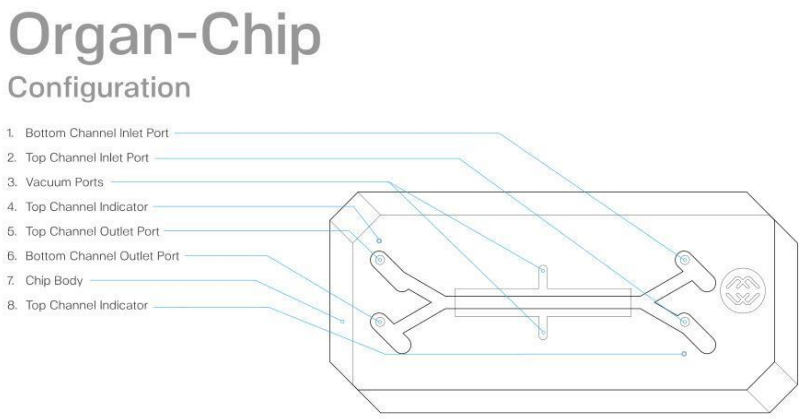

Figure 11. *Structure of the Liver-Chip [33]*

As seen in Figure 11, the chip body has inlet and outlet ports for top and bottom channels, vacuum ports to create a vacuum and inert environment and top channel indicators. In Figure 12 the top channel is colored blue while the bottom channel is purple. Emulate

Inc. provides a human emulation system, which includes accessories to achieve the desired properties inside the chip and to avoid contamination and deterioration of the tissue. These accessories contain the Pod, Zoë Culture Module and Orb Hub Module. The Pod supplies media and enables a compatible connection with microscopes and other equipment used for analyzing. [33] The pump system is handled with the Zoë Culture Module, which provides the dynamic media flow and mechanical forces to mimic the nutrient rich media and blood flow inside the liver. [35] Orb Hub Module is the central hub that provides the necessary CO_2 concentration and vacuum as well as the power to operate the whole system. Inside the Liver Bio-Kit, Emulate sends a patch of pre-qualified primary human hepatocytes and liver sinusoidal endothelial cells in a cryogenic storage. Even though the Liver Bio-Kit includes a set of primary human liver cells, the co-culture protocol doesn't explicitly forbid using other liver cell types in conducted experiments. [33] In a publication done to showcase the design and manufacture of an Emulate organ-on-chip microdevice, a step-by-step protocol presents the fabrication, chemical etching, sterilization, fluid channel connection, cell culture cultivation and data processing of the chip system. The protocol is specified to apply on lung-on-a-chip and gut-

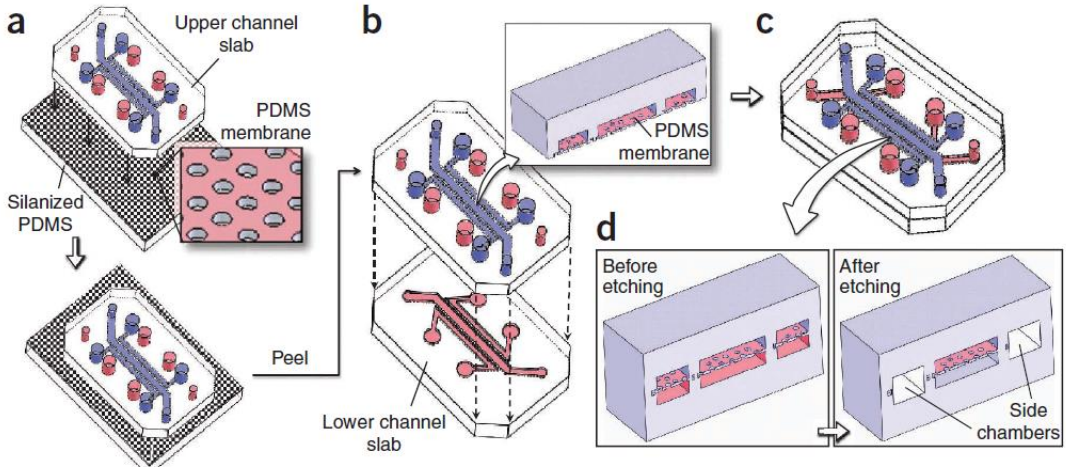

Figure 12. *Organ-on-a-chip slabs combined together a), b) forming a united chip c). In d) the PDMS membrane is etched off. [36]*

on-a-chip but

it is possible to extend the model to almost any organ, including liver-on-a-chip. [34] Emulate's Liver-Chip was used in a research aiming to compare rat, dog and human derived primary hepatocytes drug toxicity responses. Tests were done by measuring albumin secretion and its decline. Drug response was measured in a system with added liver NPCs, liver sinusoidal endothelial, hepatic stellate and Kupffer cells. Hepatocytes are attached on the top channel with nutrient rich media, whereas NPCs are placed on the bottom channel with blood replicating media flow. This sandwich structure, which

creates a greater biocomplexity and showcases more realistic results, is presented in Figure 13.

