Centre for Drug Research
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DEVELOPMENT OF ORGANOTYPIC LIVER CELL CULTURES IN THREE-DIMENSIONAL BIOMATERIAL HYDROGELS

Melina Malinen

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Pharmacy of the University of Helsinki, in lecture hall 1, Infocenter Korona, Viikinkaari 11, on September 1st, 2014, at 12 noon.

Helsinki 2014

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Cover: Accumulation of a fluorescent probe (green) into the intercellular vacuoles of HepaRG cell aggregates cultured in the hyaluronan-gelatin hydrogel. Bile canaliculi-like structures (green) and nuclei (red).

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ABSTRACT

New organotypic liver cell cultures are needed to predict the metabolism, excretion, and safety of chemical compounds. Liver cell models are particularly important since the liver largely regulates the ultimate fate of compounds in the body. Approximately 70% of the drugs administered to the body are metabolized or excreted by the liver.

Animal models, cell cultures, and cell-free assays are the most common liver models. However, animal models and animal cells do not represent humans due to the interspecies differences in drug metabolizing enzymes and transporters. Instead, the most common cell-free methods, microsomes, are appropriate for drug metabolism studies, but the lack of drug transporters and transcription machinery prevents the complete evaluation of compounds. Primary human hepatocytes are capable of both drug metabolism and drug transport, and are, therefore, considered the gold standard to assess metabolism and toxicity of compounds *in vitro.* Primary hepatocytes, however, suffer limited availability, high functional variability, and difficulty with maintaining differentiated phenotypes and functions in cell cultures. Therefore, continuous human liver cell lines, such as HepG2 and HepaRG, have been widely used to evaluate drugs and chemicals even though they have defects in their biotransformation functions. The advantages of cell lines are their good availability, easy maintenance, and inducible drug metabolism.

Generally, these cells are cultured in a two-dimensional (2D) manner that deviates from the physiological morphology and functions of the hepatocytes. The flattened 2D phenotype leads to reduced polarization and loss of important signaling pathways; this is likely to be a major reason for the failure in the prediction of drug metabolism, pharmacokinetics, and hepatotoxicity. It is believed that for more predictive *in vitro* models, the liver cells should be maintained in a three-dimensional (3D) microenvironment that allows reconstruction of polarization, and cell-cell and cell-extracellular matrix (ECM) contacts. The 3D cell cultures have been generated by different methods, such as cultures in matrices, scaffolds, bioreactors, and microfluidic platforms. Biomaterial hydrogels have demonstrated great potential for 2D liver cell culturing, but their potential to generate functional 3D liver cell cultures is largely unknown.

The main goal of this thesis was to establish improved 3D liver cell cultures with biomaterial hydrogels. Particular attention was focused on the effects of 3D hydrogels on drug metabolism and excretion, cytoarchitecture, and cellular differentiation of HepG2 and HepaRG cell lines. As a starting point, we studied the suitability of wood-derived nanofibrillar cellulose (NFC) hydrogel as a cell culture matrix. NFC hydrogel has not been studied in cell culture before; however, as a novel, defined, animal-free, and

abundantly available material, it evoked interest for testing. Herein, the wood-derived NFC was proven to own rheological and structural characters that allow 3D cell culture. Moreover, the NFC was compatible with the HepG2 and HepaRG cells, allowing for the formation of 3D multicellular aggregates with increased apicobasal polarity. When compared to commercial hydrogels, the NFC supported the albumin secretion, an indicator of hepatocellular synthetic function, from HepG2 and HepaRG cells as well or even better. These results demonstrate the potential of wood-derived NFC to function as an ECM analogue, and present the first HepaRG aggregate cultures.

Next, the effect of the RAD16-I peptide hydrogel on the HepG2 cell line was investigated in more detail. Immunofluorescence staining and vectorial transport showed formation of tissue-like arrangements including bile canaliculi-like structures and polar distribution of canalicular efflux transporters, multidrug resistance-associated protein 2 (MRP2), and multidrug resistance protein 1 (MDR1), in the spherical HepG2 cell aggregates. The study clearly demonstrated that the peptide hydrogel increases the apicobasal polarity and appearance of bile canaliculi structures in HepG2 cell cultures.

The plasticity of HepaRG liver cells was exploited to investigate the impact of 3D NFC and hyaluronan-gelatin (HG) hydrogel cultures on the phenotype of both undifferentiated HepaRG cells (early liver progenitors) and differentiated HepaRG cells (hepatocyte-like cells together with cholangiocyte-like cells). Based on the expression and activity of hepatic markers, drug metabolizing enzymes, and drug transporters, the 3D NFC and HG hydrogels promoted the differentiation of HepaRG liver progenitor cells when compared to the standard 2D technique. Instead, the 3D hydrogel cultures could not really improve the properties of differentiated HepaRG cells.

In conclusion, these findings reveal the capability of the NFC, RAD16-I peptide, and HG hydrogels to improve the properties of HepG2 and HepaRG human liver cells. The new spheroid cultures of HepG2 and HepaRG cells may represent added value for pharmacokinetic and toxicity predictions, showing a liver-like cytoarchitecture and demonstrating applicability for drug metabolism and transport studies. Overall, the results deepen our knowledge of the 3D liver cell cultures.

ACKNOWLEDGEMENTS

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Helsinki, 2014

Melina Malinen

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Bhattacharya M*, Malinen MM*, Lauren P, Lou Y-R, Kuisma SW, Kanninen L, Lille M, Corlu A, Guguen-Guillouzo C, Ikkala O, Laukkanen A, Urtti A, and Yliperttula M. Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture. Journal of Controlled Release 164: 291-298, 2012. http://dx.doi.org/10.1016/j.jconrel.2012.06.039

* Equal contribution

II Malinen MM, Palokangas H, Yliperttula M and Urtti A. Peptide nanofiber hydrogel induces formation of bile canaliculi structures in 3D hepatic cell culture. Tissue Engineering. Part A 18: 2418-2425, 2012. http://dx.doi.org/10.1089/ten.tea.2012.0046

III Malinen MM, Kanninen L, Corlu A, Isoniemi H, Lou Y-R, Yliperttula M, Urtti A. Differentiation of liver progenitor cell line to functional organotypic cultures in nanofibrillar cellulose and hyaluronan-gelatin hydrogels. Biomaterials 35: 5110-5121, 2014. http://dx.doi.org/10.1016/j.biomaterials.2014.03.020

The publications are referred to in the text by their roman numerals.

AUTHOR´S CONTRIBUTION

Publication I: Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture

The author designed the experiments together with the supervisors and co-authors. The author performed and analyzed the experiments concerning fluorescence properties of nanofibrillar cellulose, cell viability, cellular morphology and albumin secretion. The first cell cultures, injectability, absorbance and permeability studies were performed by Dr. Madhushree Bhattacharya and undergraduate student Patrick Lauren. The other experiments were designed and performed by the other co-authors. The author wrote her part in the manuscript and actively participated in finishing the article.

Publication II: Peptide nanofiber hydrogel induces formation of bile canaliculi structures in 3D hepatic cell culture

The study was designed together with supervisors and co-authors. The experiments were started with the guidance of Dr. Harri Palokangas, from whom the cell culturing and immunostaining protocols originate. Then cell viability, RT-PCR and functional polarity studies were performed by the author. The author analyzed the results and wrote the first draft of the manuscript and actively participated to complete the final form.

Publication III: Differentiation of liver progenitor cell line to functional organotypic cultures in nanofibrillar cellulose and hyaluronan-gelatin hydrogels

The author designed the experiments together with supervisors and co-authors. The author performed the assays of cell viability (live/dead), RT-PCR, gDNA and functional polarity. The cell culturing, phase contrast microscopy, cell viability (alamarBlue), CYP3A4 activity, CYP3A4 induction and immunostaining were done with the help of undergraduate student (later MSc) Liisa Kanninen. The author analyzed the data and wrote the first draft of the manuscript and actively participated to complete the final form.

ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ABC	ATP-binding cassette
ABCB1	ATP-binding cassette sub-family B member 1, also known as Multidrug resistance protein 1 and P-glycoprotein
ABCB11	ATP-binding cassette sub-family B member 11, also known as Bile Salt Export Pump (BSEP)
ABCC2	ATP-binding cassette sub-family C member 2, also known as Multidrug resistance-associated protein 2 (MRP2)
ABCG2	ATP-binding cassette sub-family G member 2, also known as Breast cancer resistance protein
BCRP	Breast cancer resistance protein, also known as ATP-binding cassette sub-family G member 2
BSEP	Bile salt export pump, also known as ATP-binding cassette sub-family B member 11 (ABCB11)
CAR	constitutive androstane receptor
CLh	Drug clearance occurring in the liver, also known as hepatic clearance
CLr	Drug clearance occurring in the kidney, also known as renal clearance
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	Food and Drug Administration
FMO	Flavin-containing monooxygenase
GST	Glutathione S-transferase
HA	Hyaluronan, hyaluronic acid
HCS	High-content screening
HG	Hyaluronan-gelatin
HNF	Hepatocyte nuclear factor
HTS	High-throughput screening
iPSC	induced pluripotent stem cell
KRT19	Keratin 19
MATE1	Multidrug and toxin extrusion protein 1, also known as solute carrier family 47 member 1 (SLCO47A1)
MCT	Monocarboxylate transporter
MDR1	Multidrug resistance protein 1, also known as P-glycoprotein and ATP-binding cassette sub-family B member 1
MRP	Multidrug resistance-associated protein sub-family

MRP2	Multidrug resistance-associated protein 2, also known as ATP-binding cassette sub-family C member 2 (ABCC2)
NAT	N-acetyltransferase
NFC	Nanofibrillar Cellulose
NTCP	Na^+ -taurocholate cotransporting polypeptide
OCT	Organic cation transporter
OATP	Organic anion transporting polypeptide, also known as Solute carrier organic anion transporter (SLCO)
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PXR	Pregnane X receptor
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SLCO	Solute carrier organic anion transporter, also known as Organic anion transporting polypeptide
SULT	Sulfotransferase
UGT	Uridine Glucuronide Transferase
US	United States
UV	ultraviolet

1 INTRODUCTION

Drug discovery and development is a complicated process that takes an average of 10–15 years. The entire process is comprised of several stages from target identification through preclinical studies to clinical trials in humans. Only 10% of the compounds that enter clinical trials qualify through the process and reach the market (Bunnage, 2011; Castellani, 2011). This means that preclinical studies are not able to properly predict the behavior of novel compounds in the human body. Especially, the predictive ability and reproducibility of preclinical tests need improvement (Mandenius et al., 2011; Begley and Ellis, 2012; Henderson et al., 2013).

Animal experiments do not mirror humans due to the interspecies differences in drug biotransformation and, therefore, may fail to predict the drug´s safety or effectiveness (Dash et al., 2012; Pfeifer et al., 2014). The validity of the current *in vitro* models is not high either. Genetic variations between the donors and the loss of polarized architecture together with the loss of appropriate functions decrease the reproducibility and predictive power of primary human cells (Hewitt et al., 2007; Hewitt, 2010; Sahi et al., 2010). Further, the short lifetime of primary human cells in the culture prevents long-term experiments. Cell lines offer more stable models, but generally, the data cannot be extrapolated to humans as the drug biotransformation differs from the native human cells (Anene-Nzelu et al., 2011; Dash et al., 2012). In addition, cell-free systems have been revealed as too simplified to predict the *in vivo* safety and effectiveness of drugs (Jia and Liu, 2007; Hillgren et al., 2013).

Innovations are clearly needed to develop more reliable preclinical tests. In fact, the Food and Drug Administration (FDA) of the United States (US) has emphasized the need for improved preclinical cell models for drug development in their “Critical Path Initiative” (US FDA, 2004, 2010), while the European Union (EU) has supported several research projects to establish new *in vitro* models for investigating human drug metabolism and toxicology properties of compounds. The need for new preclinical models is further increased by the recent chemical safety regulation that the EU released in 2007 (European Parliament and Council, 2006; European Chemicals Agency, 2009). The “Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)” regulation requires that the safety properties of all chemicals that are manufactured and marketed in the EU be assessed and collected. This means that the effects and safety of approximately 140,000 chemical substances must be tested (European Chemicals Agency, 2008). Especially interesting is that the REACH promotes alternative methods for the assessment of chemical safety in order to reduce the number of animal tests.

