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BIOMATERIALS FOR 3D *IN VITRO* LIVER MODELING

ABSTRACT

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The liver is complex and the largest internal organ in the human body. It has multiple important functions such as filtration, metabolism, removal, storage, and synthesis of several compounds. Therefore, it is important to be able to model it *in vitro*. Liver models can be used in several experiments, such as in drug and toxic studies. This thesis introduces *in vitro* liver models, what they consist of, and how the liver can be modeled using a chip.

Various *in vitro* liver models have been developed and many cell lines have been used in these liver models, including primary human hepatocytes (PHH), different liver cell lines, such as HepG2 and HepaRG, and induced pluripotent stem cell-derived hepatocyte-like cells (iPSC-HLCs). All these cell lines have their own advantages and disadvantages. In addition, several biomaterials have been used as scaffolds, including collagen, fibrin, Matrigel, GrowDex and poly (ethylene glycol) (PEG). The function of biomaterial is to mimic the extracellular matrix (ECM) and therefore support and give attachment sites for the cells. To be able to achieve this, the biomaterial must be cytocompatible and have suitable mechanical properties. Moreover, many different three-dimensional (3D) cultivation systems have been established, including a microfluidic device, such as a chip. The chip allows perfused culturing conditions with the control of media flow containing oxygen and other nutrients. Observation and imaging during cultivation are also possible because of the transparent material of the chip.

As a combination of all these, a liver model system containing iPSC-HLCs in a biomaterial on a chip is created. This model allows the personalized study and better maturation of the iPSC-HLCs. In addition, this cell culture model mimics the liver cells native environment by enabling the co-culture of different cell types and perfused cell culture conditions. Therefore, this cultivation system could be the key to *in* vitro liver modeling by enabling longer cell cultivation time and more mature liver functions.

Keywords: Liver model, Microfluidic device, Liver cell, iPSC-HLC, 3D, biomaterial

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TIIVISTELMÄ

Kati Rinnekari: Biomateriaalit maksan 3D *in vitro* mallinnuksessa
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Maksa on monimutkainen ja lisäksi ihmiskehon suurin sisäelin. Maksa on vastuussa monista kehon toiminnoista, esimerkiksi, maksa huolehtii veren suodattamisesta, metaboliasta, vieraiden aineiden poistamisesta, varastoinnista ja useiden aineiden syntetisoimisesta. Maksa on tärkeä osa monia kehon perustoimintoja ja siksi sen mallintaminen on tärkeää. Maksamalleja voidaan käyttää monissa tutkimuksissa, kuten lääke- ja myrkyllisten aineiden tutkimuksissa. Lisäksi maksamallien avulla voidaan saada tärkeää tietoa maksan toiminnasta. Tämän kandidaatin työn tavoitteena oli tutkia maksamalleja, ja sitä mistä ne koostuvat ja miten maksaa voidaan mallintaa siruilla.

Monia in vitro maksamalleja on kehitetty ja monia eri solulinjoja on käytetty niissä. Näitä solulinjoja ovat muun muassa, primaariset ihmisen hepatosyytit (PHH, engl. primary human hepatocyte), erilaiset maksasolulinjat, kuten HepG2 ja HepaRG, sekä indusoiduista pluripotenteista kantasoluista erilaistetut hepatosyyttien kaltaiset solut (IPSC-HLC, engl. induced pluripotent stem cell-derived hepatocyte-like cell). Näillä kaikilla solulinjoilla on omat vahvuutensa ja heikkoutensa. Lisäksi monia biomateriaaleja on käytetty skaffoldeina, muun muassa, kollageenia, fibriiniä, Matrigeeliä, GrowDex:iä ja polyeteeniglykolia (PEG, engl. poly (ethylene glycol)). Biomateriaalin tehtävä on matkia soluväliainetta (ECM, engl. extracellular matrix) ja siten tukea ja antaa kiinnittymispintoja soluille. Jotta tämä olisi mahdollista, materiaalien pitää olla yhteensopivia solujen kanssa ja niiden mekaanisten ominaisuuksien on oltava sopivat. Monia kolmiulotteisia (3D, engl. three dimensional) soluviljelysysteemejä on myös luotu, joista yksi esimerkki on mikrofluidistiset laitteet, kuten sirut (engl. chip). Sirut mahdollistavat perfuusio-viljelyolosuhteet, joita voidaan kontrolloida hapen ja muiden ravintoaineiden saannin lisäksi. Lisäksi solujen havainnointi ja kuvaaminen ovat mahdollisia soluviljelyn aikana, koska siru on valmistettu läpinäkyvästä materiaalista.

Yhdistämällä nämä asiat: solut, biomateriaalit ja sirut, saadaan luotua maksamalli, joka sisältää iPSC-HLC:t biomateriaalissa sirulla. Tämä malli mahdollistaa yksilöidyn tutkimuksen ja iPSC-HLC:n paremman kypsymisen. Lisäksi tämä soluviljelymalli matkii maksasolujen luonnollista elinympäristöä mahdollistamalla eri solutyyppien yhteisviljelyn ja perfuusio-soluviljelyolosuhteet. Näin ollen tämä viljelymalli voi olla avainasemassa maksan mallintamisessa mahdollistamalla pidemmän soluviljelyajan ja kypsemmät maksan funktiot.

Avainsanat: Maksa malli, Mikrofluidistinen laite, maksasolu, iPSC-HLC, 3D, biomateriaali

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
3T3-J2	Murine fibroblasts cell line
ACMS	Additive combined mold systems
ADSC	Adipose-derived stem cells
AFP	Alpha-fetoprotein
cRGD	Cyclic arginyl glycyl aspartic acid
CYP	Cytochrome P450 enzyme family
CYP2E1	Cytochrome P450 2E1
DE	Definitive endoderm
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarm
ESC	Embryonic stem cell
G	Modulus
GST	Glutathione S-transferase
HA	Hyaluronic acid
HA-BCN	Cyclooctyne-modified hyaluronan
HA-PEG	Hyaluronic acid-polyethylene glycol
HepaRG	Hepatoma cell line
HepG2	Hepatoma cell line
HLC	Hepatocyte-like cell
HMEC-1	Human microvascular endothelial cell line
in vitro	Outside the body, for example, in a laboratory
in vivo	Inside the body, in its normal environment
iPSC	Induced pluripotent stem cell
iPSC-HLC	Induced pluripotent stem cell-derived hepatocyte-like cell
linRGD	Linear arginyl glycyl aspartic acid
MPCC	Micropatterned coculture
MRP1	Multidrug resistance-associated protein 1
NAT	N-acetyltransferase
PBS	Phosphate buffered saline
PCLS	Precision-cut liver slices
PDMS	Poly-dimethyl-siloxane
PEG	Poly (ethylene glycol)
PEGDA	Polyethylene glycol diacrylate
PHH	Primary human hepatocytes
PLGA	Poly (dl-lactic-*co*-glycolic acid)
RGD	Arginyl glycyl aspartic acid
SOD	Superoxide dismutase
THP-1	Human monocytic cell line
UV	Ultraviolet

1. INTRODUCTION

The liver is the biggest internal organ in the body, and it is also a complex one. The liver has many significant functions. It, for example, plays a role in glucose balance, the control of metabolism, the control of cholesterol synthesis and transport, urea metabolism, and secreting plasma proteins. In addition, the liver stores many substances, including blood, glycogen, iron, and vitamins. [1, 2, 3] Furthermore, the liver consists of many parenchymal and non-parenchymal cells that work in collaboration. The most important parenchymal cell type is hepatocytes which take care of most of the liver functions. [1, 2, 3] The liver structure, cell types and functions are discussed more in Chapter 2.

An *in vitro* liver model mimics liver functions, and it can be used to study how different drugs are metabolized and how toxic substances are removed. All in all, the liver is very important in basic body functions, so it is also important to be able to model it outside the body in the laboratory, in other words, *in vitro*. Several different liver models have been developed but none of these can fully mimic all liver functions. Different hepatocytes have been used to create *in vitro* liver models, such as primary human hepatocytes (PHH), different liver cell lines, such as HepG2 and HepaRG, and also induced pluripotent stem cell-derived hepatocyte-like cells (iPSC-HLC) [4]. These cell types are discussed more in Chapter 3.

There are also many cultivation systems for liver cells in two-dimensions (2D) and three-dimensions (3D). The 3D cultivation system has shown to be a better option for liver modeling because it allows more cell-cell interactions and contacts. There are many different ways to create a 3D cultivation system, including 3D bioprinting, scaffolds, spheroids, hydrogels, microfluidic culture systems or chips and organs-on-a-chips [5, 6]. The cultivation systems that contain biomaterial that mimics the extracellular matrix (ECM) allows cell-ECM interactions. These interactions are also important for maintaining liver functions. The biomaterials used in 3D culture systems often mimic the ECM, are cytocompatible and create a suitable environment for cells. This thesis concentrates more on a few biomaterials: collagen, fibrin, Matrigel, GrowDex, and poly (ethylene glycol) (PEG), which are discussed in Chapter 4.