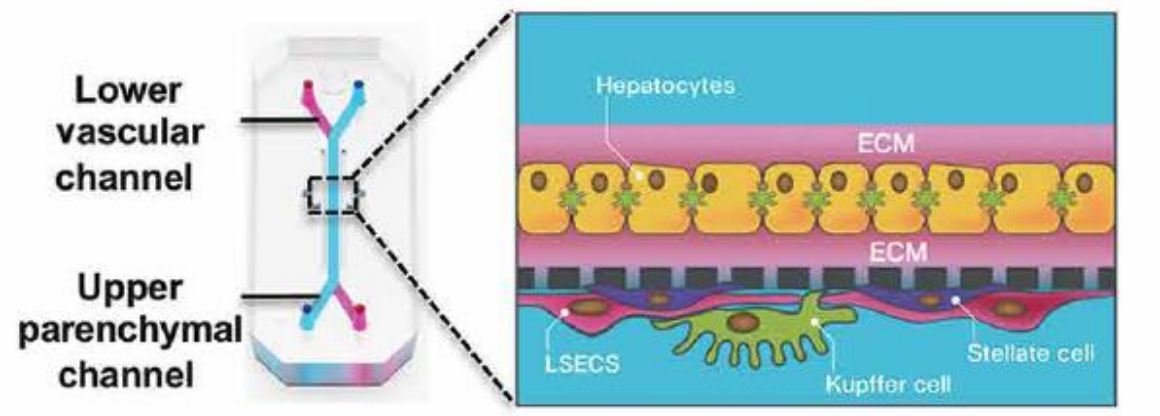

Figure 13. *Liver-Chip structure with hepatocytes on the top channel and NPCs on the bottom channel. [37]*

Moving away from nonclinical animal toxicity models is one of the biggest ambitions in drug testing and in liver tissue research. Animal tests fail to predict complications and in human bodies leading to drug-induced liver injuries (DILI) and causing the drugs to be removed from the market. This research suggests that a chip with primary hepatocytes and liver NPCs is viable to use for screening drug responses and hepatotoxicities instead of animal testing. One benefit of using a continuous flow, is that all cells were exposed to the drug and the drug concentration is easy to adjust with the flow rate. With this test it is possible to avoid DILI responses and increase human safety as well as lower the risk for drug removal from the market. It is important to factor the possible outcomes on how DILI form instead of only evaluating cell viability. [37]

4.3 Perfusable 3D Microvascular Bed

Paek et al. created a perfusable 3D microvascular bed that starts forming a 3D vascular network with a micropatterned hydrogel inside an elastomeric microdevice. A coculture of fibroblasts and primary human vascular endothelial cells induces vasculogenesis and works as a base for organ-specific tissue formation. With the chip's open-top feature, it renders the possibility to integrate liver cell spheroids on top of the vascular ECM hydrogel. In their experiment, Paek et al. injected lipopolysaccharide (LPS)-laden microbeads on top of the hydrogel, causing a sought inflammation reaction at the injection location. This proofs that the injected microbeads adhered on the hydrogel and began to self-assemble vascular perfusion between the nearby blood vessels. In their experiment, they also managed to develop stem cell-derived models of vascular adipose tissue and a blood-retinal barrier. [38]

The design of the model is simple and its made from three pieces of PDMS with standard soft lithography tecniques. The PDMS base is a mixture of PDMS polymer and curing agent with the ratio of 10:1 with the majority being polymerbase. A PDMS slab containing constructed microchannel features is used as a base for the cell culture chamber, which has a 1600 µm wide and 400 µm high chamber. Side microchannels have a 400 µm diameter and the whole piece is 3D-printed. The media reservoir PDMS ring was molded 12 mm wide, 12 mm long and 4 mm high. This open-top area inside the ring functinos as a co-culture surface area, as it is the location where the hepatocytes would be implanted. After all parts had been prepared, the models were degassed and hardened in an oven at 65 ºC. The hardened pieces were removed from their molds and two inlet as well as outlet ports were made. External reservoirs are required for each of the ports, making it possible to alter the media output. Both of the channels are independent but they can be used for a perfusion test to see if the ECM in the cell culture chamber enables throughout perfusion of the medium. This test is done by blocking one inlet port from one side and one outlet port from the other side. The test is proceeded by connecting a reservoir with fluorescent microsized beads and observing if the beads travel to the outlet port on the other side of the bed. The separate PDMS pieces are attached with thin spin-coated uncured PDMS layers. The assembled device is fully sealed together with PDMS adhesive layers baked in 65ºC. ECM in the cell chamber combines organ specific endothelial cells, fibroblasts and other cells necessary for a vasculatory system. [38] In the case of liver vascularity, possible implanted cell types could be liver sinusoidal endothelial cells (LSECs), primary liver fibroblasts and hepatic