Liver models are particularly important for drug discovery and chemical testing since the ultimate fate of drugs and chemicals in the body is largely regulated by hepatic uptake, metabolism, and excretion. Approximately 65% of drugs are predominantly eliminated by metabolism in the liver, and an additional 6% of drugs are eliminated by excretion to the bile (Williams et al., 2004; RxList, 2012). Liver parenchymal cells, hepatocytes, carry out drug elimination with their metabolizing enzymes and membrane transporters. The metabolizing enzymes convert the compounds to the metabolites that can be more easily excreted from the body, whereas membrane transporters are responsible for the vectorial transport through the hepatocyte cell membranes, thereby enabling drug elimination from the blood circulation to the bile. To predict vectorial drug elimination in humans, the liver model should comprise polarized cells and express both metabolizing enzymes and transporters at the correct cellular positions at relevant levels.

Human hepatocyte cultures and human liver microsomes are well-accepted *in vitro* test systems. They are used to predict *in vivo* human drug metabolism (US FDA, 2012), but the prediction of drug transport has not been satisfactory (Giacomini et al., 2010). Also the prediction of human hepatotoxicity remains very challenging (EMA, 2008). This failure may result from the fact that the current methods are based on two-dimensional (2D) hepatocyte cultures, where cells grow as a monolayer on a flat surface. These cells do not represent either the structure or the functions of the three-dimensional (3D) liver tissue (Bissell et al., 2005; Pampaloni et al., 2009). Therefore, novel cell culturing techniques for liver cells need to be developed. Particularly, 3D cell culture methods have raised interest, since they may offer better prospects for modelling of liver tissue by increased cell-cell and cell-extracellular matrix (ECM) signaling properties (Mandenius et al., 2011; LeCluyse et al., 2012; Godoy et al., 2013).

This thesis aimed to develop 3D liver cell cultures that mimic the polarized cellular structure and drug elimination functions of the liver. The 3D culturing environment was generated with hydrogels based on nanofibrillar cellulose (NFC), peptide nanofibers, and block copolymers.

2 REVIEW OF THE LITERATURE

2.1 THE ROLE OF THE LIVER IN DRUG METABOLISM AND EXCRETION

The liver plays an essential role in the human body being responsible for metabolizing and excreting of endogenous and exogenous solutes. In addition, the liver produces bile, urea, and the majority of plasma proteins, and controls the homeostasis of glucose, glycogen, and cholesterol. To be capable of performing all these functions, the liver has a high blood supply from both the hepatic artery and the hepatic portal vein, and is composed of functionally different zones and cell types (Figs. 1 and 3). Parenchymal cells (hepatocytes) represent almost 80% of the liver´s total volume, and non-parenchymal cells, such as sinusoidal endothelial cells, Kupffer cells, and biliary epithelial cells (i.e. cholangiocytes) contribute approximately 10% of the total volume (Kmiec, 2001). The remaining 10% of the liver volume consists of extracellular space compartments, such as sinusoids (the capillaries of the liver) and bile canaliculi.

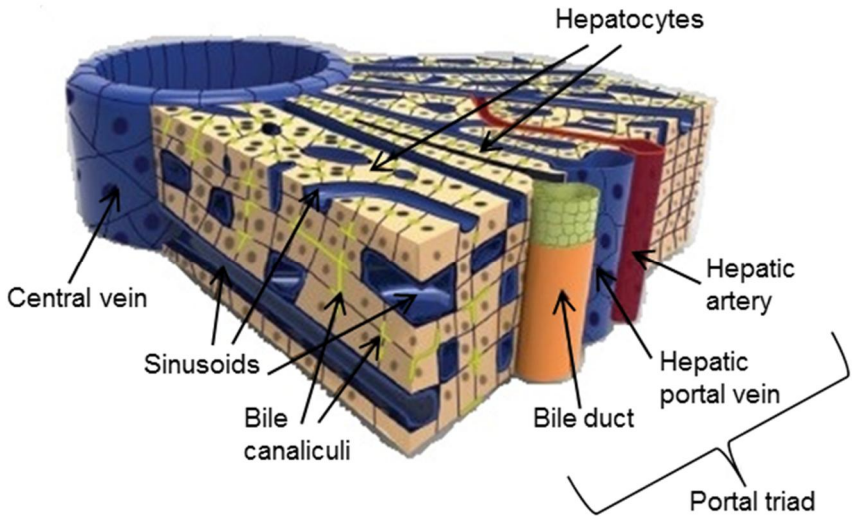

Figure 1 The architecture of a liver functional unit. The central structure is the central vein, which is radially surrounded by liver parenchymal cells i.e. hepatocytes (beige), sinusoidal capillaries i.e. sinusoids (blue), and bile canaliculi (yellow-green). The unit receives blood from the hepatic portal vein and the hepatic artery from where the blood flows through sinusoids to the central vein. The bile, secreted by hepatocytes, flows to the opposite direction than the blood, from bile canaliculi to the bile duct. The image is modified from (LeCluyse et al., 2012).

2.1.1 METABOLISM AND EXCRETION OF DRUGS IN THE LIVER

The liver is the major organ of drug elimination. Approximately 65% of the drugs administered to the body are metabolized in the liver (RxList, 2012). In addition, about 6% of drugs are eliminated through biliary excretion. The rest of the drugs are eliminated predominantly through renal excretion (25%) or are metabolized/excreted elsewhere in the body (4%). Because drugs are predominantly metabolized in the liver or excreted in the urine and bile, drug elimination (total body clearance) is described as a summary of the drug clearance occurring in the liver (CLh) and the kidney (CLr) (Rowland and Tozer, 2011).

Hepatic clearance (CLh) is a complex process that depends on passive drug diffusion into the hepatocytes, drug metabolism by the enzymes, and drug transporting activity of the membrane transporters (Fig. 2). (1) The drug enters the hepatocyte either by passive diffusion across the sinusoidal plasma membrane or by active transport via uptake transporters at the sinusoidal plasma membrane. (2) Conversion of the drug to the metabolite takes place within the hepatocytes by phase I and II reactions. Phase I enzymes catalyze the formation of a functional group or cleavage (e.g., oxidation, reduction, hydrolysis), and phase II enzymes induce drug conjugation with an endogenous substance (e.g., glucuronidation and sulfation). In general, drug metabolites are more hydrophilic compounds than the parent drug and cannot enter the plasma membrane easily. (3) Instead, hydrophilic metabolites may be actively transported from the inside of the hepatocyte to the bile or the blood by efflux transporters located at the canalicular and basolateral membranes, respectively (Fig. 2). The metabolites that are excreted to the blood are effectively excreted through the urine due to their reduced re-absorption in the kidney.

The roles of the phase I and II metabolizing enzymes in drug elimination has been known for decades. On the contrary, the clinical importance of drug uptake transporters and drug efflux transporters is only currently being revealed (Giacomini et al., 2010; EMA, 2012; Hillgren et al., 2013). The best-known transporter, P-glycoprotein (P-gp, MDR1, ABCB1) was discovered in the 1970s (Juliano and Ling, 1976), but its role in pharmacokinetics and drug-drug interactions was understood later. P-glycoprotein, which belongs to the ATP-binding cassette (ABC) superfamily, acts as an efflux transporter at the canalicular membrane of the liver. The other transporters of emerging importance include: canalicular efflux transporters, multidrug resistance-associated protein 2 (MRP2, ABCC2), breast cancer resistance protein (BCRP, ABCG2), bile salt export pump (BSEP, ABCB11), and multidrug and toxin extrusion protein 1 (MATE1, SLC47A1), and sinusoidal uptake transporters, organic anion transporting polypeptide 1B1 (OATP1B1, SLCO1B1), organic anion transporting polypeptide 1B3 (OATP1B3, SLCO1B3), and organic anion transporting polypeptide 2B1 (OATP2B1, SLCO2B1) (Hillgren et al., 2013).

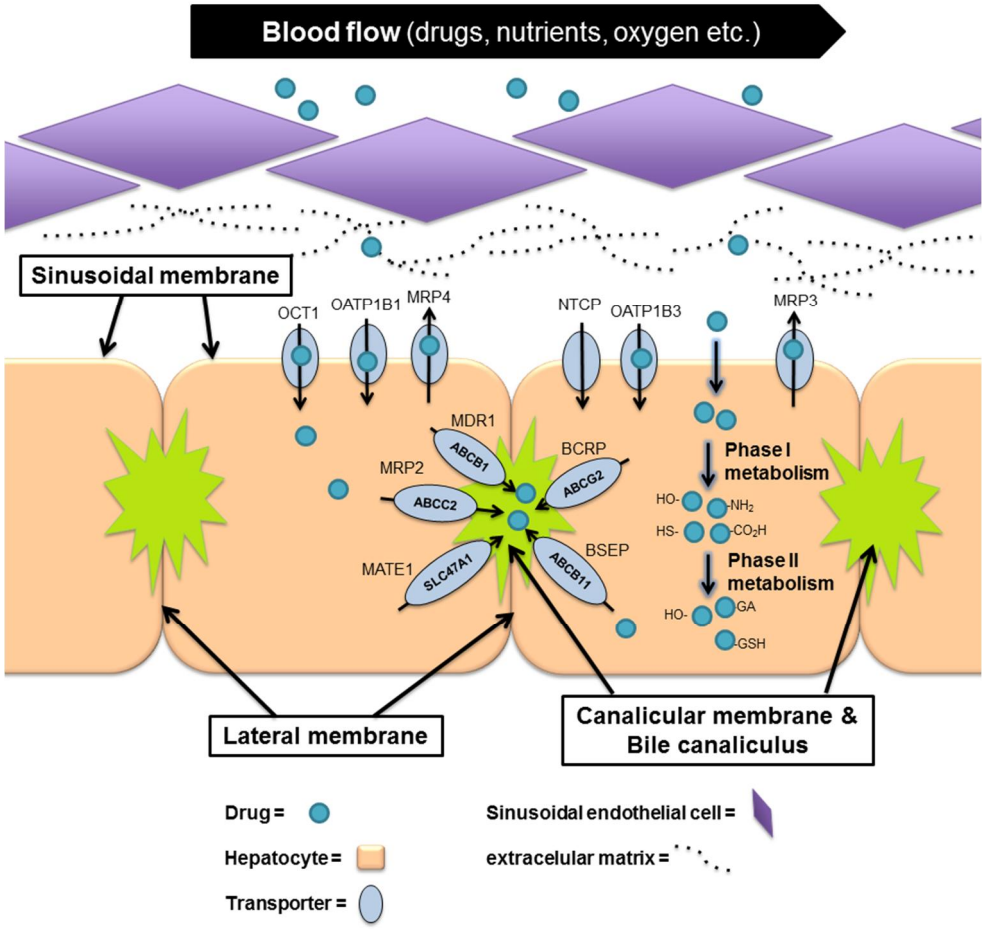

Figure 2 Schematic diagram of drug transport and metabolism in the hepatocytes. Hepatocytes receive endogenous and exogenous substances from the blood and can convert these compounds, such as drugs, to the metabolites that are excreted to the bile or back to the blood. The substances enter and exit the hepatocytes by passive diffusion or active transport. Uptake transporters (e.g. OATP) locate at the sinusoidal membrane and efflux transporters both at the canalicular and sinusoidal membranes (e.g. MDR1, MRP2, MRP3).