The microfluidic device or the chip is a 3D cell culture device, which allows the control of culture condition on a microscale [7]. The chip is made of transparent material, for example, poly-dimethyl-siloxane (PDMS), that allows good light permeability. This allows the observation and imaging of cells in real-time [8]. The chips have become a more and more useful culture method because of their good properties.

Human iPSC-HLCs have shown good properties for *in vitro* liver modeling. Culturing these cells in the microfluidic chip could be the answer for liver modeling and therefore to drug and toxicity studies. Chapter 5 concentrates on the cultivation of iPSC-HLCs with biomaterial on the chip. The aim is to find, what kind of biomaterials have been used or tried and which methods have shown to be a good option for the liver models.

Many studies have been done by using iPSC-HLCs on a chip and some of them have used biomaterial as a coating on the inner walls of the chip's channel. However, only a few studies have been done by using a 3D biomaterial scaffold with iPSC-HLCs on a microfluidic chip. These studies have shown that the perfused cultivation systems support the maturation of iPSC-HLCs. These cultivation systems and materials are discussed more in Chapter 5.

This thesis is not only going to concentrate on the hepatocytes which are responsible for many liver functions, but also how the liver can be modeled *in vitro*. First, there is an overview of liver functions, structure, and cell types. Second, the different cell types that have been used when modeling liver functions are examined. This is followed by a discussion of biomaterials that have been used in liver models. The review concludes with an examination of iPSC-HLCs with biomaterial on a chip.

2. THE LIVER

The liver has many significant functions, for example, it has both endocrine and exocrine properties. Endocrine properties appear in the ability of the liver to secrete many hormones, such as angiotensinogen and insulin-like growth factors. Exocrine properties, on the other hand, appear in their ability to form bile. [1] The liver itself stores many important substances, such as glycogen, blood, iron, and vitamins. It also removes toxic substances from the blood circulation by turning them into a water-soluble form. In addition, the liver plays a role in glucose balance, the control of metabolism, the control of cholesterol synthesis and transport, urea metabolism, and secreting plasma proteins, such as albumin. [1, 2] As can be seen from this wide range of functions, the liver is an important regulator of many routine bodily processes.

2.1 Liver structure

The liver can be divided into five different tissue systems according to its histology and structure. These five groups are a vascular system, hepatocytes (aka hepatic or liver lobule), hepatic sinusoidal cells, biliary system, and stroma. [3] The liver's basic structural and functional unit is the liver lobule, which consists of hepatocyte plates and sinusoidal capillaries leading to the central efferent vein [1]. The liver lobule's structure is shown in Figure 1.
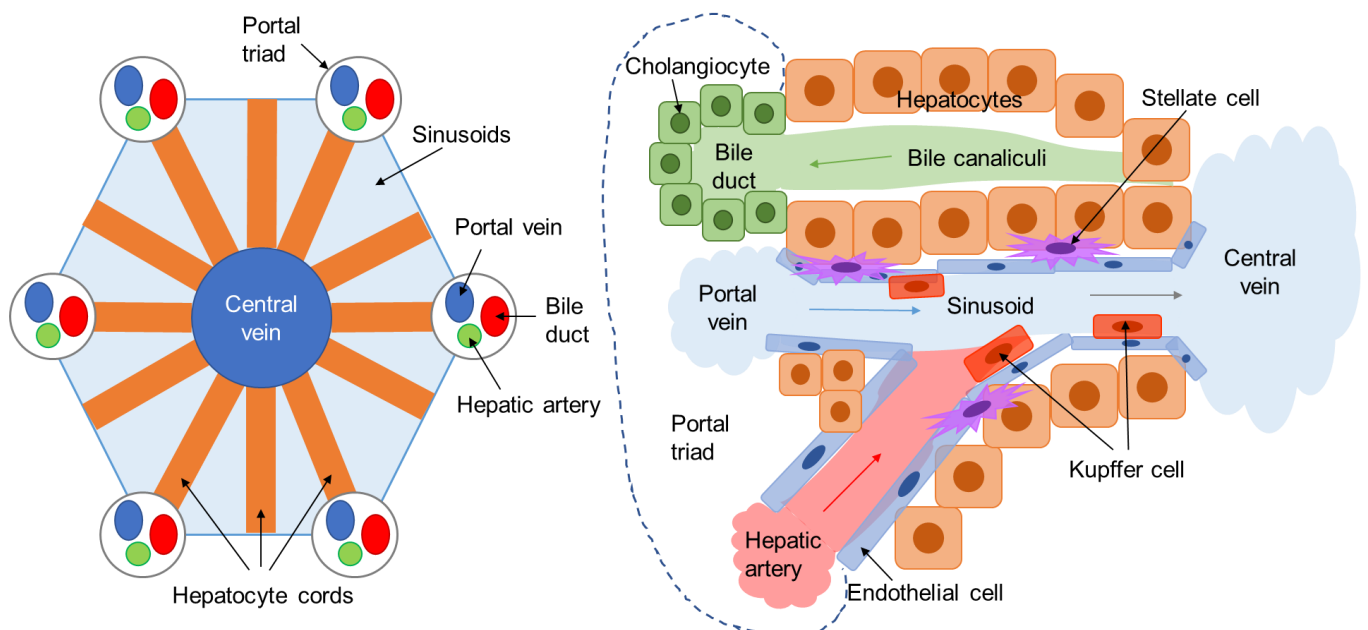

Figure 1. The liver lobule's structure. Adapted from [2]

The left side of the figure (Fig. 1) shows the liver lobule, which includes the central vein, and the portal triad, which itself consists of the hepatic artery, portal vein, and biliary duct. Hepatocytes are separated by sinusoids. The right side of the figure shows the organized structure of the parenchymal and non-parenchymal cells in the liver lobule. The blood is transported from the portal triad to the central vein by sinusoids, while the secreted bile moves towards the bile ducts that is, it moves in the opposite direction to the blood.

The liver is a highly vascularized organ, and it has a dual blood supply. The arterial blood comes from the hepatic artery and venous blood from the hepatic portal vein. The arterial blood comes straight from the heart bringing oxygen-rich blood to the liver. The venous blood, on the other hand, comes from the gastrointestinal tract bringing oxygen-poor blood containing several substances, including hormones, nutrients, and toxins. [9]

2.2   Liver cell types

Hepatocytes cover up to 78 % of the liver volume, and they are also the most important parenchymal cell type of the liver. They work in conjunction with many other cell types, including cholangiocytes or biliary epithelial cells, endothelial cells, sinusoidal endothelial cells, Kupffer cells, pit cells, and hepatic stellate cells, which all are non-parenchymal. [1, 3] Cholangiocytes can also, according to some sources, be perceived as parenchymal cells [4]. Hepatocytes take care of most of the liver functions [1, 3]. The main liver cell types and their features and functions are shown in Table 1.

The hepatocytes seem to be the most metabolically active cells in the entire body. This is because they have many mitochondria, peroxisomes, lysosomes, Golgi complexes, and aggregates of smooth endoplasmic reticulum in their cytoplasm. Therefore, the hepatocytes need for oxygen is very high compared to other cell types. [9]

Hepatocytes are highly differentiated cells and therefore they do not usually divide in an adult individual under normal conditions. However, the liver has a unique ability to regenerate after partial hepatectomy. This process depends on the proliferation of the hepatocytes and non-parenchymal liver cells. [11, p. vii]

Table 1. Main liver cell types and their features and functions [1, 10].

CELL	TYPE	DIAME-TER (µm)	PROPORTION	FEATURES AND FUNCTIONS
PARENCHYMAL				
HEPATOCYTES	Epithelial	20-30	60-65 %	Protein and bile secretion, cholesterol, glucose/glycogen and urea metabolism, and detoxification Mostly double nuclei
NON-PARENCHYMAL				
BILIARY EPITHELIAL CELLS (CHOLANGIOCYTES)	Epithelial	~10	Little	Form bile ducts, control bile flow rate and bile's pH, secrete water and bicarbonate, acute phase response and blood clotting Distinct basement membrane, contain unique proteoglycans, adhesion glycoproteins
HEPATIC STELLATE CELLS	Fibro-blastic	10.7-11.5	8 %	Storing, especially vitamin A and retinoid, maintaining of ECM, control microvascular tone, secretion of cytokines and contributes toward a regenerative response to injury
KUPFFER CELLS	Macro-phages	10-13	~15 %	Scavengers of foreign material, secretion of cytokines and proteases and other mediators Irregularly shaped and mobile cells
LIVER SINUSOID ENDOTHELIAL CELLS	Epithelial	6.5-11	16 %	Form sinusoidal plexus, highly specialized, allow transfer of molecules and proteins between serum and hepatocytes, scavenger of macromolecular waste, secretion of cytokines, antigen presentation and blood clotting SE-1, CD31, fenestrations and no basement membrane

The next chapter concentrates more on the cell types that have been used in *in vitro* liver modeling. These cell types mimic the functions of hepatocytes, which take care of most of the liver functions.