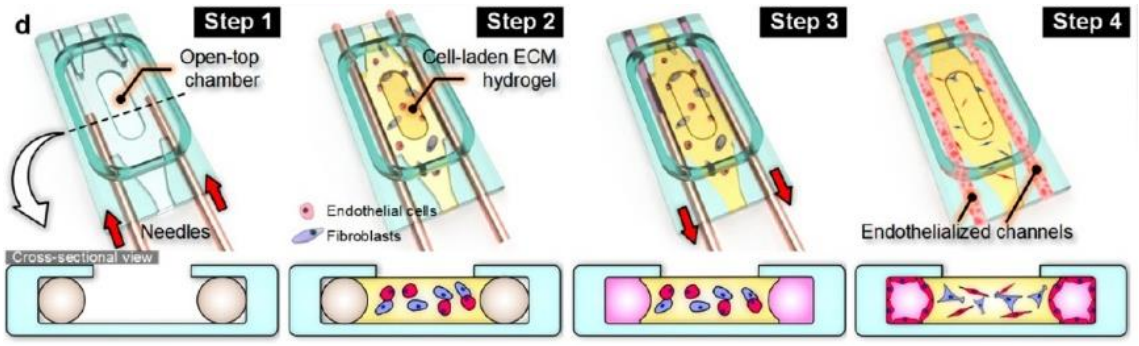

Figure 14. *Microvascular bed formation and a cross-section of the bed. (Step 1) Needles are inserted inside the microvascular channels to keep them open. (Step 2) ECM hydrogel solution is injected inside the chamber. (Step 3) Needles are pulled out, leaving an empty canal for (Step 4) vascular endothelial cells. These cells create an externally accessible channel for endothelial cells inside the ECM, which interconnect and form a microvascular structure inside the chamber. [38]*

differentiated hiPS. [8] The design and the vascular microculture formation is presented in Figure 14. [38]

Both of the sidechannels are filled with 400 µm diameter needles to keep them open for flow channels. After a 15 min gelation, the needles were removed and two solutions, one containing fibronectins and another containing human umbilical vein endothelial cell (HUVEC) suspension, were injeceted into both channels. The HUVECs were allowed to attach for 3 hours. After the attachment, a medium flow of 70 µl/h was generated with a Chemyx pump to form a solid monolayer of endothelial cells on the surface of the channels. The monolayer was induced to establish anastomosis between hydrogel's vasculature system and the endothelial layer. [38]

4.4 Liver-on-a-chip summary

There are many more articles on both commercial and non-commercial liver-on-a-chips. The three chips that were chosen for this thesis differentiated from each other in many aspects such as size, design, manufacture and flow system. Characteristics and properties of all three chips are presented in Table 1. All the chips were made from PDMS but as mentioned in the bioprinting chapter, an alternative material could be PCL or some other natural polymer. Comparison between 3D and 2D cell culturing was conducted only with the VLSLL-on-a-chip-device but the cause for a better cell culture and cell yield results are verifiably connected to the advances of 3D tissue growth and microvascular fluid flow. Any comparison between the three chips on cell growth couldn't be made as there was no compatible evidence. To examine the effects of chamber design, flow rate or chamber volumes influence in the chips, a single research should be made. Cross-examining results between two research results would produce too many variables between the performed experiments.