2.1.2 THE IMPORTANCE OF HEPATOCYTE ARCHITECTURE

For drug metabolism and excretion, the hepatocytes must be well-organized and polarized. The plasma membrane of the hepatocyte consists of structurally distinct domains: sinusoidal, lateral, and bile canalicular (Hubbard et al., 1983) (Fig. 2). The sinusoidal domain faces blood vessels, whereas the lateral and bile canalicular domains face the adjacent hepatocytes. The bile canalicular domain is separated from the lateral domain by tight junctions and expresses a different function than the lateral domain (Fig. 2). All the domains express different transporter proteins and are functionally very different, enabling transcellular vectorial transport from the blood to the bile. The sinusoidal membrane mediates an intense solute transfer with blood plasma, whereas the lateral membranes are specialized for cell attachments, cell-cell communication, and function as barriers

between the sinusoidal and the canalicular domains, while the canalicular membranes secrete bile to the canaliculi (Jansen, 2000). The bile canaliculi functions also as a pathway for the excretion of endogenous wastes and drug metabolites.

2.1.3 REGULATION OF HEPATOCYTE PHENOTYPE

Hepatocytes are subject to signals from each other, other cell types, blood and bile flow, and ECM. The biochemical and mechanical signals direct the polarity, expression patterns, and functions of hepatocytes (Fig. 3). The direct physical contact between the cells is known to maintain the hepatocyte polarity and the normal phase I metabolism (Hamilton et al., 2001). The soluble signals enable the communication between the non-parenchymal cells and the hepatocytes in the liver. For example, the non-parenchymal cells can mediate induction of the efflux transporters in the hepatocytes (Campion et al., 2008).

Blood flow generates mechanical effects to the hepatocytes, and its unidirectional drainage between portal and central veins results in local concentration gradients of oxygen, nutrients, and growth factors. These gradients regulate the spatial expression of genes that encode for drug metabolizing enzymes, thereby establishing zones with different metabolic activities (Fig. 3). Most drug metabolizing enzymes are preferentially expressed in perivenous hepatocytes with the lowest oxygen levels (Godoy et al., 2013).

Also, the liver´s ECM plays a pivotal role in determining the phenotype of hepatocytes even though it occupies only a small fraction of the total liver volume (Kleinman et al., 1986; Dutta and Dutta, 2009; Bowers et al., 2010). The composition of the ECM is zonally distributed in the liver and follows the oxygen gradient between the portal vein and the central vein (McClelland et al., 2008). Type I collagen, type III collagen, hyaluronan, and laminins are most prominent near the portal vessels, whereas type IV collagen, type VI collagen, and fibronectin are in the central vein zones (Martinez-Hernandez, 1984; Martinez-Hernandez and Amenta, 1993; Pinkse et al., 2004) (Fig. 3). The fibrous proteins, such as collagens, self-assemble and form nanofibrillar networks (Kleinman et al., 1982; Fernandes et al., 2009) that offer structural support to hepatocytes and guidance for cell attachment, growth, migration, and differentiation. In contrast, proteoglycans such as heparan sulphate, which is also located in the liver´s ECM, function as signaling receptors (Wells, 2007). In addition, the stiffness of the ECM has been reported to direct the differentiation and proliferation of hepatocytes (Wells, 2008; Lozoya et al., 2011). If the stiffness surpasses the typical levels in the liver (approximately 1–2 kPa) (Asbach et al., 2008; Chatelin et al., 2011), the hepatocytes start to proliferate and dedifferentiate (Semler et al., 2000; Fassett et al., 2006; Hansen et al., 2006), whereas on a soft surface, such as

collagen hydrogel and laminin-rich basement membrane hydrogel, the hepatocytes remain differentiated and do not proliferate.

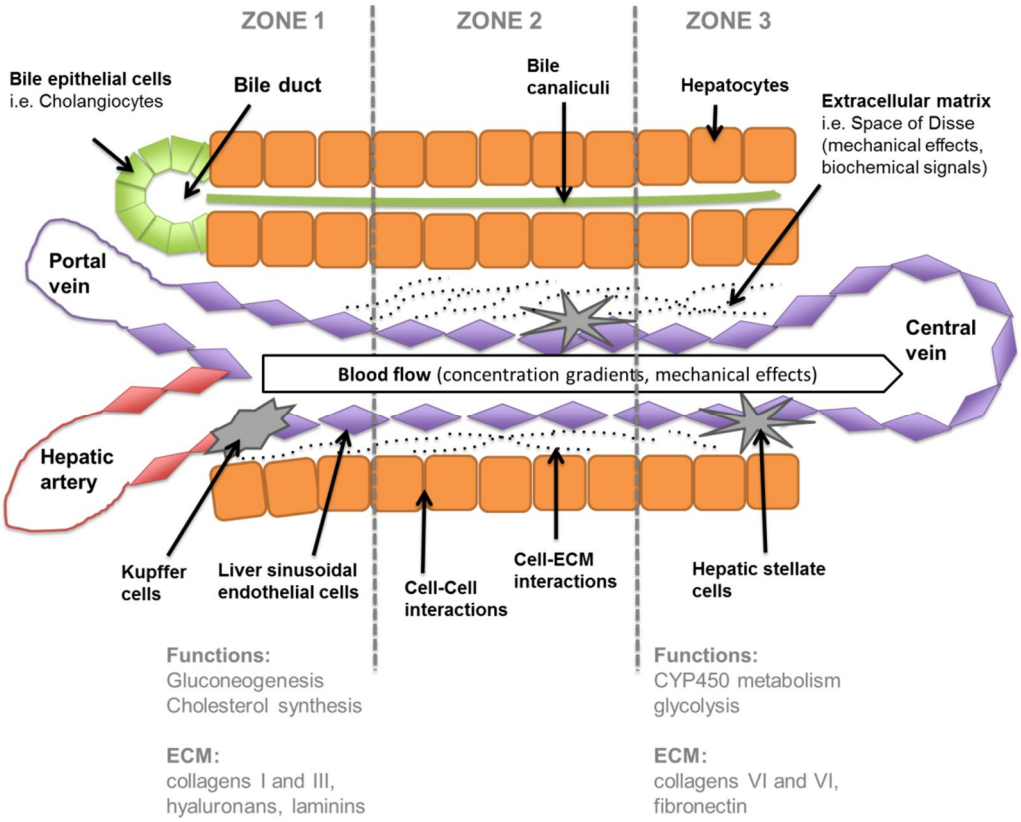

Figure 3 Diagram illustrating the microenvironmental factors that maintain the hepatic phenotype in the liver. The phenotype and functions of the hepatocytes are regulated by biochemical and mechanical signals from neighboring cells (e.g. sinusoidal endothelial cells, Kupffer cells, and cholangiocytes), ECM (space of disse), and blood flow. The local differences in these signals divide the functional unit of the liver into distinct zones. The hepatocytes close to the hepatic portal vein and the hepatic artery (zone 1) receive the blood richer in oxygen and nutrients than those located near the central vein (zone 3), hereby inducing functionally different hepatocytes into the zones. Hepatic stem cells and hepatoblasts locate in the zone 1, whereas metabolically active hepatocytes populate the zone 3. Also the non-parenchymal cells and ECM are different between the zones. The figure bases on the following publications (McClelland et al., 2008; Dash et al., 2012; Godoy et al., 2013).

2.2 CURRENT *IN VITRO* LIVER MODELS FOR DRUG DISCOVERY AND CHEMICAL TESTING

Laboratory animals are used as *in vivo* models to study drug metabolism and toxicity. Nevertheless, interspecies differences in drug metabolism and transport impair the clinical predictability of the animal experiments. The US

FDA and the European Medicines Agency (EMA) have expressed dissatisfaction with the predictability of animals and encourages the use of alternative, non-animal tests for pre-clinical safety studies (EMA, 1997; US FDA, 2004, 2006). These revelations have increased the interest and use of *in vitro* models for drug and chemical testing (Table 1). Another motivation for *in vitro* models is the growing ethical and economical pressure to reduce, refine, and replace the use of animals. In 2011, the EU established a reference laboratory for alternatives to animal testing (EURL ECVAM), with the aim of validating the alternative methods for drug, chemical, and cosmetics testing.

Table 1 *Current liver models for drug discovery and chemical testing: Advantages and Disadvantages.*

Model	Advantages	Disadvantages
Mice and rats	• *in vivo* • 3D cytoarchitecture • functional drug metabolizing enzymes, transporters and bile canaliculi	• ethical concerns • not HTS method ▪ not human drug metabolizing enzymes and transporters
Liver slices	▪ *in vivo* microenvironment and 3D cytoarchitecture ▪ functional drug metabolizing enzymes ▪ transporters and bile canaliculi	▪ short viability (24 h) ▪ limited nutrient and oxygen diffusion to inner cell layers ▪ human liver slices are rather difficult to get ▪ not HTS method
Primary hepatocytes	▪ both phase I and II drug metabolizing enzyme activities at physiological level ▪ suitable for HTS ▪ possible to screen for toxicity	▪ difficult to maintain ▪ not liver-like cellular architecture ▪ limited growth activity and life-span ▪ rapid loss of drug metabolizing enzymes and transporters ▪ large inter-individual variation ▪ limitedly available
Hepatic cell lines	▪ unlimited life-span ▪ reproducible ▪ stable phenotype ▪ some liver specific functions ▪ suitable for HTS ▪ easily available ▪ some cell lines suitable for long-term studies	▪ many liver-specific functions are lost or expressed at non-physiological levels ▪ not liver-like cellular architecture

Stem cell derived hepatic cells	▪ unlimited source of hepatocyte-like cells ▪ possible to establish cell panels reflecting the variation in population (with iPSCs)	▪ the quality of stem cells varies ▪ heterogeneous populations of liver-like cells ▪ low yield of differentiated cells ▪ low levels of liver-specific functions ▪ no liver-like cellular architecture
Liver microsomes	▪ part of the phase I and phase II drug metabolizing enzymes ▪ all CYP enzymes ▪ well characterized ▪ easily available ▪ suitable for HTS	▪ transporters are absent or not functional ▪ lack of nuclear receptors (prevents CYP induction studies) ▪ not suitable for toxicity studies ▪ interspecies differences with animal-derived microsomes
Recombinant drug metabolizing enzymes	▪ suitable for single enzyme studies HTS ▪ suitable for drugs eliminated primarily by CYPs	▪ not *in vivo* relevant ▪ no transporters ▪ no toxicity studies

3D, three-dimensional; CYP, cytochrome P450; HTS, high throughput screening; iPSC, induced pluripotent stem cell; UGT, uridine glucuronosyl transferase

2.2.1 LIVER SLICES

Human liver slices are a model for metabolic and toxicity studies with an intact *in vivo* cellular microenvironment (Table 1). The cellular architecture of the slices is similar with the intact tissue and the slices retain cell-cell and cell-ECM interactions (Thohan and Rosen, 2002). Liver slices with thicknesses of 100–250 µm are optimal, enabling good drug permeation into the inner layers of the slice (Olinga et al., 2001; de Graaf et al., 2006). Drug metabolism, transport, and hepatotoxicity can only be studied for less than 24 hours due to the loss of metabolizing activity in the slice (Elferink et al., 2011). The liver slice approach is also limited by the problems of slice availability and low throughput of the method.

2.2.2 PRIMARY HEPATOCYTES

Primary human hepatocytes are considered the gold standard of *in vitro* liver models in drug development (Thohan and Rosen, 2002; Wells, 2007). In the late 1960s, Berry and Friend (1969) harvested viable rat hepatocytes using collagenase perfusion. Thereafter, primary hepatocytes have been studied both in suspension and monolayer cultures. Human hepatocyte cultures became more common in the beginning of 1980s (Guguen-Guillouzo et al.,

1982; Guguen-Guillouzo and Guillouzo, 2010). Later developments in cryopreservation and commercial availability have contributed further to the adaptation of primary hepatocytes for routine use.

However, primary hepatocytes have well-known disadvantages, such as limited availability of the liver tissue for cell harvesting, high functional variability between hepatocytes from different donors, and difficulty maintaining differentiated phenotype and functions in cell cultures. Primary hepatocytes lose the liver-specific functions within a few days after their isolation. As a result, a range of methodological improvements has been tested to stabilize the hepatocyte phenotype in cultures. See the Section 2.3.