3. LIVER CELLS FOR *IN VITRO* MODELING

Hepatocytes are the major parenchymal cell types in the liver. They take care of most of the liver functions. Hepatocytes work in conjunction with many non-parenchymal liver cells, including cholangiocytes, endothelial cells, sinusoidal endothelial cells, Kupffer cells, pit cells and hepatic stellate cells. [1,3]

Liver *in vitro* cell culture models have become important in pharmacological and toxicological research. This is because they can model liver functions and metabolic pathways, which are important in such research. The main requirements of the liver model are that the model can express the primary liver functions. These include endogenous substrates and the metabolism of exogenous compounds, such as cell products and drugs, regulation of nutrients like amino acids, carbohydrates and fatty acids, and protein synthesis, including albumin and transferrin. They also include the activation of immune reactions and inflammation during liver injury. [4]

There are many cell sources for *in vitro* liver cell culture models, including primary human hepatocytes (PHH), liver cell lines like HepG2, and pluripotent stem cells like induced pluripotent stem cell-derived hepatocyte-like cells (iPSC-HLCs). These different cell sources and their processing paths are shown in Figure 2. [4] In addition, the advantages and disadvantages of these cell types are summarized in Table 2.
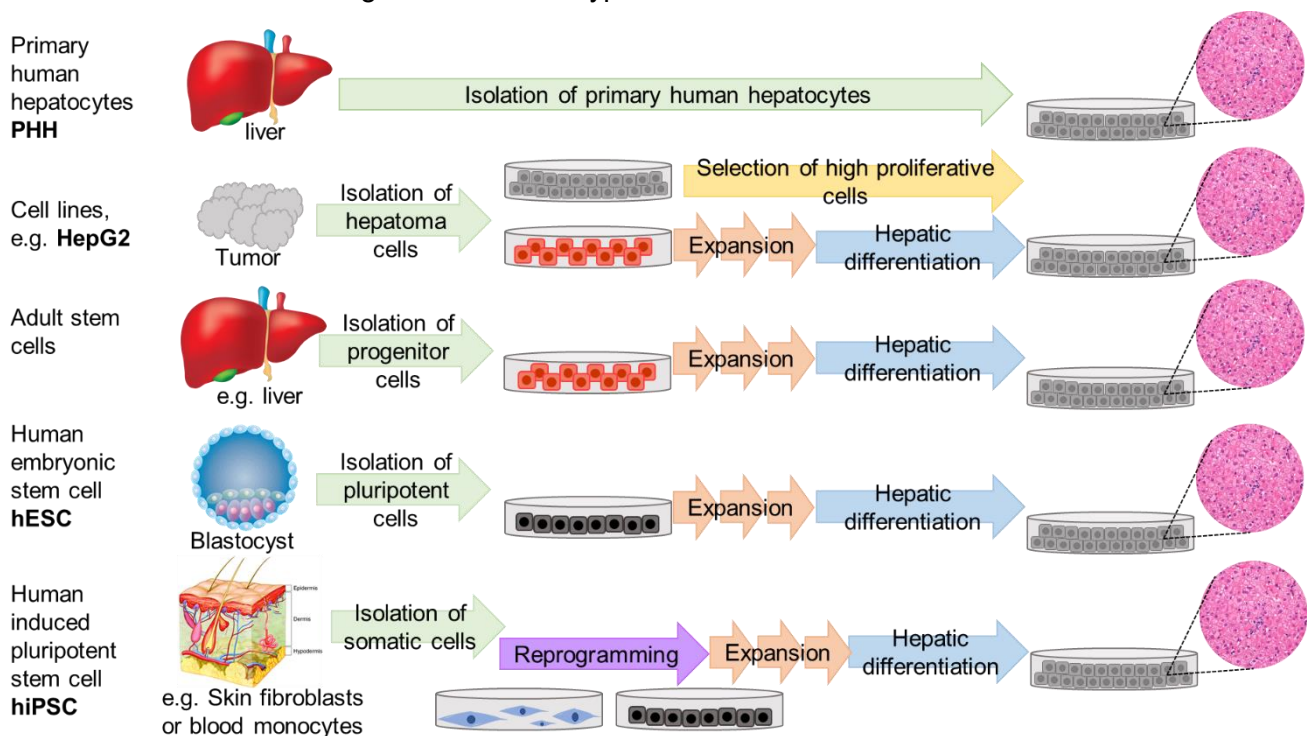

Figure 2. Different cell types used for liver cell culture modeling. Adapted from [4, 12-15]

Table 2. Summary of the different cell type used for liver *in vitro* modeling. [4]

CELL TYPE	ADVANTAGES	DISADVANTAGES	APPLICATIONS

PRIMARY HUMAN HEPATOCYTE	- High functionality
	- Mimic human metabolism
	- Possible to detect genetic polymorphism between donors
		- Limited availability
		- Variability between donors
		- Rapid de-differentiation *in vitro*
		- No proliferation *in vitro*
			- Toxic studies
			- Investigation of
			  o metabolism
			  o drug effects
			  o diseases

HEPATOMA CELL LINES	- High proliferation activity - Good availability - Stable metabolic performance	- Alterations in liver-specific functions	- Tumor biology studies - Anticancer drug testing - metabolic/toxicologic analysis
→HepG2 CELL LINE	- Well-characterized - A lot of information available	- Different metabolic properties from primary human hepatocytes - Isolated from one donor →no variation between individuals	
→HepaRG CELL LINE	- Maintain many liver-specific functions and many cytochrome P450s - Stable karyotype - Able to differentiate into either hepatocyte or biliary cells	- Isolated from one donor →no variation between individuals	
PLURIPOTENT STEM CELLS	- Unlimited growth	- Lack of standardized methods for cell differentiation and characterization	- Drug metabolism & toxicity studies - Regenerative medicine research
→iPSC-HLC	- Good availability - Less ethical issues - Many options for differentiation	- Epigenetic memory may inhibit hepatic differentiation - Not fully matured	- Genetic polymorphism analysis - Disease models - Drug testing for personalized medicine

The next chapters concentrate more on PHH, two liver cell lines, HepG2 and HepaRG, and iPSC-HLCs, which are the most commonly used cell types in *in vitro* liver modeling.

3.1 Primary human hepatocytes

The PHHs (primary human hepatocytes) are cells that have been isolated from the native liver. They mimic the whole liver functionality *in vitro*, because of their origin, and that is why they give very predictable results in different research. Also due to their origin, every donor has their own genetic information, which can cause a variation in the results. On the other hand, the variation in genetic information gives more information about different polymorphism. [4] In addition, the PHHs express the drug transporter enzymes, such as cytochrome P450 (CYP) family's enzymes, more than HepG2s. This is because the PHHs mRNA level of CYPs is significantly higher than HepG2. [16]

The PHHs are usually isolated from livers that are not used for transplantation or resected liver parts. Because of their origin and their ability to mimic liver functions, PHHs are more commonly used in studying, for example, liver diseases or drug and hepatoxicity. The PHHs are therefore a good option for liver modeling, but there are problems with tissue availability. There is simply too little quality tissue for the research. In addition, when the quality tissue part is found, there also must be cooperation between the surgeon and scientist to make the tissue available for research. Cryopreservation is one solution for cell availability by storing cells. This is because cooling the sample allows the cells to be used later without being wasted. [4]

2D cell culture systems are widely used with the PHHs because it is easily achieved. There is usually collagen-coated plastic where the PHHs are plated directly. Collagen allows cells to attach and form a confluent monolayer. [4] The plates can also be coated with Matrigel.

The PHHs mimic *in vivo* hepatic conditions best when cultured short-term. This is because they lose their hepatocellular phenotype, in terms of both morphology and functionality, when cultured for too long periods. The de-differentiation of hepatocytes starts during isolation. This is because the cell-cell and cell-matrix interactions are disturbed, which leads to the loss of hepatocytes polarization. [4]

The de-differentiation of the PHH *in vitro* can be prevented by forming a similar microenvironment as in the liver. The 3D culture system enables the cells to adhere three-dimensionally which resembles hepatocytes *in vivo* environment. That also allows hepatocytes to interact with other cells and ECM and these interactions can prevent the de-differentiation of PHH. The 3D cell culture environment can, for example, consists of

two ECM layers and one cell layer in the middle. These sandwich structures are usually repeated, so there are many cell layers and ECM or biomaterial layers in one culture. [4] In these sandwich hepatocyte cultivations, the ECM substitute can, for example, be collagen I or Matrigel, which will be discussed later in this thesis.