VLSLL-on-a-chip was the only chip that had any mentions on stresses that are applied on the cells or walls of the chip. The amount of shear stress applied to the cells were only about 1% of the shear stress that hepatic sinusoids can sustain. [28]

Table 1. *Characteristics and properties of VLSLL-on-a-chip, Emulate Inc. Liver-Chip and Perfusable 3D microvascular beds [29], [33], [36], [38]*

Characteristic	***VLSLL -on-a-chip***	***Emulate Inc. Liver-Chip***	**Perfusable 3D micro-vascular beds**
Diffusion channel size	*2 µm x 2 µm (4 µm^2)*	*Ø 7 µm (38.5 µm^2)*	*< 1µm (<0.8 µm^2)*
Feed channel width x height	*92 x 92 µm*	*1000 x 1000 µm and 1000 x 200 µm*	*400 x 400 µm*
Chamber count	*18 pcs*	*1*	*1*
Co-culture surface area	*3 cm^2 comprised from 18 lobules with 1.2-2.4 mm Ø*	*17.1 mm^2 area of membrane*	*3 mm^2 area, opening to the cell culture chamber*
Chamber volume	*0.2 µl*	*-*	*20 µl*
Maximum medium volume	*3.6 µl + 7.3 µl (10.9 µl)*	*50 µl + 20 µl (70 µl)*	*< 576 µl (12mm*12mm*4mm)*
Inlet and outlet reservoirs volume	*2 ml and 2 x 1 ml*	*2 x 4 ml and 2 x 4 ml*	*External reservoirs*
Flow rate at the feed	*1.00 µl*min^{-1}*	*0.50 µl*min^{-1} (15-1000 µl*hr^{-1})*	*1.17 µl*min^{-1} (Used to create a monolayer on the channels)*
Maximum velocity of the liquid	*0.6 mm s^{-1}*	*-*	*Pump dependent*
Maximum shear stress in feed channel	*0.004 Pa*	*-*	*-*
Maximum shear stress alongside diffusion channel	*0.001 Pa*	*-*	*-*
Maximum shear stress in the tissue chamber	*5*10^{-5} Pa*	*-*	*-*
Flow system	*Internal syringe pump-driven and gravity-driven flow*	*The Zoë™ Culture Module applies a controlled and automated flow*	*Eternal Chemyx syringe pump withdraws media from channel outlets*
Building materials	*Silicon wafer, spin-coated photoresists, PDMS, ECM hydrogel cell culture*	*Silicon wafer, spin-coated photoresists and PDMS, ECM hydrogel cell culture*	*Soft lithography PDMS slab, 3D-printed PDMS cell culture chamber, molded PDMS reservoir, ECM hydrogel cell culture, HUVEC coated microchannels*
Monitoring methods	*Transmitted light and fluorescent microscopies to monitor viability, morphology and functionality, ELISA analysis*	*The Pod is compatible with microscopes and other equipment of analysis*	*Fluorescent microbeads and Fluorescence Microscope, Immunostaining analysis, Scanning electron microscopy, Atomic force microscopy*
Possibility to culture liver-cell or other cell types	*Several cell types are possible, but liver endothelial has the best chance to prosper*	*Hepatocytes on top channel and liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells on bottom channel*	*Several types of endothelial cells, adipose cells, epithelial cells and fibroblasts*

5. CONCLUSIONS

As of writing this thesis, new articles were published covering organ-on-a-chips and 3D bioprinting. This shows that new research on the subject is being done widely all around the world. The benefits of creating a more user-friendly, profitable and industry customizable chip are extensive just on the medical field. Liver diseases are relatively common as modern living habits and aging population seem to increase the number of liver related diseases. Harmful alcohol consumption, obesity and adverse drug reactions induce more liver failures than there are liver donors. Drug-induced liver injuries occur with almost every medication, making liver drug response testing pivotal for any drug company. Drug-induced liver injuries have to be taken very carefully into account when multiple drugs are being used simultaneously. The lack of healthy primary hepatocyte is also slowing down liver disease and drug response research. hiPSC have the potential to generate a limitless supply of hepatocyte-like cells, which recapitulate human liver function more accurately than animal cells. By adding other liver cells types, like Kupffer, hepatic stellate and liver sinusoidal endothelial cells, the drug reaction and liver functions can be better replicated. In combination with human organ environment mimicking 3D models and non-parenchymal liver cells, hiPSC-derived hepatocytes are needed for realistic *in vitro* liver research.

By moving away from 2D liver models and more towards 3D models, better hepatocyte differentiation, maturation and cell yield results cause an increase in the quality of liver research *in vitro*. In 3D cell culturing, cells receive more stimulation, are able to maintain their natural shape and produce more *in vivo* like responses towards different pharmaceutical reagents, compared to 2D models. The possibility to treat liver diseases with autologous cells taken from the patient with a biopsy, might be possible in the future. But for now, finding the most crucial parameters with 3D liver models like liver-on-a-chips and the best practices to create a realistic liver environment in a laboratory are the next steps towards this future.

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