2.2.3 IMMORTAL LIVER CELL LINES

Hepatoma-derived cell lines, including HepG2, Hep3B, Huh7, and HepaRG, are potential alternatives to human hepatocytes in drug metabolism and hepatotoxicity studies (Kanebratt and Andersson, 2008). As cell lines, the hepatoma-derived cells may lack many features of the hepatocytes. However, they have also obvious advantages: good availability, unlimited growth, easy handling and absence of donor variability (Table 1). Moreover, the regulatory agencies accept the use of immortal liver cell lines for drug metabolism studies if normal induction of cytochrome P450 (CYP) enzymes can be demonstrated (EMA, 2012; US FDA, 2012).

The most frequently used liver cell line, HepG2, was derived from the liver tissue of a male patient with hepatocellular carcinoma (Aden et al., 1979). This cell line has been shown to be equal to the primary hepatocyte in assessing parent compound toxicity in the endpoint assays (Ekwall et al., 1998; Noor et al., 2009; Mandenius et al., 2011), but it is important to note that HepG2 cells could not metabolize the tested drugs (Noor et al., 2009; Gerets et al., 2012), showing that this particular cell line does not suite the study of metabolites, at least, when it is cultured conventionally on 2D plastic or glass surfaces. Fortunately, improvements in the culture environment have shown to induce both drug metabolizing enzymes and transporters in HepG2 cells (Westerink and Schoonen, 2007; Horiuchi et al., 2009; Nakamura et al., 2011; Oshikata et al., 2011).

A recent hepatoma cell line, HepaRG, was derived from a liver tumor of a female patient who suffered from the hepatitis C virus and hepatocarcinoma (Gripon et al., 2002). HepaRG is considered a superior cell line compared to the other immortal hepatic cell lines. It expresses the metabolizing enzymes at a level comparable to the primary human hepatocytes (Aninat et al., 2006; Lubberstedt et al., 2011). In addition, HepaRG cells have an intact nuclear receptor pathways (pregnane X receptor [PXR] and constitutive androstane receptor [CAR]), and the induction of CYP enzymes is comparable with the primary hepatocytes (Kanebratt and Andersson, 2008). This cell line also expresses several active drug transporters (Le Vee et al., 2006). The other advantageous properties of HepaRG are the stability of the phenotype, which

extends to several weeks in duration (Antherieu et al., 2010), and its suitability for toxicity studies (Le Vee et al., 2006; Josse et al., 2008; Kanebratt and Andersson, 2008; Antherieu et al., 2010; Lubberstedt et al., 2011). HepaRG is routinely cultured on standard 2D tissue culture plastics with media containing a corticosteroid and insulin.

HepaRG cells are commercially available (Life Technologies) as a fully differentiated form that is suitable for drug metabolism studies, whereas the original HepaRG hepatic progenitor cell line is a proprietary cell line that is provided only to a few research laboratories. The versatile HepaRG progenitor cell line expresses bipotent differentiation. At low density, the HepaRG resembles liver progenitors, and in the confluent state, the culture is composed of hepatocyte-like and cholangiocytes-like cells (Aninat et al., 2006; Cerec et al., 2007). Furthermore, the differentiated cells show reverse differentiation to the progenitor phenotype while seeded at low density. The xenobiotic metabolism of the HepaRG progenitor is initially low, but is induced after several weeks of treatment with dimethyl sulfoxide (DMSO) (Aninat et al., 2006).

2.2.4 CELL-FREE METHODS

Liver microsomes are widely used in drug metabolism studies, especially in CYP metabolism studies (Table 1). Microsomes are prepared from liver homogenate and consist of the membranes of endoplasmic reticulum. Thus, they contain phase I and II metabolizing enzymes that are bound to the membranes (CYPs, uridine glucuronide transferases [UGTs], and flavin-containing monooxygenases [FMOs]). Therefore, the “non-membrane bound” phase II metabolism is missing (Björnsson et al., 2003). In addition, microsomes host incomplete transport (uptake and efflux), and the existing enzymes (CYP and UGT enzymes) are not active as such, but require “activation” or supplementation of co-substrates and/or co-enzymes (Jia and Liu, 2007). Microsomes are particularly favorable in industrial drug discovery, because they are amenable to high throughput studies (HTS); in addition, they are economical and convenient to use. The results from microsomes are used to predict intrinsic hepatic drug clearance in humans (Jia and Liu, 2007).

Recombinant drug metabolizing enzymes (e.g., CYPs and UGTs) have been exploited as an alternative to microsomes in drug metabolism studies (Table 1), but the data derived from recombinant enzymes is generally not clinically relevant (Stringer et al., 2009). So-called S9 subcellular fraction is also used in the metabolism studies together with microsomes. This liver fraction consists of the cytosols and microsomes, thus containing phase I enzymes (CYPs and FMOs) and phase II enzymes (glutathione S-transferases [GSTs], sulfotransferases [SULTs], N-acetyltransferases [NATs], and UGTs). S9 fraction, like microsomes, requires additional cofactors to maintain metabolic activities. It is worth noting that utilization of the human liver S9

fraction is diminished by the interdonor differences in drug metabolizing enzyme activities.

Drug transport (influx and efflux) can be investigated with cell membrane vesicles. The membrane vesicles are isolated from the cells that overexpress a transporter, and are, therefore, useful in determining when a transporter transports a particular substrate. Inside-out-oriented membrane vesicles are used to study efflux transport directly, and allow for determining transport kinetic parameters, such as the inhibition constant. Inside-out vesicles are actually recommended *in vitro* models to study the function of efflux transporters (Giacomini et al., 2010; Hillgren et al., 2013). However, the membrane vesicles are not complete liver models, and do not allow *in vitro* to *in vivo* extrapolation, because the role of a transporter in the presence of other competing transporters remains unclear.

2.2.5 HEPATOCYTE-LIKE CELLS DERIVED FROM PLURIPOTENT CELLS

During the last few years, pluripotent stem cells have been actively investigated as a possible cell sources for drug testing due to their high self-renewing capability and potential to differentiate into a wide variety of somatic cell types, including hepatocytes (Snykers et al., 2009; Guguen-Guillouzo and Guillouzo, 2010). Recently, several research groups have demonstrated that embryonic stem cells or induced pluripotent stem cells (iPSCs) can be differentiated in mature hepatocyte-like cells that express liver-specific functions, including drug metabolism and drug transport (Duan et al., 2007; Si-Tayeb et al., 2010; Chen et al., 2012; Hannan et al., 2013; Shan et al., 2013), but quantitative and systematic evidence of success is still missing.

The inefficient differentiation protocols, the low yield of metabolically competent hepatocyte-like cells, and high costs are still major challenges and prevent the use of stem cell-derived hepatocytes as alternatives to primary hepatocytes. Future experiments will determine the role of pluripotent cells as platforms for drug discovery.

2.3 METHODS TO IMPROVE LIVER CELL CULTURES

The dissatisfaction with current liver cell models has promoted the development of numerous new cell culture techniques (Fig. 4). The new techniques are designed to retain hepatocyte phenotype and functions by re-establishing physiological microenvironment stimuli (Fig. 3). The following paragraphs introduce the most widely adopted technologies.

2.3.1 CULTURE MEDIUM MODIFICATIONS

The simplest approach to improve liver cell cultures is to modify the culture medium. In the 1970s, Williams et al. developed a culture medium to support the growth in long-term cultures of adult liver epithelial cells (Williams and Gunn, 1974; Laishes and Williams, 1976). Later, the culture media was supplemented with cytokines and other chemicals. Improved stability was achieved using additives, such as DMSO and insulin or glucocorticoid supplements (Isom et al., 1985; Dich et al., 1988). To optimize culture conditions further, the effect of medium components to the drug metabolizing enzymes and hepatobiliary transporters have been investigated in more detail (Dich et al., 1988; Turncliff et al., 2006).

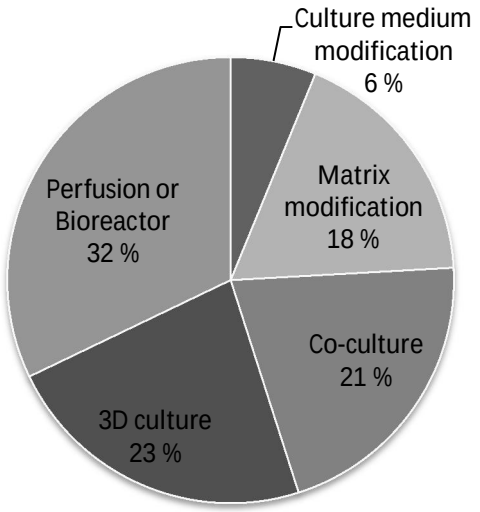

Figure 4 The cell culture techniques for liver cells to retain hepatocyte phenotype and functions. The chart was drawn upon the number of references available for the search terms “liver cell culture medium modification”, “liver cell culture scaffold OR liver cell culture sandwich OR liver cell culture hydrogel OR liver cell culture printed surface OR liver cell culture micropatterned surface”, “liver cell co-culture”, “3D liver cell culture OR three-dimensional liver cell culture OR spheroid liver cell culture OR hanging drop liver cell culture”, and “liver cell culture perfusion OR liver cell culture bioreactor” in the Scopus database (24.10.2013).

2.3.2 CULTURE MATRIX MODIFICATIONS

Another relatively simple approach to maintain hepatocyte functions is to modify the culturing matrix. The matrix manipulation offers the cues missing in tissue culture plastic and may allow anchorage of cells, thus guiding the functions of the intracellular biochemical pathways. Culturing on a layer of type I collagen gel was invented in the 1970s (Michalopoulos and Pitot, 1975), and is still widely used for primary hepatocyte culture. A collagen gel has proven to support metabolism and transport activity of primary hepatocytes for 72 hours (Rippin et al., 2001; Ulvestad et al., 2011).

The cell growth environment has been further improved by culturing the liver cells between two collagen gel layers. The sandwich configuration supports the viability, polarity, and hepatocyte functions better than a layer of collagen gel (Dunn et al., 1991). The sandwich culture has resulted in extended CYP activity, *in vivo*- like distribution of actin filaments (Dunn et al., 1991), formation of canalicular network (LeCluyse et al., 1994), and recurrence of both basolateral and hepatobiliary transporter activity (Turncliff et al., 2006; Swift et al., 2010). The sandwich approach has enabled prediction of *in vivo* biliary clearance of some drugs (Liu et al., 1999). In addition, Schaefer et al. showed that protein expression levels of influx and efflux transporters in a human primary hepatocyte sandwich were comparable to human liver tissue (Schaefer et al., 2012).

Also, other matrix modifications have been tested for the hepatocytes. See Section 2.4.

2.3.3 CO-CULTURING WITH NON-PARENCHYMAL CELLS

Co-culturing of hepatocytes with non-parenchymal cells has been performed for 30 years (Guguen-Guillouzo et al., 1983; Mesnil et al., 1987). It is known that co-cultures of different cell types enhance the phenotype and functions of hepatocytes (Guguen-Guillouzo et al., 1983; Harimoto et al., 2002; Takayama et al., 2007; Khetani and Bhatia, 2008). Thus, hepatocyte phenotypic functions can be maintained for several weeks. The effect is explained by direct heterotypic cell-cell contacts, and partially due to the paracrine influences of cytokines secreted by the surrounding cells (Krause et al., 2009). Co-culture with fibroblasts, hepatic stellate cells, Kupffer cells, or liver sinusoidal endothelial cells have improved at least some of the hepatic functions (Riccalton-Banks et al., 2003; Zinchenko et al., 2006; Khetani and Bhatia, 2008; Krause et al., 2009).

Another motivation for co-culturing is that hepatotoxicity mechanisms may require collaboration between different cell types of the liver. Thus, to predict liver toxicity from *in vitro* data, hepatocytes have been co-cultured with immune system cells, such as Kupffer cells. This kind of co-culture seems promising since it enhances cytotoxicity to the hepatotoxic chemicals (Zinchenko et al., 2006; Edling et al., 2009).