One study in which the PHHs are cultured in collagen I sandwich shows that the results from 3D *in vitro* cultivation system have better comparability to the situation of *in vivo* than the 2D cultivation system. They also demonstrated that the increase in the metabolic activity of cytochrome P450 2E1 (CYP2E1) and the activity of multidrug resistance-associated protein 1 (MRP1), and the depletion of the antioxidative protein superoxide dismutase (SOD) maintained only in 3D cultures. [17]

3.2 Liver cell lines

There are two main ways to generate human hepatic cell lines: refining tumor cells or using genetic engineering for primary human liver cells. Liver cell lines are widely used in *in vitro* culture modeling because they have a high proliferation rate, stable metabolism, and their cells are readily available. The high proliferation potential can cause a loss of differentiated liver functions, which can lead to a lack of functional performance that in turn affects the research. [4]

Two main hepatic cell lines that have been used in research are the HepG2 and the HepaRG cell lines. The HepG2 cell line is the human hepatoma cell line. Whereas the HepaRG cells are generated from human hepatoma cells and they are bipotent progenitor cell line. [4, 11, 18]

3.2.1 HepG2

The HepG2 are human hepatoma cells, but they are nontumorigenic cells. The HepG2 have many good properties, including a high proliferation rate, their epithelial-like morphology, and their ability to mimic many differentiated hepatic functions. The main disadvantage of the HepG2 cells is that they have the limited function of liver metabolism compared to the PHH. The main advantages in addition to the above are that they are easy to handle, they have a nearly unlimited lifespan and stable phenotype without variation between donors because there is only one donor. The HepG2 cell line is the most widely used hepatoma cell line and it originates from a 15-years-old Caucasian male's liver biopsy with differentiated hepatocellular carcinoma. This cell line is especially used in drug metabolism and hepatotoxicity studies even though their expression of metabolic enzymes is limited. [18, p. 77]

The HepG2 cells can show many differentiated liver functions, including synthesizing and secreting plasma proteins, bile acid and glycogen, metabolism of cholesterol, triglyceride, and lipoprotein, and also insulin signaling. The main downside is the limited expression of drug-metabolizing enzymes and transporters, such as the cytochrome P450 (CYP) enzyme family. The HepG2 cells expression levels of phase II-enzymes, including glutathione S-transferase (GST), N-acetyltransferase (NAT), sulfotransferase and uridine diphosphate glucuronosyltransferase, are lower in HepG2 cells than in PHHs but GTS's and NAT's mRNA levels are quite the same as the PHH's. These differences are less marked than CYP's. In addition, transporters like bile salt export pump, organic anion transporting polypeptide C and sodium-taurocholate cotransporting polypeptide, are poorly expressed in the HepG2 cells or there is no expression. [18, p. 78] These transporters are characteristic of hepatic cells, so lack of their expression indicates poor modeling properties for these liver functions.

The HepG2 cells can be cultivated in a collagen I scaffold. According to a study by Pruksakorn et al., the HepG2 cells metabolism changes during cultivation in 3D. The environment seems to become hypoxic which can be caused by the space between collagen scaffold's fibers that have been filled with the HepG2 colonies and their ECM. All in all, the 3D cell culturing system seems to be more suitable for the HepG2 cells than a 2D culturing system. [19] In addition to collagen sandwich culture, also Matrigel matrix has been used in the cultivation of HepG2 cells [20].

3.2.2 HepaRG

The HepaRG cell line is generated from human hepatoma cells and they are bipotent progenitor cell line, which means that the cells from this cell line can differentiate either to hepatocytes or biliary cells [4], [11, p. 261]. The HepaRG cell line originates from an Edmonson grade I well-differentiated liver tumor of a female patient who suffered in addition to cancer also chronic hepatitis C infection and macronodular cirrhosis. The HepaRG cell line has similar characteristics and properties to adult hepatocytes. [11, p. 261, 270]

The HepaRG cells are not tumorigenic, and they do not have changes in the expression of common cancer genes, like p53, pRb and β-catenin. The HepaRG cells have a pseudodiploid karyotype, which means that the cells have diploid karyotype, but there are chromosomal translocations. The translocation is located in chromosome 12 position 22, where the short arm has a loss and remodeled has been added to chromosome 7. It has also been shown, that the HepaRG cells are not able to grow in the serum-deprived medium and they can form moderate size colonies in soft agar. [11, p.264]

Due to their ability to express cytochrome P450 enzymes, like CYP1A1 and CYP4A11, nuclear receptors, including PXR and CAR, the major hepatic transporters, for example, bile salt export pump, phase II-enzymes, such as UGT1A1 and GSTA1, and antioxidant enzymes, the HepaRG cells have shown to be a very valuable option for *in vitro* liver modeling. Especially, they are a good option for drug metabolism and toxicity studies. [11, p. 267]

There are a few 3D cultivation systems in which the HepaRG cells are cultivated with biomaterial scaffolds. The HepaRG cells have, for example, been mixed with Arginyl–Glycine–Aspartic (RGD)/galactose conjugated material, a functional polymer containing a polysaccharide gellan gum, a nonadherent polyethylene glycol diacrylate (PEGDA) microscaffold, and an adherent gelatin microscaffold. These scaffold-based cultivation systems have shown improved precision and accuracy for hepatotoxic drug screening. They have also shown improved control of the model mimicking physiological assay conditions. [21]

3.3   Induced pluripotent stem cell-derived hepatocyte-like cells

Pluripotent stem cells have two main sources: human embryonic stem cells (ESC) and human-induced pluripotent stem cells (iPSC). Human iPSCs can be generated by reprogramming adult cell types, such as blood cells or skin fibroblasts. These cells have many advantages, for example, they have limitless availability, they show almost limitless proliferation capacity, and there are less ethical issues than with ESCs. Human iPSCs are similar to ESCs because they both are able to differentiate into all three primary germ layer derivatives, which are ectoderm, mesoderm, and endoderm. In addition, they both can show a nearly limitless capacity for proliferating. [4]

Human iPSCs can be derived from various individuals with different genomic information, which opens opportunities to research specific diseases and therapeutic approaches for every individual. In addition, several protocols have been developed to differentiate hepatocytes from iPSC. These differentiating methods try to mimic the development of the liver from the embryonic. This is achieved by adding the different growth factors that are important for each development stage, and as a result, hepatocyte-like-cells (HLCs) are obtained. [4]

HLCs are a quite new approach for modeling liver functions, and when compared to PHH, they have shown immature phenotype with reduced hepatic functionality [4]. However, human iPSC-HLCs can, for example, secrete albumin, triacylglycerol, and urea. They can also express some characteristic hepatic drug transporters, CYPs

enzymes and apolipoprotein genes, including APO1, APOC and APOE. In addition, these cells are able to produce, secrete and uptake cholesterol and respond to statin treatment by decreasing the cholesterol secretion *in vitro*. Furthermore, HLCs have an advantage over some other cell types like hepatoma cells HepG2 and HepaRG since they are sensitive to hepatitis C virus infection and they also support viral replication. PHHs have the same advantage. In addition, liver modeling with iPSC-HLCs have lots of possible uses, including modeling hepatic metabolism inborn errors, studying, and understanding liver cell differentiation at the molecular level and disease mechanisms and perhaps the main use is discovering new and safe drugs. [22]

Differentiating iPSC-HLCs still entails some difficulties as none of the existing differentiating methods produces fully mature hepatocytes because there is often a persisting expression of fetal liver markers like alpha-fetoprotein (AFP). Besides, there is a lot of variability in differentiation capacity between iPSC lines, which are generated from different donors or in different laboratories. [22]

3D cultivation methods with cell-cell and cell-ECM interactions have shown to improve the maturation of iPSC-HLCs. Collagen and Matrigel have been used as a scaffold when culturing iPSC-HLCs. [23, 24] iPSC-HLCs inside the collagen matrix have also shown to induce polarization, formation of bile canaliculi and increased cultivation time over 75 days. In addition, maintaining cell-cell junctions have significantly changed the phenotype of iPSC-HLCs closer to PHH's one. [23]

4. BIOMATERIALS USED FOR CULTURING LIVER CELLS IN 3D ON A CHIP

A 2D cell culture has been widely used for the past few decades. It is easier than 3D cell culture, but it cannot model all the functions of the cell. [25, p. 1] The main problem with culturing liver cells in 2D is that the cells lose some of their functions over time. In addition, the 3D culture conditions model better the cells' normal environment in the body than the 2D conditions. The 3D culturing methods that have been developed include cell spheroid culture, microcarrier cultures, polarized epithelial cell culture, organotypic explant culture, and tissue-engineered models. [26] In addition, the tissue-engineered models include 3D bioprinting, scaffolds, hydrogels, micropatterned coculture (MPCC), microfluidic culture systems or chips and organs-on-a-chips [5, 6]. This thesis is concentrating on 3D cell cultures, especially on chips.

Microfluidic culture devices or microfluidic chips or just chips are 3D cell culture devices, which allow the control of culture condition on a microscale [7]. The controlled parameters include fluid flow and particles in the chip [7, 8]. The chip is made of transparent material, for example, poly-dimethyl-siloxane (PDMS), that allows good light permeability. This allows the observation and imaging of cells in real-time. [8]

Usually, there are microscale chambers for the biomaterial-cell complex and the culture media. Cell culture medium contains oxygen and nutrients that are important for cells. On the other hand, metabolites are also removed via culture media. There is a pressure difference between media channel inlets, so the media flows through the channels and the cells get a fresh medium all the time. The medium flow can also be made possible with the help of a pump. In addition, these chip devices can be monitored with different sensors and there is also a possibility for automatization. [7] Figure 3 shows the structure of a chip described in three directions.
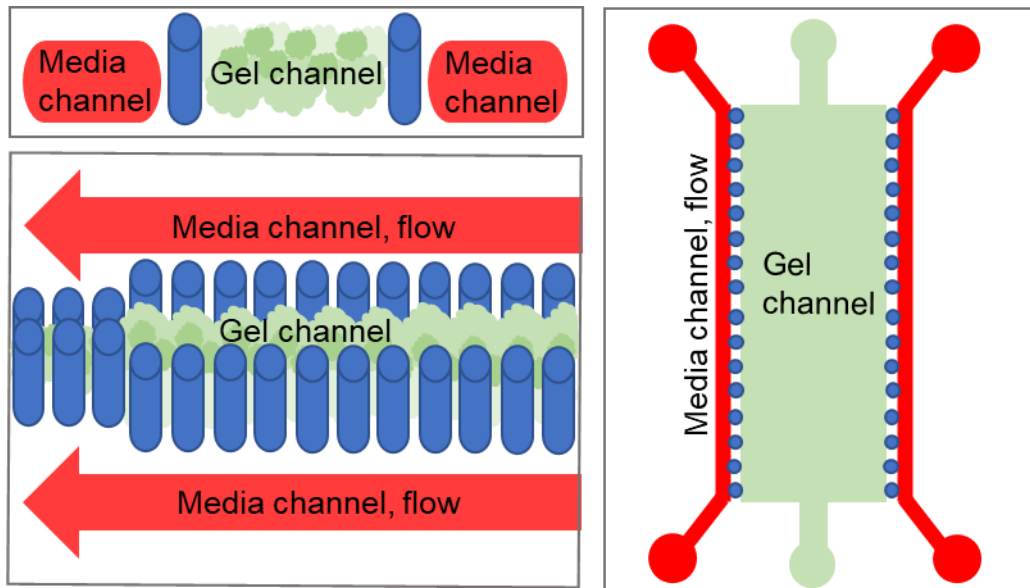
Figure 3. Structure of a chip described in three directions.

The cell culture chip can model the physiological functions of the tissues or organs, which cells are cultured. The simplest cultivation system contains only one cell type, but more complex devices can include two or more cell culture chambers that can be connected with porous membranes or linked fluidically. [8] These co-culturing systems can contain different cell types from one tissue or organ, so they model one tissue or organ's functions. For example, hepatocytes can be cultured with endothelial cells or other non-parenchymal cells, when trying to model liver functions. It is also possible to co-culture cells from two different models, for example, the heart model and the liver model.

Culturing cells in 3D can be done by using a scaffold, which is a structure made of biomaterial that supports cell adhesion and growth. Porous scaffolds enable the exchange of gases, nutrients, and metabolites, in the 3D cell culture. The main requirement for the biomaterial scaffold is that it mimics the functions and environment of ECM. [25, p. 4] The scaffold must be cytocompatible too because it is in contact with living cells. In addition, the scaffold's structure must enable cell intrusion, support cell growth, stress transfer and load-bearing. All in all, there are few requirements for scaffold structure, for example, they must have high porosity, high surface area, a fully interconnected geometry, structural strength, and a specific three-dimensional shape. [25, pp.18-19] These structure properties create a suitable environment for cells.

Both natural and synthetic polymers can be used in 3D cell culture. Collagen, chitosan, and fibrin are examples of natural polymers that have been used in 3D models. These polymers can be isolated from plants, animals, or human tissues, so they have good biocompatibility and low toxicity. The main problems with using natural polymers are

batch-to-batch variation, limited mechanical strength, and processing difficulties, which makes them expensive. Synthetic polymers' main advantages, on the other hand, are their good workability, properties that can be modified, reproducibility and high versatility. They have also an easier manufacturing process than natural ones. However, synthetic polymers biocompatibility and bioactivity properties are inferior to those of natural polymers. [25, pp. 20, 24] An example of a synthetic polymer is PEG.

Hydrogels are hydrophilic polymers that form crosslinks between the polymer chains. They can also absorb a large amount of water. Hydrogels are a valuable option for scaffold material for cells because they mimic the ECM. In addition, hydrogels' processing conditions are usually mild, and they can be delivered in a minimally invasive way. [27] As can be seen from these properties, hydrogels are a great option as a scaffold on a chip. Despite all these good properties, hydrogels' mechanical strength is limited, and their degradation rate must be considered.

This thesis is concentrating on the biomaterials or hydrogels that have been used especially with hepatocytes, which are cultured on microfluidic chips. These biomaterials on which to focus next are collagen, fibrin, Matrigel and GrowDex as natural polymers and PEG as a synthetic one. The advantages and disadvantages of these biomaterials are summarized in Table 3.

Table 3.   *Summary of different biomaterials used in 3D liver modeling.*

MATERIAL	ADVANTAGES	DISADVANTAGES
NATURAL POLYMER		
COLLAGEN	- Attachment site for cells - Biocompatible - Fibrous - Mimics ECM - Natural	- Batch-to-batch variation - Processing difficulties - Limited mechanical strength - Rapid degradation - Slow gelation (>30 min)
FIBRIN	- Biocompatible - Deformability - Extensible - Natural - Viscoelastic - Promotes cell adhesion - Cell-mediated degradation kinetics	- Batch-to-batch variation - Processing difficulties - Limited mechanical strength
MATRIGEL	- Basement membrane - Biocompatible - Improve differentiated phenotype - Mimics ECM	- Batch-to-batch variation - Processing difficulties
GROWDEX	- Biocompatible - Mimics ECM - Nanofibrillar structure allows diffusion of small substances - Natural - No crosslinks - Transparent - Viscosity can be modified - Xeno-free	- Batch-to-batch variation - Processing difficulties - Viscosity when mixed with cells
SYNTHETIC POLYMER		
POLY (ETHYLENE GLYCOL)	- Specific cell attachment when desired ligands are used - Neutral charge - Biocompatible - Easy to process - Properties can be modified - Hydrophilic - Non-immunogenic	- Inert and protein-repellent without ligand - Poor degradability - Weak mechanical properties

4.1 Collagen

Collagen is a commonly used natural polymer. It is a fibrous polymer consisting of a long and stiff triple-stranded helical structure. [25, p.20] Collagen is a trimeric ECM protein, and it has three α chains that form the triple helical structure. The α chains consist of three repeating amino acids, glycine, and two others, which usually are proline and hydroxyproline. Proline-hydroxyproline-glycine is the most common triplet in the collagen. [28, 29] Collagens main function is to provide mechanical support and to be a template for cell distribution and capillary formation. [25, p.20], [28, 29]

There are many types of collagens, even 28 different types have been found, but the main types are I, II and III. Collagen type I can be found from skin and bone, type II from cartilage and type III from blood-vessel walls and skin. [25, p. 20], [28, 29] Collagen I is the most common collagen type and almost every connective tissue contains it. It is a heterodimeric molecule consisting of two α1 chains and one α2 chain. [30, p. 1] Collagen II is a homodimeric molecule consisting of three identical α1(II) chains. It belongs to fibrin-forming collagens and is synthesized from procollagen. Collagen II usually occurs with collagen XI and it forms covalent crosslinks with collagen IX. [30, pp. 13-14] Collagen III is also a homodimeric molecule consisting of three α1(III) chains. It is secreted by fibroblasts and other mesenchymal cell types and takes part in inflammatory reactions. Collagen III usually occurs with collagen I but not in the bone. [30, pp. 21–22]

Collagen is usually isolated from animals' tissues so there is batch-to-batch variation in collagen. When using collagen in contact with cells, the collagen's source, concentration, solubility, polymerization temperature and polymerization pH must be considered. This is because the material needs to be suitable for cell adhesion and growth.