There are several experimental approaches to perform co-culturing. This includes direct co-culturing on standard tissue culture plastic (West et al., 1985), co-culturing with biomaterials, where hepatocytes and non-parenchymal cells are separated typically by a collagen layer (Bader et al., 1996), or culturing in membrane inserts, where different cell types are separated by a membrane, but soluble mediators can migrate between the compartments (Kang et al., 2013). Additionally, co-culturing has been performed on biodegradable surfaces (Riccalton-Banks et al., 2003), with cell sheet technology where confluent cell monolayers of hepatocytes and non-parenchymal cells are stacked on top of each other (Ohashi et al., 2007), and

with 3D techniques where hepatocytes are co-cultured with non-parenchymal cells using a hanging drop technique to form heterotypic spheroids (Messner et al., 2013).

2.3.4 THREE-DIMENSIONAL CULTURE TECHNIQUES

While hepatocytes have a 3D polygonal shape in the body, they present flattened morphology on the standard 2D cultures, where half of the cell surface is exposed to the growth media fluid and the other half to the stiff surface. The flattened cell morphology limits cell-cell interactions, and consequently leads to reduced polarization, loss of important signaling pathways, and, thus, reduced liver functions. Therefore, researchers have been interested in retaining the physiological cell-cell and cell-ECM contacts by various 3D cell-culture techniques.

Cellular spheroids are simple 3D systems, which take advantage of the natural tendency of many cell types to aggregate. Spherical multicellular aggregates, spheroids, are formed when the cells self-assemble and re-establish the cell-cell contacts in three dimensions. Formation of 3D spheroids was first reported by using non-adherent plastic (Landry et al., 1985). Since then, spheroids have been generated by different methods, such as culturing on non-adherent materials (Timmins et al., 2005), agitation/ rotating vessels (Li et al., 1992; Surapaneni et al., 1997), hanging drops (Timmins et al., 2005; Müeller et al., 2011), and gel entrapment (Semino et al., 2003). All these techniques regulate the extracellular environment of the cell culture.

Many spheroid cultures of liver cells show prolonged survival (Landry et al., 1985; Tostoes et al., 2012), *in vivo*-like morphological characteristics, such as polarity (Müeller et al., 2011; Oshikata et al., 2011; Tostoes et al., 2012), enhanced secretion of albumin and urea, and formation of bile acids (Walker and Woodrooffe, 2001; Yang et al., 2002). The beneficial effects of spheroid culture are believed to result from the retention of the 3D cytoarchitecture, an increased number of cell-cell contacts and the possible presence of ECM components (Landry et al., 1985; Luebke-Wheeler et al., 2009; Sakai et al., 2010).

Although the spheroid technique has been applied for years, its use in drug and chemical testing is in its early stages, and only a few research groups have aimed to develop liver spheroids for drug toxicity, efficacy, or metabolism testing (Müeller et al., 2011; Fey and Wrzesinski, 2012). The other challenge needing further evaluation is the use of human liver cells because researchers are mainly using animal cells to generate spheroids.

Various matrices, scaffolds, bioreactors, and microfluidic platforms can support 3D cell growth and improve liver-specific behavior and morphology. More on these systems is discussed in the following paragraphs.

2.3.5 BIOREACTOR AND PERFUSION TECHNIQUES

Generally, liver cell cultures are static. To mimic blood and fluid circulation in the liver tissue, methods where cell culture fluids are in motion have been developed. The well-known example of perfused liver models is the liver slices. Recently, 3D cultures have been combined with perfusion or bioreactor systems with the aim of overcoming possible nutrient transport limitations. Cells can be cultured either as aggregates, such as spheroids in stirred bioreactors, or assembled on the surfaces of a compartmental bioreactor (Mandenius et al., 2011). To further improve the culturing environment, cells can be encapsulated in a hydrogel or microcarrier before populating in a stirred bioreactor; alternatively, the bioreactor can be surfaced with microstructures (Miranda et al., 2010).

Bioreactors have been shown to support 3D architecture of the cells and to improve hepatocyte-specific functions of liver cells (Miranda et al., 2009; Darnell et al., 2011). The reasons for improved phenotype could be that fluid flow mimics the physiological gradients, allows control of oxygen, and assists the nutrient and waste product exchange. Additionally, dynamic cultures generally improve the formation of cell-cell contacts. However, the shear stress alone cannot maintain the phenotype of hepatocytes, but it can affect the cells too strongly, above the levels of physiological hydrodynamics (Roy et al., 2001). The hepatocytes, which are in direct contact with the fluid motion, are gradually damaged by shear stress, suffer from a short lifespan, and are thereby only appropriate for short-term studies (Kim et al., 1998; Cervenkova et al., 2001).

When Park et al. (2008) compared the effect of shear flow on the hepatocytes in the scaffolds of different pore sizes, they noticed that the cells, which were better protected from the shear stress, expressed higher viability than those exposed to a higher shear force. The studies where hepatocytes are protected from direct contact, for example gel entrapment, have given promising results (Tostoes et al., 2012; Dash et al., 2013). Tostoes et al. (2012) showed that a bioreactor can maintain CYP enzyme expression and biotransformation of xenobiotics for a longer duration than the corresponding plated 2D cells.

2.4 BIOMATERIALS AS A PART OF THE LIVER CELL CULTURE

As early as the 1950s it was realized that traditional culturing on the rigid polystyrene or glass surface might not serve its purpose (Leighton, 1951). The importance of the cell culture matrix for the formation of hepatocyte polarity and directional functions has been known since the 1980s (Chambard et al., 1981; Chen et al., 1985; Dich et al., 1988). Thereafter, several articles have

testified that ECM resembling biomaterials are suitable for liver cell culturing (Fig. 5).

Generally, biomaterials are used as surrogates of the missing physiological ECM. The biomaterial must be biocompatible, provide support, and allow for cell growth. At its best, the biomaterial represents the geometry, chemistry, and signaling environment of a natural ECM. Furthermore, an optimal biomaterial would support cell adhesion, proliferation, differentiation, and ECM production. Since hepatocytes are known to be anchorage dependent (Underhill et al., 2007b), adhesion ligands on the biomaterial might facilitate the cell culture.

A completely different approach to support a liver cell culture with biomaterials is to use non-adherent materials to minimize the cell-ECM contacts and hereby support the cell-cell contacts (Landry et al., 1985; Timmins et al., 2005). These kinds of biomaterials do not particularly mimic the physiological ECM, but must be biocompatible and allow for cell growth.

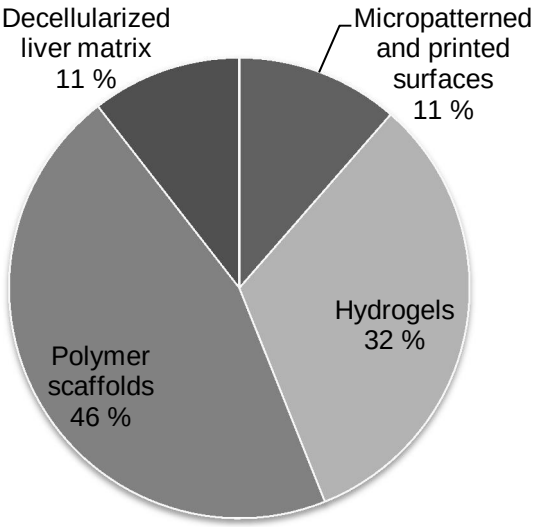

Figure 5 Biomaterials are widely used for liver cell culturing. Synthetic polymer scaffolds are the most typical biomaterials, whereas acellular liver matrix, which is obtained by decellularization of the liver tissue, is not so common, but has attracted interest lately. The chart was drawn upon the number of references available for the search terms “liver cell culture scaffold, “liver cell culture hydrogel”, “liver cell culture printed surface OR liver cell culture micropatterned surface”, and “liver cell culture acellular matrix OR liver cell culture decellularized matrix” in the PubMed, Web of Science, and Scopus databases, and Google scholar (14.4.2014).

2.4.1 PREFABRICATED POLYMER SCAFFOLDS

Biomaterial scaffolds have been produced as synthetic ECM to enable the 3D organization of liver cells. Scaffolds are designed to be porous and their mechanical properties should be viscoelastic like natural tissue. In practice,

the cells are seeded in prefabricated scaffolds where they grow on the surface of the pores and may form 3D cell assemblies. Especially, biodegradable scaffolds have been widely investigated since they may be further suitable for tissue engineering purposes (Griffith et al., 1997).

The most successful synthetic polymers include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA). The PLA, PGA, and their derivatives have supported 3D hepatocyte growth for up to two weeks (Hasirci et al., 2001). More recently, a cross-linked polystyrene scaffold, Alvetex™ (Reinnervate), has been shown to support hepatocyte morphology and upregulation of CYP enzymes better than the conventional 2D polystyrene plasticware (Schutte et al., 2011; Burkard et al., 2012).

The scaffold based on the natural alginate polymer, AlgiMatrix (Invitrogen), has shown to increase the aggregation of primary hepatocytes (Glicklis et al., 2000), to maintain albumin synthesis of primary hepatocytes (Rowley et al., 1999; Glicklis et al., 2000), and to increase CYP and UGT enzyme activity of C3A hepatocyte cells (Elkayam et al., 2006).

A disadvantage of prefabricated polymer scaffolds is that they have micrometer-based openings that generally induce flat 2D geometries of the cultured cells and limit the number of cell-cell contacts. The other drawback of synthetic scaffolds is that they are rather rigid, and do not allow the cells to remodel and adapt their microenvironment as they do in the *in vivo*. Prefabricated scaffolds may have more potential for tissue engineering applications than for the *in vitro* cell models. In fact, the scaffolds have proven to be ideal in tissue engineering of bone and cartilage.

2.4.2 MICROPATTERNED AND PRINTED SURFACES

The most recent approach to apply biomaterials in hepatocyte culturing is to use lithography or printing technologies to generate patterns and surfaces that mimic the geometry of natural ECM. Microscale surfaces resemble natural dimensions and offer control over cell-ECM and cell-cell interactions with micrometer precision. Biomolecules, such as collagen I, have been patterned on glass or polystyrene substrates (Khetani and Bhatia, 2008; Ukairo et al., 2013), generating patterns that are perfectly suited for co-culturing because of the control on the proximity of one cell type to another (Zinchenko et al., 2006; Wang et al., 2010; Ukairo et al., 2013).

The other main advantage of micropatterned and printed surfaces is that they can be produced and utilized in the HTS format. Microscale cultures would be especially useful for drug discovery because both the amount of the drug candidate and the cells are limited, and screening is highly appreciated. Recently, Hepregen Corporation started to market patterned hepatocyte cultures (HepatoPac™) for metabolism and toxicity studies (Chan et al., 2013; Ukairo et al., 2013). It has been reported that these micropatterned hepatocyte fibroblast co-cultures maintain liver-specific functions, including phase I and phase II metabolism (CYP2A6, CYP2B6, CYP3A4, some UGTs

and SULTs) and active efflux transport of 5-(and 6)-carboxy-2´,7´-dichlorofluorescein diacetate for several weeks (Khetani and Bhatia, 2008; Ukairo et al., 2013).

The disadvantage of these cultures is that they represent more of a 2D than a 3D environment, and thus do not support the natural 3D organization of hepatocytes. In addition, the localization and expression of drug transporters in this system is not known, or at least, this has not been published.

2.4.3 DECELLULARIZED LIVER MATRICES (BIOMATRICES)

Hepatocytes have been successfully cultured on decellularized liver-derived ECM since the 1980s (Rojkind et al., 1980; Enat et al., 1984). However, the interest to use a fully physiological biomatrix as cell culture platform has arisen again.