Collagen can be used as a scaffold in the chip. One option is to create a layer-by-layer structure, where one layer is positively charged collagen and the other is negatively charged collagen and between these collagen layers are the hepatocytes. This structure is deposited into the microfluidic device to obtain the constant flow of media. This kind of culturing method enables the hepatocytes to maintain their morphology and functions for 14 days. [31] As discussed before, PHHs start to de-differentiate during isolation. By comparison, 14 days cultivation time is quite average, because the PHHs have shown to maintain their morphology for 11 days in a collagen-coated 2D culture plate [17].

Bavli et al. used HepG2 cells mixed with collagen I biomaterial and oxygen-sensing beads, in their real-time monitored liver-on-a-chip microdevice. Mixing was done at low temperature to prevent the polymerization of premature collagen I. After the cell-collagen-beads suspension has been injected into the chip, they were incubated in the

incubator for 5 min in 37 °C to polymerize the collagen. Bavli et al. aimed to study the dynamics of mitochondrial dysfunction by monitoring metabolic functions of the liver. They used a microfluidic platform that combined a microfluidic liver-on-a-chip device and automated microfluidic analysis of glucose metabolism and mitochondrial function. The group used medical-grade commercial sensors and tissue-embedded microsensors which gave information about the amount of glucose, lactate, and oxygen in the cultivation. The HepG2 cells showed long-term gene expression, mitochondrial function, and polarization for over 28 days *in vitro*. [32]

4.2 Fibrin

Fibrin is formed by its preform fibrinogen, which is a soluble macromolecule. Fibrinogen typically occur in human blood plasma and it takes part in, for example, angiogenesis, hemostasis, inflammation, and wound healing. When it reacts with serine protease thrombin, the cascade is activated, and as a result, fibrin is formed. Fibrin is insoluble, so it forms a clot or gel, which is essential for its functions, including in wound healing the clot stops the bleeding. However, there is an activation cascade that regulates fibrin formation. This activation cascade is carefully controlled, so the blood does not clot without a reason. [33, p. 407] In addition, fibrinogens consist of three pairs of polypeptide chains, Aα, Bβ and γ, and it is mostly synthesized in liver hepatocytes. [33, pp. 408-409]

Fibrin is a viscoelastic polymer, so it has both elastic and viscous properties. These properties show in deformation. Elastic properties are shown by reversible mechanical deformation, which means that the material can revert to its original form after deformation. Because of viscosity, irreversible deformation occurs slowly under the influence of force. [33, p. 432] Fibrin is also a very extensible polymer, so it can stretch before breaking under stress. In addition, its stiffness can be modified, for example, by modifying the Factor XIIIa's express. This is because the Factor XIIIa catalyzes fibrin's crosslinking and increases its elastic modulus. [33, p. 433]

Fibrin has unique biological and physiological characteristics, which makes it widely used in tissue engineering scaffolds. These characteristics include biodegradability, deformability, elasticity, and porosity. Fibrin has been used as a biological scaffold with many different cell types, like cardiac tissue and liver cells. Fibrin can be used alone in scaffolds, but it can also be used in combination with, for example, collagen or gelatin, to achieve a suitable environment for the cells to differentiate, grow, and proliferate. [33, pp. 437-438] Fibrin is also biocompatible because it is a natural protein of the body. All in all, fibrin hydrogel is a suitable option for various bioartificial organ manufacturing, because of its excellent biocompatibility and 3D processing features [34].

Many studies using rat hepatocytes with fibrin on a chip has been done. In one study, fibrin was used in additive combined mold systems (ACMS) with rat hepatocytes and adipose-derived stem cells (ADSCs). In ACMS, there is a layer-by-layer structure containing cells and fibrin hydrogel, which forms a large construct. Inside the construct, there are a branched vascular system and the whole construct is coated with poly (dl-lactic-*co*-glycolic acid) (PLGA) to support the structure. The cell activities are retained in that structure. ASDCs are able to proliferate into both endothelial cells and hepatocytes depending on the used growth factors. The fibrin has also an important role in differentiated liver functions, for example, because the hepatocytes laden in fibrin are capable of synthesizing albumin. [34]

No study in which fibrin was used with human liver cells on a chip is found, but a few studies using HepG2 cells on a fibrin scaffold is found. In one study, they optimized a protocol for the 3D cultivation of HepG2 cells in a fibrin scaffold. The fibrin scaffolds resulted in the development of effective hepatic tissue. The scaffold also showed suitable long-term stability by providing enough time for the formation of hepatic tissue. This study also indicated that the fibrin isolated from blood plasma for the scaffold is a better option than purified fibrinogen because the process is easier. [35]

4.3   Matrigel

Matrigel is a combination of ECM proteins, such as laminin, collagen IV, and enactin, that have been isolated from mice's Engelbreth-Holm-Swarm (EHS) chondrosarcoma tumors. Matrigel is a basement membrane, so it forms specialized ECM that surrounds many cell types, including epithelial and endothelial cells. Furthermore, Matrigel forms a 3D gel at approximately 37 °C, so its structure supports cell morphogenesis, differentiation and even tumor growth. In addition, Matrigel has shown good properties for differentiation and outgrowth of many different cell types, including hepatocytes. Usually, primary cells do not proliferate, but they can differentiate when they are in contact with this matrix. Cells that are in contact with Matrigel, have shown more differentiated phenotype, both in their morphology and gene expression. For example, when hepatocytes are in contact with the Matrigel, their morphology is maintained, and they also produce albumin. [36]

Matrigel is stored as a frozen solution that usually contains 10-15 mg/ml media. Matrigel can be thawed overnight at 4 °C and it gels in 30 min at 24-37 °C temperature. In addition, the gel does not dissolve easily when cooling. Cells can be mixed with the Matrigel matrix before gelling or they can be just plated on top of the gelled Matrigel. [36]

There is a model, where HepG2 cells have been mixed with Matrigel and the mixture is injected into a microfluidic chip. There is not a physical barrier between the gel and media channels of the chip, there are only phaseguides between the channels. There is also media flow on the chip, which allows the exchange of oxygen, nutrients, and metabolites. That study shows that culturing HepG2 cells in perfused 3D microfluidic device mimics better *in vivo* conditions than 2D and other 3D cultures. In addition, that study shows that the HepG2 cells maintain their hepatic functions at least for 2 weeks with that culturing method. [37]

4.4 GrowDex

GrowDex is a hydrogel developed by UPM Biomedicals. It mimics ECM and therefore it supports cell growth and differentiation [38, p. 2]. GrowDex is a nanocellulose hydrogel, so it is a natural solution, which is extracted from birch trees derived from sustainable and responsibly managed forests [38, p. 2], [39]. GrowDex has many ideal properties for 3D cell culture and organ-on-a-chip model applications. Firstly, it is xeno-free, so it does not contain endotoxins. The GrowDex does not form crosslinks or contain gelatin, so it does not need any other steps to get ready than mixing with media and cells. Perhaps most importantly, the GrowDex is biocompatible and its nanofibrillar network allows small molecules, such as nutrients and oxygen, to diffuse easily, so the cells get needed substances. The GrowDex is also transparent without any fluorescent substances, so imaging and microscoping are possible. In addition, the GrowDex can be stored, transported, and used at room temperature, which makes it easy to use. Furthermore, the GrowDex hydrogel's viscosity can be modified by dilution with media, so the viscosity of the material can be adjusted to fit the requirements of different cell types. [38, p. 2]

In principle, the use of GrowDex is very simple: cells, media and GrowDex need to be mixed and then dispense the mixture into a culture platform or a chip. In practice, the suspension could be hard to inject into the chip because of the material's viscosity. The material's viscosity depends on the dilution with culture media as mentioned above. Cells can be mixed with the hydrogel or they can be placed on top of it. GrowDex's thixotropic nature allows it to be dispersed into gel channels of the microfluidic chip. The GrowDex is also able to retain its structure when it has been placed into the channel. In addition, there is also a GrowDase enzyme that digests the GrowDex when incubated at 37 °C, so only the cells remain in a solution. This makes it easy to collect the cells for research or re-plating. [38, pp. 3-4]

PHHs have been cultured on top of the GrowDex hydrogel for 35 days. The cells form 3D spheroid structures. They also have good viability and functionality of liver cells. [40]

Furthermore, the HepG2 cells have been mixed with GrowDex and cultured in the wells as 3D. As a result, GrowDex showed good properties for 3D cell culture-based drug screening and discovery. [41] There is not an article where GrowDex hydrogel has been used with liver cells on a chip but there is a protocol for using the GrowDex on a chip [42].

4.5   Poly (ethylene glycol)

PEG (poly (ethylene glycol)) is a synthetic polymer whose biophysical and biochemical parameters can be controlled to create hydrogel with suitable properties. In addition, PEG is cytocompatible, so it can be used in contact with cells. That also makes it very suitable for various biomedical applications. PEG can form covalent crosslinks between its branches so it can become hydrogel. The crosslinks can be formed by three different mechanisms: chin-growth, step-growth and mixed-mode chain and step growth. The PEG hydrogels are hydrophilic, neutral charged, and biocompatible. They have an inert and protein-repellent nature which allows specific cell attachment by using desired ligands. A copolymer containing PEG and biologically active moieties such as peptides support cell activity. [43, 44]

PEG can be used with other polymers in order to achieve the desired type of material. PEG can, for example, be mixed with diacrylate to form PEGDA to make the polymer's molecular weight large enough [44]. PEG can also be mixed with hyaluronic acid (HA) to form HA-PEG. This is done by mixing cyclooctyne-modified hyaluronan (HA-BCN) and a multiarmed azide-modified PEG [45].

Christoffersson et al. used HepG2 and iPSC-HLCs with agarose, alginate, and HA-PEG hydrogels on a liver-on-a-chip device. HepG2 cells were mixed separately with all three hydrogels and the suspensions were injected into the chips. The HepG2 cells formed spheroids in all hydrogels. The cells remained viable and maintained their function over 9 days. HepG2 secreted albumin and urea during the cultivation. According to the analysis, cells in alginate secreted the most amount and cells in agarose the least amount of both albumin and urea. [45]

5. CULTURING IPSC-HLCS IN BIOMATERIAL ON CHIP

Multiple microfluidic systems containing PHH cells have been developed. The problems with using PHHs are lack of cell availability and de-differentiation of the PHHs when cultured too long. The iPSC-HLCs could solve these problems because they can be differentiated from iPSCs originating from skin fibroblasts or blood cells. The main problem with the use of iPSC-HLCs is the fetal morphology, which can be solved with better differentiating methods and culturing environment. This chapter describes in more detail, which kind of microfluidic systems have been developed for modeling liver functions by using iPSC-HLCs.

Microfluidic devices or chips are microscale 3D culturing systems, so they need less media, biomaterial, and cells to carry the experiment than other 3D cultivation systems. This lowers the prices of needed substances and allows research to be done even if there are few cells available. Furthermore, in microfluidic systems, there is a medium flow that mimics the blood flow *in vivo*. This perfused flow enables control of oxygen, stable supply of nutrients, and removal of waste metabolites. [46]

Hydrogels mimic ECM and are therefore a good option as a scaffold on a chip. They create a 3D environment with a selective structure that enables the cells to cluster together without surface adhesion. In addition, hydrogel's structure enables the cells to be co-cultured without artificial membranes, but the membranes can also be used to separate different cell types. [46] These co-culture systems where multiple cell types can be cultured together enables the normal interactions between hepatocytes and non-parenchymal cells.

Many studies have been done by using iPSC-HLCs on a chip but only a few of them used 3D biomaterial scaffold. Usually, the chip has been coated with biomaterial and the cells are mixed with a culture medium and then the suspension has been injected on top of the coated channel of the chip to create perfused 2D cell culture system. The inner surface of the chip's gel channel may have been coated, for example, with collagen [47] or Matrigel [48, 49]. Next some studies, where the iPSC-HLCs in 3D biomaterial on a chip are presented.

Schepers et al. have developed a human liver-on-a-chip model which allows perfusion at different flow rates. They used both PHHs and iPSC-HLCs along and with the 3T3-J2 murine fibroblasts cell line. The iPSC-HLCs were resuspended in PEGDA pre-polymer

to encapsulate the cells to prevent the aggregation and overgrowth of the cells when placed on the chip. They placed the cell-hydrogel suspension on the C-trap chip architecture made of PDMS that enables robust loading of encapsulated cells and perfused cultivation with different flow rates. The pre-polymer was polymerized on-chip by exposure to ultraviolet (UV) light. [50]

On cultivation day 22, the iPSC-HLC aggregates showed the secretion of hepatocyte markers HNF4α and albumin. They also showed biliary marker HNF1β, which means that the aggregates are highly differentiated into several cell types. The activity of CYP-enzymes at day 22 was lower with iPSC-HLCs than with PHHs. The albumin secretion of iPSC-HLCs on a chip was robust up for 28 days. [50] As these results show, this microfluidic tissue model is an opportunity for patient-specific studies such as drug screening. However, the maturity of the hepatocytes used in the study is not known because the expression of AFP is not known and because their CYP activity was lower than PHHs'. This study still shows that the encapsulated cell aggregates withstand media flow over a relatively long time of cultivation.

Christoffersson et al. studied HepG2 and iPSC-HLCs with agarose, alginate, and HA-PEG hydrogels on a liver-on-a-chip device. There were two kinds of HA-PEG, one with linear arginyl glycyl aspartic acid (linRGD) and one with cyclic arginyl glycyl aspartic acid (cRGD). The iPSC-HLCs were mixed with each hydrogel separately by mixing $5 \times 10^6 \frac{cells}{ml}$ of viable cells with hydrogel. The suspensions were loaded on the commercial chips (μ-slide III 3D Perfusion, Ibidi) by injecting 25 μl of one suspension directly to two channels so that there were 4 wells in total for each condition. The study was set up to compare the different hydrogels, so 1 % agarose, 1 % alginate, and 2 % HA-PEG were used because they had the same range of modulus (G). [45]

Small viable iPSC-HLC aggregates were found separately in agarose, alginate, and HA-PEG with linRGD hydrogels, but the cells encapsulated in the HA-PEG with cRGD formed large structures of viable cells. These large structures had migrated all over the hydrogel. In addition, there was albumin expression and secretion only in chips, where the hydrogel was HA-PEG with cRGD. Nevertheless, the secretion level of albumin did not reach the secretion level of the corresponding 2D culture system. This study showed that the iPSC-HLCs need cell adhesion motifs in the hydrogel to function and survive over 13 days. [45] When considering future options for hydrogels used with iPSC on chips, the crosslinking methods and cell attachment sites must be considered to get better results in the future.

Starokozhko et al. used a highly porous PDSM as a scaffold in the microfluidic device during the differentiation of iPSC-HLCs from iPSC-derived definitive endoderm (DE) cells. The PDSM scaffolds were treated with oxygen plasma to make the surface of the scaffold hydrophilic. The scaffolds were also coated with Hepatocyte Coating and washed with phosphate-buffered saline (PBS) solution. The scaffolds were left in media for 2 h at 37 °C before loading. [51]

The coated PDSM scaffolds were placed into holes of a custom-made tray platform. A 2.5×10^6 freshly thawed iPSC-DE cells were resuspended into 30 µl of Hepatocyte Thawing and Seeding Medium and the suspension was pipetted into each PDSM scaffold. The cells had 3 h to adhere at 37 °C under 95 % air 5 % CO_2 before the trays were inverted and placed vertically in 4 different positions. The reversal of posture was done to allow the cells to distribute through the whole scaffold during 3 h. Then the scaffolds were placed in the 4 × 4 bioreactor array of the fluidic platform. The media flowed through the scaffold at flow rates of either 1 or 5 µl/min. The entire system maintained in the incubator at 37 °C under 95 % air 5 % CO_2 and the cells were cultured and differentiated for 28 days. [51]

Starokozhko et al. had a few comparison cultures: they differentiated iPSC-HLCs in 2D plates both in polystyrene wells and PDMS-coated well plates and they also had fresh human precision-cut liver slices (PCLS) in the same perfused conditions as to where iPSC-HLCs were differentiated. As a result, the differentiated iPSC-HLCs' activities of CYP, metabolism of Phase II, and synthesis of albumin, urea and bile acids were on the same level as PCLSs. This indicates better maturation of iPSC-HLCs. When comparing the static 2D culturing conditions to perfused 3D culturing conditions, the perfusion induced Phase II metabolism which therefore decreased the expression of AFP. However, the activity of some Phase I metabolism enzymes was a little lower in perfused conditions than in static. Also, according to gene expression, the iPSCs differentiated into both hepatic and biliary cells. As can be seen from the results, perfused culture conditions support the differentiation of iPSCs expressing a wide range of different liver functions. [51] This study shows good opportunities for future studies and also personalized medicine. In addition, this study shows that the iPSC-HLCs can also be cultured in a scaffold that is not a hydrogel and still the results are as good as hydrogel scaffolds have shown.