A liver biomatrix is prepared by perfusion of the tissue with enzymes and detergents to remove the cellular compartments, while preserving the structural features and biochemical components of the matrix (at least collagen type I and IV, fibronectin, and laminin β1) (Uygun et al., 2010). Generally, hepatocytes are introduced to a decellularized whole organ construct. These kinds of whole organ constructs have shown to maintain albumin and urea synthesis of primary hepatocytes up to five days, but have not been able to maintain the expression of phase I and II metabolizing enzymes (Uygun et al., 2010).

Lately, biomatrices have been processed further to produce biomatrix hydrogel. Lyophilization, grinding, and dissolution of the decellularized liver matrix have shown to produce a biomatrix hydrogel suitable for 2D and 3D cell cultures (Sellaro et al., 2010; Lee et al., 2014). The hydrogel maintained the cellular viability, albumin secretion, and urea synthesis of primary hepatocytes (Lee et al., 2014). However, the effect of the biomatrix hydrogel on drug metabolism or transport functions remains unknown.

The evident advantage of biomatrices is the preservation of liver-specific ECM and 3D architecture, providing essential signals for long-term hepatocyte function. However, biomatrices are highly variable because of the donor-donor variability.

2.4.4 BIOMATERIAL HYDROGELS

Hydrogels have demonstrated great potential for cell culture and tissue engineering applications (Drury and Mooney, 2003; Peppas et al., 2006; Kopecek, 2007). They are a promising class of biomaterials consisting of cross-linked hydrophilic polymers. The cross-linked structure prevents dissolution in water, but allows water penetration between the polymer chains, causing swelling and formation of the hydrogel. The porous structure with high water content allows efficient transport of oxygen, nutrients, and

drugs, and facilitates removal of waste molecules (Drury and Mooney, 2003), making hydrogels appealing for cell culturing applications. The highly swollen and soft nature of hydrogels resembles the tissue, where cells are capable of remodeling and adapting to their microenvironment according to their developmental needs.

Furthermore, polymer networks resemble the physiological ECM networks of collagen, elastin, and proteoglycans, and they can be self-assembled from smaller counterparts (bottom-up) like physiological ECM (Zhang et al., 1995). In addition, mechanical, responsive and diffusive properties of hydrogels can be tailored and the optimal composition and crosslinking selected for each cell type (Table 2). The possibility of growing the cells within or on top of the gel further widens their usefulness.

2.4.4.1 Mammalian natural hydrogels

Due to their biocompatibility, several natural hydrogels have been used in liver cell cultures. Many natural hydrogels have been extracted from animals or cultured cells (Table 2). Collagen is the main protein of mammalian ECM. Collagen fibrils are composed of triple helixes of proteins that are capable of forming hydrogels in *in vivo* and *in vitro* environments (Fig. 6). Based on the structure and the genes used for their assembly, the collagens are divided into subtypes (e.g., I, III, IV, and VI types). Collagens are perhaps the most commonly used hydrogels in the liver cell cultures (see Subsection 2.3.2). Collagen solutions are cross-linked either chemically or physically, leading to the formation of fibrillar networks resembling the physiological counterparts (Table 2).

The commercially available laminin-, collagen IV-, and entactin-rich mammalian ECM hydrogel, Matrigel™, is cross-linked and forms networks due to the interaction of ECM components with each other. Interestingly, the structure of the network depends on the underlying material. On polystyrene, Matrigel forms globular networks, whereas on hydrophilic surfaces (glass), the networks are fibrillar (Kohen et al., 2009). The variability is increased by the fact that Matrigel contains over 1,000 different proteins (Hansen et al., 2009). Matrigel has been shown to maintain the differentiated liver gene expression (Schuetz et al., 1988) and albumin secretion (Moghe et al., 1996), and to increase CYP2C9, CYP2D6, and CYP3A4 metabolism and sensitivity to hepatotoxic compounds (Ramaiahgari et al., 2014), but it has also generated a non-physiological cell morphology (Moghe et al., 1996).

The advantage of mammalian-derived hydrogels, such as collagen, hyaluronan (HA) and fibrin, is that they contain the cell-signaling domains that are present in *in vivo* ECM, and hence, may enable *in vivo*-like behavior of hepatocytes (Fig. 6). However, mammalian-derived materials have poor mechanical properties and are subject to enzymatic degradation in human cell culture environments (Lee et al., 1995). This is advantageous in most tissue engineering applications, but it makes their use in routine cell

culturing difficult. In addition, the mammalian hydrogels suffer inherent variation of undefined components, creating significant batch-to-batch variability (Prestwich, 2007; Serban and Prestwich, 2007). This may hamper the reproducibility of the cell cultures. However, some ECM components, such as laminins and fibronectin, are available in pure format, produced by recombinant technique.

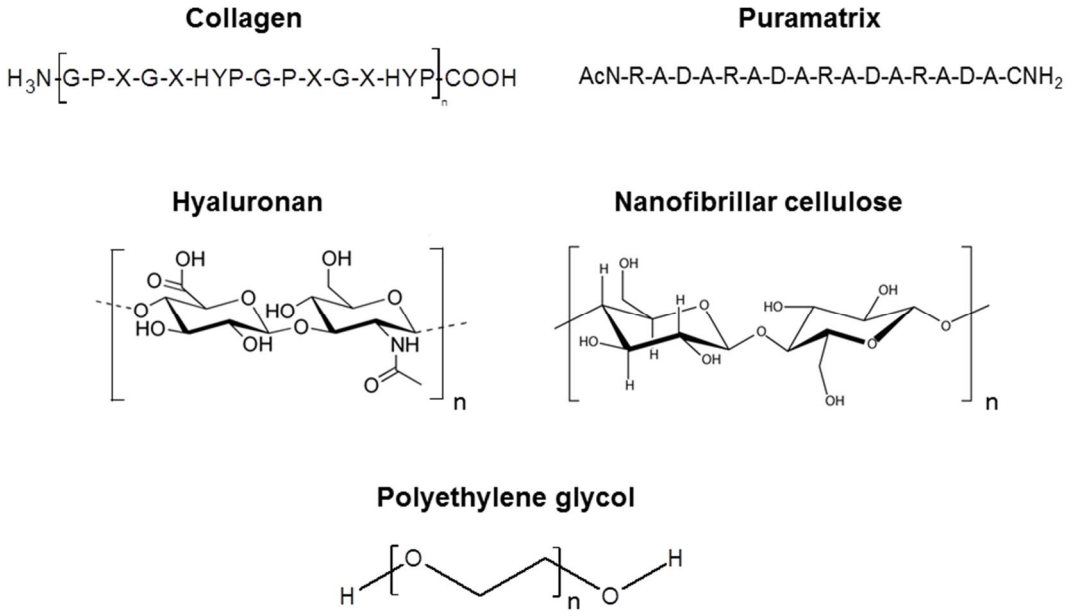

Figure 6 Chemical structures of the central hydrogel forming polymers. Collagen and Puramatrix™ consist of amino acids sequences that self-assemble to fibers, whereas hyaluronan and nanofibrillar cellulose (NFC) are polymers composed of sugar units, and polyethylene glycol (PEG) is a polymer of ethylene oxide.

2.4.4.2 Non-mammalian natural hydrogels

Alginate and chitosan are frequently used hydrogels for hepatocyte culturing (Underhill et al., 2007b) (Table 2). They are non-mammalian natural polymers that can be chemical or physically cross-linked (Table 2). Alginate is derived from marine algae sources, and chitosan from shellfish shells. Alginate has been extensively studied for hepatocyte encapsulation in both static and dynamic environments, because it is known to degrade slowly (Lee et al., 1995).

Recently, interest has grown in applying NFC in tissue engineering. The NFC consists of anhydrase glucose units building linear chains (Klemm et al., 1998) (Fig. 6), that finally form a nanofibrillar network mimicking the architecture of natural fibrils in the ECM (Klemm et al., 2011). However, NFC does not offer biochemical signals that are found in the human ECM. This

disadvantage is, however, linked to all natural non-mammalian hydrogels, and also to the widely applied alginate and chitosan.

NFC is produced by both top-down and bottom-up processes. The plant NFC is obtained when plant cellulose (top-down) is disintegrated using high shear forces, whereas bacterial NFC (bottom-up) is obtained by biosynthesis of cellulose by bacteria (Siro and Plackett, 2010). The bacterial NFC has already proven to be suitable for the tissue engineering of hard tissues such as bone and cartilage (Andersson et al., 2010; Klemm et al., 2011), but recently, interest has arisen in applying plant-derived NFC in tissue engineering. The main reasons for the growing interest in plant cellulose are the need for defined materials in tissue engineering, and at the same time the need for new applications for cellulose, as the traditional use of cellulose in the paper industry wanes.

Plant NFC differs considerably from bacterial cellulose scaffolds by its mechanical properties. The significantly lower Young's moduli of plant NFC from that of bacterial nanocellulose (15 Gpa versus 79–134 Gpa) is the result of a lower degree of polymerization, reduced crystallinity, and a lack of alignment (Klemm et al., 2006; Tanpichai et al., 2012). Thus, the plant NFC could suit the engineering of soft tissues, such as the liver, rather than the hard tissues. Moreover, the stiffness of the plant NFC hydrogel can be tuned simply by adjusting the concentration. The storage moduli (G´), which express the elastic character of the gel, has shown to range from 10^5 to 10^2 Pa with the concentrations of 0.13 and 6 % (Pääkkö et al., 2007).

Thus far, plant NFC has been applied as a reinforcing agent in diverse composites aiming for tissue engineering applications (Borges et al., 2011; Cherian et al., 2011; Eyholzer et al., 2011; Mathew et al., 2012a; Mathew et al., 2012b). However, to the best of our best knowledge, plant-derived NFC has not been studied as a cell-supportive matrix as such. To clarify, the wood-derived NFC was applied in this thesis to determine its suitability in cell cultures (I, III).

2.4.4.3 Synthetic hydrogels

Synthetic hydrogels represent attractive alternatives to naturally derived substrates, since they have a more defined structure, properties, and improved reproducibility. Poly(ethylene glycol) (PEG) and its derivatives are among the most widely studied hydrogels for biomedical applications (Table 2). Native PEG is a passive cell culturing material (Peppas et al., 2006; Zhu, 2010), but various modifications of PEG have been done to improve its cell adhesion properties (Hern and Hubbell, 1998). The pore size of PEG hydrogels is generally smaller than that of biological fibrin and collagen networks that can interfere with cell proliferation and migration (Raeber et al., 2005). Polyhydroxyethyl methacrylate (pHEMA), poly(vinylalcohol) (PVA), and their derivatives are also widely studied synthetic hydrogels in

biomedicine (Landry et al., 1985; Stol et al., 1993; Chirila, 2001; Schmedlen et al., 2002).

Because synthetic ECM analogs often fail to recapitulate essential biological features such as biological recognition (Goldberg et al., 2007), protein- and peptide-based synthetic polymeric materials have been developed. These ECM analogs can offer cell recognition sites originating from their biological structure even though it is chemically designed (Table 2). For example, the repeating sequence (RADA) of a commercially available Puramatrix™ hydrogel (BD Biosciences) mimics the well-known cell adherence motif of fibronectin (RGD). In addition, the fiber size of Puramatrix resembles the size of natural ECM fibrils (Zhang et al., 1995). The Puramatrix nanofibrillar network self-assembles when salts are introduced (e.g., culture media) in the bottom-up way, thereby mirroring the natural ECM (Fig. 6). Puramatrix has also shown to maintain differentiated functions of porcine hepatocytes (Navarro-Alvarez et al., 2006) and rat hepatocytes (Wang et al., 2008). In addition, it has promoted differentiation of rat liver progenitors into hepatocyte-like cells, shown by upregulated CYP activity and albumin secretion (Semino et al., 2003). This thesis evaluated the suitability of Puramatrix hydrogel for human liver cells (I, II).