In a study by Bircsak et al., iPSC-HLCs mixed with collagen I were injected into OrganoPlate LiverTox™ -chip. These iPSC-HLCs were cocultured with both HMEC-1 endothelial cells and THP-1 monoblasts differentiated to macrophages. The iPSC-HLCs formed clusters containing 4-16 cells during differentiation. These clusters were

resuspended into 4 mg/ml collagen I, so the density of suspension was approximately $15 \times 10^6 \frac{cells}{ml}$. Then 1.3 µl of the suspension was injected into the OrganoPlate 2-lane gel inlet. The suspension containing HMEC-1 and THP-1 cells was injected into the perfusion channel inlet and media was added after them to hydrate the chip as the cells attached to the iPSC-HLCs-collagen matrix. [52]

The cultivation method used in that study showed stable cell viability, albumin, and urea secretion for 15 days. During these 15 days, the secretion of AFP decreased and the activity of CYP3A4 increased which would suggest better maturation of iPSC-HLCs. [52] This is because AFP is a fetal or immature marker and CYP3A4 is a mature marker of the liver. This study shows also that co-culturing hepatocytes with endothelial cells and monoblasts support the maturation of iPSC-HLCs, as well as the perfused conditions.

As a result of all the studies discussed above, perfused cultivation environment would seem to support the maturation of iPSC-HLCs. This is shown in the decrease of the expression of AFP, which is a fetal marker, and the increase of albumin and CYP secretion which are mature markers. In addition, the cultivation time seems to be longer in perfused cultivation systems than in stable ones. This can be explained by the support that the 3D biomaterial scaffold gives to the cells during cultivation and because the environment mimics better the *in vivo* conditions. Table 4 summarizes the 3D cell culture systems discussed in this chapter.

The summary of different cultivation systems containing iPSC-HLCs with biomaterial on a perfused chip.

CELLS	MATERIALS	INFORMATION	YEAR	REFERENCE
IPSC-HLCS, PHH, 3T3-J2	PEGDA	Different perfusion rates Showed both hepatic and biliary markers	2016	[50]
HEPG2, IPSC-HLCS	Agarose, Alginate, HA-PEG	HA-PEG with cRGD showed the best properties for albumin secretion and viable cell aggregates	2018	[45]
IPSC-HLCS, PCLS	PDSM	A highly porous PDMS scaffold in a perfused platform enable better maturation of iPSC-HLCs	2018	[51]
IPSC-HLCS, HMEC-1, THP-1	Collagen I	Better maturation of iPSC-HLCs because the secretion of AFP decreased and the activity of CYP3A4 increased during cultivation	2021	[52]

iPSC-HLC: induced pluripotent stem cell-derived hepatocyte-like cells, PHH: primary human hepatocytes, 3T3-J2: murine fibroblast cell line, HepG2: human hepatoma cell line, PCLS: human precision‑cut liver slices, HMEC-1: endothelial cell (human microvascular endothelial cell line), THP-1: monoblasts differentiated to macrophages (human monocytic cell line)

6. CONCLUSIONS

This thesis investigated 3D *in vitro* liver models, the cell types, different biomaterials, and cell culture systems that are used. First, basic information about the liver structure and function was reviewed. Hepatocytes are the most important liver cell type, as they are responsible for performing most liver functions. Then different cell sources and materials used in the *in vitro* liver models were described. There are 4 main sources of cells used in liver modeling. These sources are PHH, liver cell lines, such as HepG2 and HepaRG, and iPSC-HLCs, which are also discussed in this thesis. Every cell source or cell type has its own advantages and disadvantages which are summarized in Table 2. The PHHs mimic the best native liver functions because of their origin, but their availability is limited. Hepatoma cell lines, including HepG2's and HepaRG's, availability is high, as well as their proliferation rate but there are alterations in liver-specific functions, such as limited metabolism functions. Human iPSC-HLCs availability is also high, their differentiation has many options and there are less ethical issues than with ESCs. However, they are not fully matured but the maturation could be solved with better differentiation methods and culture conditions.

Several biomaterials have been used as a scaffold when culturing liver cells. This thesis discussed 5 of them: collagen, fibrin, Matrigel, GrowDex and PEG. These materials' advantages and disadvantages are summarized in Table 3. All these materials are hydrogels, so they mimic ECM enabling the attachment and growth of cells in a 3D. However, the main challenges in using hydrogels are degradation and mechanical properties. In addition, most of the discussed biomaterials are natural polymers, so there are batch-to-batch variations and processing difficulties. These problems do not affect synthetic polymers which can be modified to get the wanted properties. As a downside, synthetic polymers' biocompatibility and bioactivity properties are inferior to those of natural polymers but these properties can be modified with proper additives.

Finally, in Chapter 5, the liver models containing iPSC-HLCs with biomaterial on a microfluidic chip were investigated. The current studies containing iPSC-HLCs with biomaterial on a chip are summarized in Table 4. The main result is that the perfusion supports the maturation of the iPSC-HLCs, as well as the microfluidic device helps the control of cultivation conditions. Various biomaterials have been used in perfused liver models containing iPSC-HLCs and all of them show longer cultivation time and better maturation of iPSC-HLCs.

The purpose of this thesis was to investigate *in vitro* liver models using biomaterials in 3D. Before understanding the models, it is important to understand the parts of which the model consists of which are cells, biomaterial, in other words, the ECM, cultivation device and conditions. The differences between PHHs, HepG2 and iPSC-HLCs affect the model. The PHHs start to de-differentiate quickly during cultivation so only short-term cultivation is possible. The PHHs mimic the native liver functions the best because of their origin, but on the downside, their availability is low also because of their origin. Instead, the HepG2 cells have an almost unlimited lifespan, proliferation rate and they are easy to handle, but they do not express all the metabolism factors like the native liver. In contrast, the HepG2 cells are also isolated from one donor so there is no genetic polymorphism. The iPSC-HLCs availability is high, they have less ethical issues, and there are many ways to differentiate them, but the differentiated cells are not fully matured. Because of that, the iPSC-HLCs cannot fully mimic the native liver functions. Nevertheless, the perfused 3D cultivation system supports the maturation of iPSC-HLCs which allows, with better differential methods, more ideally function of iPSC-HLCs and thus better native liver modeling.

Choosing the right biomaterial for research is also important because all the materials have their own characteristics and therefore are not suitable for every situation. All the materials discussed in this thesis are suitable for use on a chip, but other materials may not be suitable for that. There are also additional things that must be considered when choosing the biomaterial. Firstly, some hydrogels form crosslinks, so they need time to crosslink before adding media into the cultivation system. Secondly, some materials are not cytocompatible, so they can cause immune reactions to the living cells that are in contact with the material. In addition, the degradation rate of some materials may affect their properties so that the cells do not get the support they need, or the degradation products may not be cytocompatible. All these material properties can affect the model and therefore to the research.

Different microfluidic devices have also been developed and different flow rates can be used. The flow rate of a medium also affects the cultured cells and even the material. If the rate is too high, it can damage the material and therefore affect the cells' environment. All in all, there are many things to consider when doing research.

The liver is a very complex organ with many functions and thus a difficult organ to model *in vitro*. One possibility is to culture hepatic cells with non-parenchymal ones, so the environment of hepatic cells mimics their original environment *in vivo*. Combining these cells could also enable the modeling of most liver functions. Culturing hepatocytes with non-parenchymal cells has also shown better functionality and longer culturing time [10].

Usually, both parenchymal and non-parenchymal cells are needed to reflect more precisely the pharmacokinetics, pharmacodynamics, toxicity of drugs, and liver disease progression. This is because the intercommunication between different liver cell types enables better conditions for hepatocytes to mimic liver functions. [53]

The liver is an organ, so it does not perform all the functions alone *in vivo* but it takes part in other physiological functions with other organs or tissues, such as the gallbladder and pancreas. More complex microfluidic systems containing several tissues linked together gives more information about complex drug metabolism reactions that affect several tissue functions [54]. The organ-on-a-chip can model multiple organs together, so their common functions can also be studied. A body-on-a-chip is a combination of organ-on-a-chips, which can be used in studying complex mechanisms, for example, in disease or drug studies, containing multiple organs or tissues. [10] With these complex cultivation systems, almost all body functions may be modeled someday. The iPSCs may be the answer for a body-on-a-chip because they are able to differentiate into all adult cell types and therefore, they can be used to form body-on-a-chip.

Only the time will show the possibilities of using iPSCs and chips in research. The microfluidic cultivation technique has shown promising properties, but the most ideal liver-on-a-chip system, that allows long-term cultivation of liver cells with mature liver functions, has still not been found. In addition, the most ideal differentiating method of iPSC-HLCs have not been found, but the perfused conditions support the maturation and therefore could be a great option for the development of differentiating methods. Furthermore, the liver-on-a-chip devices could replace animal experiments in many fields of research because the chips are more ethical, and the results can match human body functions better than animal studies. The iPSCs could also open doors for personalized medicine and research, but the cell culture methods should evolve for the better, and the costs of the cultivation system would have to decrease in order for personalized research to become more common.

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