2.4.4.4 Composite hydrogels

To overcome the drawbacks of natural and synthetic materials, semi-synthetic hydrogels have been developed that encompass the positive features of both natural and synthetic hydrogels. For example, Hystem™ (former name Extracel™ hydrogel) (Glycosan Biosystems) is composed of chemically modified gelatin (carboxymethyl gelatin-thiopropanoyl hydrazide) and chemically modified hyaluronan (carboxymethyl hyaluronan-thiopropanoyl hydrazide), which are cross-linked with polyethylene glycol diacrylate (PEGDA) (Prestwick 2007, 2008, Shu 2004, 2006). The suitability with liver cells has been demonstrated by extended CYP enzyme activity of hepatocytes (up two weeks) (Prestwich et al., 2007). To further clarify its beneficial properties on liver cells, the gelatin-hyaluronan hydrogel (Extracel™, Hystem™) was used in this thesis (I, III).

Table 2 *Properties of biomaterial hydrogels.*

Biomaterial type	Material	Biochemical cue	Physical cue	Composition	Gelification mechanism	Character	Reference
Self-assembling synthetic polymers	Peptides	Y	Y	defined	divalent cations	self-assembled nanofibrillar network offering cell attachment motif, degradable in humans	(Semino et al., 2003; Genove et al., 2009)
	Peptide amphiphiles	Y	Y	defined	pH or temperature change, divalent cations	self-assembled nanofibrillar network, degradable in humans	(Hartgerink et al., 2002; Luo and Tong, 2011)
Synthetic polymers	Poly(ethylene oxide), Poly(ethylene glycol) and derivatives	N	Y	defined	photoinitiator and UV exposure	network of macromolecule chains, not fibrillar, non-degradable in humans, FDA approved biomaterial	(Raeber et al., 2005; Liu Tsang et al., 2007; Underhill et al., 2007a)
	Poly(vinyl alcohol) and derivatives	N	Y	defined	repeated freeze-thawing, chemical crosslinking with aldehydes, γ-irradiation	rigid scaffold, high mechanic strength, non-degradable in humans, FDA approved biomaterial	(Lu et al., 2011; Fejerskov et al., 2012)
Natural polymers	Alginate	N	Y	defined	divalent cations or chemical crosslinking	polysaccharide, relatively rigid scaffold, the gel structure depends on the	(Glicklis et al., 2000; Lan et al., 2010;

						cross-linking agent, non-degradable in humans, FDA approved biomaterial	Miranda et al., 2010; Bierwolf et al., 2012)
	Chitosan	N	Y	defined	pH or temperature change, UV irradiation, or chemical crosslinking	polysaccharide, degradable in humans (enzymes mainly lysozyme)	(Verma et al., 2007; Feng et al., 2009; Kim and Rajagopalan, 2010)
	Nanofibrillar cellulose	N	Y	defined	colloidal dispersion, no gelification	polysaccharide, nanofibrillar entangled networks, non-degradable in humans	(Klemm et al., 2011)
	Silk fibroin	Y	Y	defined	pH or temperature change, divalent cations	protein, fibrous, high mechanic strength, non-degradable in humans (or very slowly by enzymes), FDA approved biomaterial	(Vepari and Kaplan, 2007; She et al., 2008; Yang et al., 2012)
	Fibrin	Y	Y	defined	self-assembles when fibrinogen reacts with thrombin	protein, fibrillar network, low mechanical strength, degradable in humans, contain arginine-glycine-aspartic acid (RGD) sites	(Hokugo et al., 2006; Ahearne and Kelly, 2013)
	Hyaluronan (Hyaluronic acid)	Y	Y	defined	chemical crosslinking	ribbon-like entangled network, polysaccharide, hydrophilic, polyanionic surfaces, degradable in humans, FDA approved	(Lozoya et al., 2011; Prestwich, 2011)

						biomaterial	
	Laminin-111	Y	Y	defined	temperature change	web-like fibrillar network, generally combined with other hydrogel component, degradable in humans	(Bissell et al., 1987; Yurchenco et al., 1992; Pinkse et al., 2004)
	Fibronectin	Y	N	defined	chemical or physical crosslinking	not fibrous, generally combined with other hydrogel component, degradable in humans	(Pinkse et al., 2004)
	Gelatin (soluble derivative of type I collagen)	Y	Y	nondefined	chemical or physical crosslinking or mixing with other polymer	not fibrous structure, partially hydrolysed collagen product, degradable in humans	(Yang et al., 2012)
	Type I and IV collagens	Y	Y	defined	chemical or physical crosslinking or mixing with other polymer	fibrillar network, degradable in humans	(Pinkse et al., 2004)
Extracts from animals/cells	Animal ECM components	Y	Y	poorly defined	temperature change	globular or fibrillar network depending on the surface substrate, may contain also growth factors, degradable in humans	(Kleinman and Martin, 2005; Lee et al., 2014; Ramaiahgari et al., 2014)
	Human ECM components	Y	Y	poorly defined	temperature change	degradable in humans	(Maas-Szabowski et al., 2005)

3 AIMS OF THE STUDY

The general aim of this thesis was to develop new organotypic *in vitro* liver cell culture platforms. The study aimed to clarify the potential use of biomaterial hydrogels in the development of 3D liver cell cultures. Especially, the effects of a 3D hydrogel culture on cytoarchitecture, drug metabolism and excretion, and cellular differentiation were examined. The specific aims of the study were:

1) To study the suitability of wood-derived NFC in a 3D cell culture (I).

2) To construct 3D liver cell cultures with HepG2 and HepaRG cell lines and biomaterial hydrogels of peptide nanofibers, cellulose nanofibers, hyaluronan-gelatin (HG) composite or natural ECM extract (I-III).

3) To establish analytical methods for evaluation of 3D hepatocyte cell cultures in terms of cell morphology, polarity, growth, gene and protein expression and directional transport (I-III).

4) To investigate the effect of 3D hydrogel culturing on expression, localization and function of selected drug metabolizing enzymes and efflux transporters in HepG2 and HepaRG cell cultures (II, III).

5) To evaluate the impact of biomaterial hydrogels on the phenotype of both liver progenitor cells and differentiated hepatocytes by seeding undifferentiated HepaRG cells and differentiated HepaRG cells to the NFC and HG hydrogels (III)

4 OVERVIEW OF THE MATERIALS AND METHODS

The effect of cell culture in 3D hydrogels on cellular organization and functions was investigated using HepG2 and HepaRG human liver cell lines (II-III). The influence of several hydrogels (Table 3) on the cellular proliferation, viability, polarity (I-III), the membrane transporter proteins (II-III), and also on the cytosolic metabolizing enzymes (III) was investigated. Especially, the effect of biomaterial hydrogels on the expression, localization, and activity of the efflux transporters was explored (II-III). In addition, the suitability of a novel NFC hydrogel in cell culturing on the whole was studied (I). The experimental methods are summarized in Table 4. The materials and methods are described in detail in the following sections.

Table 3 *The biomaterial hydrogels in the publications I-III.*

Biomaterial hydrogel	***Producer***	***Character of hydrogel***	***Publication***
Nanofibrillar cellulose (Growdex™)	UPM – The Biofore Company	nanofibrillar network, colloidal dispersion	I, III
Human basement membrane extract (Maxgel™)	Sigma-Aldrich	N.A.	I
Hyaluronan-gelatin (Hystem™, Extracel™)	Glycosan Biosystems	fibrillar network	I, III
Self-assembling synthetic peptide (Puramatrix™)	3-D Matrix	nanofibrillar network	I, II
Self-assembling synthetic peptide (Hydromatrix™)	Sigma-Aldrich	nanofibrillar network	I

N.A., no answer

Table 4 The experimental methods in the publications I-III.

Study objective	*Method*	*Cell/Tissue type*	*Gene/Protein/ Probe*	*Publication*
Morphology	Phase-contrast microscopy	HepG2, HepaRG	-	I-III
	Staining of filamentous actin	HepG2, HepaRG	alexa594-phalloidin	I-III
Viability and proliferation	Mitochondrial activity	HepG2, HepaRG	Resazurin	I-III
	Live cell imaging	HepG2	Fluorescein diacetate	I
	Live/Dead cytotoxicity assay	HepaRG	Calcein-AM/ Ethidium homodimer-1	III
	Quantification of genomic DNA	HepaRG	PicoGreen	III
	Quantification of total protein	HepG2, HepaRG	Bicinchoninic acid	I, III
mRNA expression	Quantification of RNA	HepG2, HepaRG, liver tissue	absorbance at 260/280	II-III
	Quality of RNA	Human liver tissue	electrophoretic analysis with RNA integrity number algorithm	III
	RT-qPCR with SYBR green	HepG2, HepaRG, liver tissue	ABCB1	II-III
		HepaG2	ABCB11	II
		HepG2, HepaRG, liver tissue	ABCC2	II-III
		HepaG2	ABCG2	II
		HepaG2	SLCO1B1 and SLCO1B3	II
		HepG2, HepaRG, liver tissue	CYP3A4	II-III
		HepaG2	CYP2D6	II
		HepG2, HepaRG, liver tissue	ALB	II-III
		HepaRG, liver tissue	HNF4α	III
		HepaRG, liver tissue	KRT19	III

Protein expression	ELISA	HepG2, HepaRG	ALB	I
Protein localization	Immunocytochemistry and confocal microscopy	HepG2, HepaRG	ABCB1	II-III
		HepG2	ABCB11	II
		HepG2, HepaRG	ABCC2	II-III
		HepG2	ABCG2	II
Functionality of MDR1 and MRP2 canalicular efflux transport	Efflux assay and confocal microscopy	HepG2, HepaRG	Fluorescein diacetate/ Fluorescein	II-III
		HepG2	Rhodamine123	II
		HepaRG	Calcein-AM/ Calcein	III
CYP3A4 enzyme activity and induction	Quantification of luminescent compound	HepaRG	Luciferin isopropyl acetate	III

ABC, ATP-binding cassette; ALB; Albumin; AM, acetoxymethyl; CYP, cytochrome P450; ELISA, Enzyme-linked immunosorbent assay; HNF, Hepatocyte nuclear factor; KRT19, Keratin 19; MDR1, Multidrug resistance protein 1; MRP2, Multidrug resistance-associated protein 2; PCR, polymer chain reaction; SLCO, Solute carrier organic anion transporter; RT, real-time

5 NANOFIBRILLAR CELLULOSE HYDROGEL PROMOTES THREE-DIMENSIONAL LIVER CELL CULTURE

Reprinted with permission of Elsevier B.V.: Bhattacharya M, Malinen MM, Lauren P, Lou Y-R, Kuisma SW, Kanninen L, Lille M, Corlu A, Guguen-Guillouzo C, Ikkala O, Laukkanen A, Urtti A, and Yliperttula M. Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture. Journal of Controlled Release 164: 291-298, 2012. Copyright 2012 Elsevier B.V. http://dx.doi.org/10.1016/j.jconrel.2012.06.039

6 PEPTIDE NANOFIBER HYDROGEL INDUCES FORMATION OF BILE CANALICULI STRUCTURES IN THREE-DIMENSIONAL HEPATIC CELL CULTURE

7 DIFFERENTIATION OF LIVER PROGENITOR CELL LINE TO FUNCTIONAL ORGANOTYPIC CULTURES IN NANOFIBRILLAR CELLULOSE AND HYALURONAN-GELATIN HYDROGELS

8 SYNOPSIS OF THE MAIN RESULTS

The main results of this thesis are summarized in the Table 5.

Table 5 *Summary of the main results in the publications I-III.*

Characterization of nanofibrillar cellulose hydrogel		***Publication***
Gel structure	NFC hydrogel is based on nanofibers with average fiber diameter of 20-30 nm resembling the natural ECM (e.g. fibrin fibers and the smallest collagen fibrils).	**I**
Rheology	Rheological measurements revealed that NFC hydrogel is a weak gel with storage modulus (G') of 10 Pa (0.5 % hydrogel). The storage modulus (G') is almost 10-fold higher than the loss moduli (G'') indicating a rather strong fiber network. The viscosity of the hydrogel depends strongly on the NFC concentration. NFC hydrogels show reversible gelation: it behaves as fluid under high stress and solidifies at rest. The high resting viscosity (circa 20000 Pas for 1% hydrogel) may be useful in 3D cell culture.	**I**
Optical properties	Lack of autofluorescence allows fluorescence imaging with low background. NFC causes some light scattering.	**I**
Diffusion	FITC-dextrans (mw range 20-250 kDa) had high diffusion coefficients of $3x10^{-8}$-10^{-7} cm^2/s in the NFC hydrogel (0.5 % hydrogel) demonstrating that the hydrogels show only modest resistance to the diffusion of macromolecules. Diffusibility decreased with the increasing molecular weight.	**I**
Influence of 3D biomaterial hydrogel culture on the hepatic cells		***Publication***
Cell proliferation	The growth of HepG2 was slower in NFC and PM hydrogels when compared to 2D environment. Similarly, the growth of HepaRG was lower in NFC and HG hydrogels than in the 2D cultures.	**I-III**
Cell viability	NFC hydrogel did not show cytotoxicity at concentrations of 0.1-1 %. Viability of HepaRG and HepG2 cells in NFC and 2D cultures was comparable. HepG2 aggregates were viable in PM hydrogel at least for seven days. Viability of HepaRG aggregates in NFC and HG hydrogels was maintained at least for two weeks. Mitochondrial activity of HepaRG cells was lower in HG hydrogel than in the NFC hydrogel and 2D cultures.	**I-III**

Cell morphology, shape	HepG2 and HepaRG formed multicellular aggregates in NFC, HG, PM and HM hydrogels. HepG2 aggregates were regular and spherical in NFC and PM hydrogels. The shape of HepaRG aggregates was more diverse and included both regular spherical aggregates and less organized aggregates, in PM, HG and NFC gels. The shape of single HepG2 cells is flattened in 2D cultures but in PM hydrogel HepG2 had polyhedral shape. HepaRG appears in three different morphologies in 2D cultures: flat elongated progenitor-like cells, granular hepatocyte-like cells and flattened cholangiocyte-like cells, but in hydrogels HepaRG has cuboidal shape.	**I-III**
Cell morphology, Actin filaments	Stress fibers were seen on the interface of glass or plastic, but not in the hydrogel cell cultures. Accumulation of actin filaments on the apical side of HepG2 and HepaRG spheroids asserts the *in vivo*-like apical-basal polarity and bile canaliculi formation.	**I-III**
Formation of bile canaliculi structures	HepG2 cells formed bile canaliculi in PM and NFC hydrogels. The appearance of bile canaliculi structure was more evident in PM hydrogel than in the 2D cultures. Instead no clear difference in the bile canaliculi appearance between the NFC and 2D HepG2 cultures were observed. Localization of canalicular resident transporters, MRP2 and MDR1, confirmed the formation of bile canaliculi-like structures in PM hydrogel. MRP2 was correctly localized also in the 2D HepG2 cultures. HepaRG liver progenitor cells formed bile canaliculi structures both in NFC and HG hydrogels and 2D surfaces. Instead, differentiated HepaRG cells formed bile canaliculi in 2D and HG, but not in NFC. In 2D cultures, the canaliculi structures were located in granular hepatocyte-like areas. MDR1 and MRP2 were localized on the canalicular membrane of HepaRG progenitors in NFC, HG and 2D. Also differentiated HepaRG cells expressed MRP2 correctly in NFC, HG and 2D, but MDR1 was detected only in 2D cultures.	**I-III**
mRNA expression of liver specific markers	PM hydrogel did not change the albumin expression of HepG2. The albumin expression increased over the culture time in all the culture conditions of HepaRG progenitors and was higher in NFC and HG hydrogels than in 2D cultures. On the contrary, albumin expression was lower in NFC and HG hydrogels than in the 2D cultures of differentiated HepaRG. The HNF4 expression was lower in NFC than in 2D and HG, both in the cultures started with HepaRG progenitors and differentiated HepaRG cells.	**II, III** **III**

	The KRT19 expression was low in the progenitor cultures in HG hydrogel. In addition, KRT19 expression decreased over the time in the NFC hydrogel and 2D cultures of progenitors. Differentiated HepaRG cells expressed KRT19 more in NFC than in the EC and 2D. Overall the KRT19 expression was higher in HepaRG compared to human liver.	**III**
mRNA expression of biotransformation markers	PM hydrogel did not change the expression of CYP3A4, CYP2D6, OATP1B1, OATP1B3, MDR1, BCRP, MRP2 or BSEP in HepG2 cells.	**II**
	NFC and HG hydrogels increased CYP3A4 expression in HepaRG progenitor cells. HG maintained the CYP3A4 expression in differentiated HepaRG cells, but in NFC cultures the CYP expression decreased.	**III**
	HG increased both the MDR1 and MRP2 expression in HepaRG progenitors. On the contrary, NFC and HG decreased the MDR1 and MRP2 expression of differentiated HepaRG cells.	**III**
Albumin secretion	HepG2 cells secreted albumin at similar level in NFC, PM, HM and EC hydrogels. HepaRG cells increased albumin secretion during the culture. The highest secretion was observed in three-dimensional NFC and HG hydrogels.	**I**
CYP3A4 activity	The CYP3A4 activity of HepaRG progenitors increased over the time in NFC and HG hydrogels. Instead the activity of standard 2D HepaRG cultures remained at same level for the culture time. Among HepaRG progenitor cultures the activity level was the highest in NFC hydrogel. On the contrary, cultures started with differentiated HepaRG showed higher CYP3A4 activity in 2D environment than in NFC and HG hydrogels. In addition, the activity of NFC culture was circa 100-fold higher than the corresponding EC cultures.	**III**
CYP3A4 induction	Rifampicin, phenobarbital and DMSO induced the CYP activity of HepaRG cells in all tested culture environments. Instead dexamethasone initiated induction was not observed.	**III**
Efflux transporters activity	Canalicular efflux transporters were active in PM cultured HepG2 spheroids shown by MRP2 and MDR1 substrates. Canalicular efflux transporters were active in all HepaRG progenitor cultures including NFC, HG and 2D. On the contrary, only a few spheroids in differentiated HepaRG cultures showed active efflux. Canalicular efflux was more common in low-density HepaRG spheroids than in the high-density ones.	**II, III**

3D, three-dimensional; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; HG, hyaluronan-gelatin; HM, Hydromatrix™ peptide matrix; NFC, nanofibrillar cellulose; PM, peptide nanofiber

9 GENERAL DISCUSSION AND FUTURE PROSPECTS

9.1 TWO-DIMENSIONAL VERSUS THREE-DIMENSIONAL LIVER CELL CULTURE

The current *in vitro* liver models are generally based on the primary hepatocytes that are cultured on polystyrene plastic as thin 2D monolayers. These 2D cultures are suitable for routine analyses, and in high content screening (HCS) and HTS procedures. However, 2D liver cell cultures poorly mimic the conditions in the liver tissue, and therefore, are not good for the prediction of drug metabolism and transport in the human body where the cells are interacting in a 3D environment.

As the need for predictive liver models is increasing, especially in drug transport and toxicity screening, there is strong demand for technologies that enable fully functional liver cell cultures. The techniques that can create three dimensions in the cell culture have especially evoked attention during the last decade (Fig. 7).

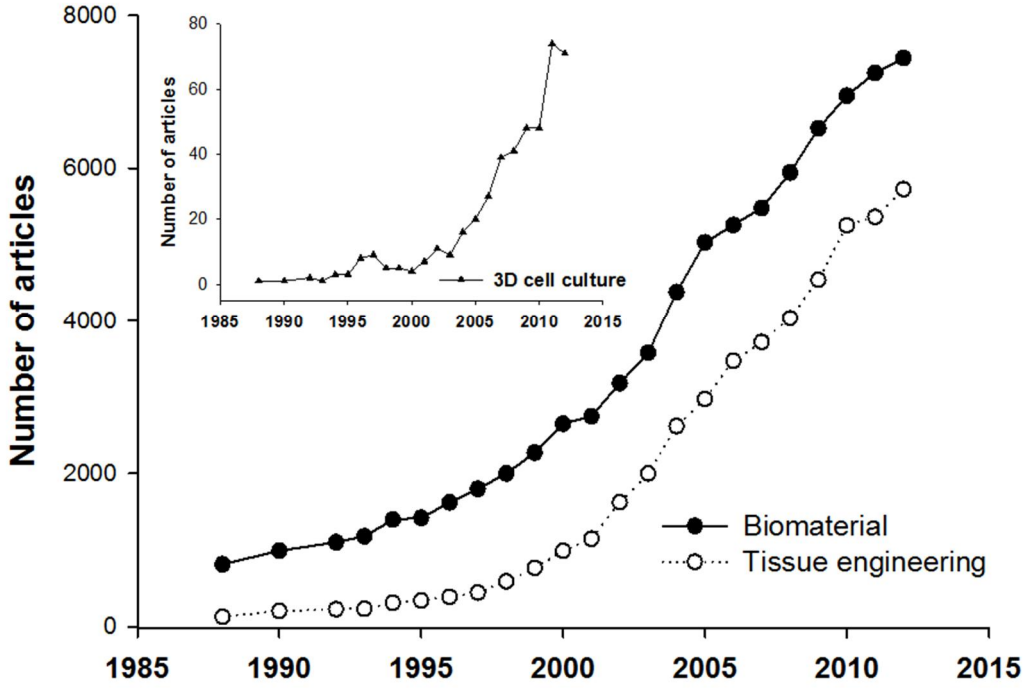

Figure 7 The interest on 3D cell culture has emerged during the on-going decade while biomaterial science and tissue engineering have evolved. The number of publications was visualized based on the search performed with the terms "Biomaterial", "Tissue engineering", and "3D cell culture OR 3-D cell culture OR three-dimensional cell culture" in the PubMed database (24.10.2013). The search was limited to in title/abstract fields.

What makes 3D liver cell culture so interesting? Liver parenchymal cells, hepatocytes, need to be highly polarized to be able to perform the vectorial transport from the sinusoidal domain to the canalicular domain; therefore, the hepatocytes could especially benefit from the multidimensional culture environment that enhances the number of cell-cell contacts. Sandwich configuration, which settles between the 2D and 3D culturing, has improved both the architecture and the functions of the liver cell cultures. However, sandwich cultures have not been fully satisfactory due to the batch-to-batch variability and poor availability of hepatocytes, as well as the limited lifetime.

Our studies together with earlier publications show that a true 3D culture can rebuild the hepatic polarity as well as promote drug metabolism and transport (Dash et al., 2012; LeCluyse et al., 2012; Godoy et al., 2013) (I-III). It is clear that 3D culture techniques can create more relevant *in vitro* models compared with the conventional 2D technique. 3D minitissues with a polarized architecture could offer improved models for the simultaneous study of drug metabolism, vectorial transport, and toxicity. Currently, all these properties must be studied with separate models: metabolism is studied with microsomes and primary hepatocytes, vectorial transport with inverted vesicles, cell cultures and animals, and toxicity with animals and primary hepatocytes.

An important advantage of 3D cultures is that they can be formed from a small number of cells, which is very beneficial when the cell source is limited, such as stem cell-derived hepatocytes. Hence, a 3D culture may enable more data points with a limited amount of cells, but then the smaller culture can also be a challenge because a more sensitive analysis technique is needed.

However, there is no validated 3D cell model. A few commercial 3D liver cell systems exist in 96-well plate format, which may enable HTS of metabolism or hepatotoxicity (Chan et al., 2013; Messner et al., 2013; Ukairo et al., 2013). All of these systems are based on the co-cultures of primary hepatocytes with fibroblasts/non-parenchymal liver cells and have been shown to maintain the CYP450 activity for 4–5 weeks, allowing for long-term toxicity studies (Messner et al., 2013; Ukairo et al., 2013). These 3D formats also support the reconstruction of bile canaliculi structures. However, the same micropatterned system did not show a clear advantage over the hepatocyte suspension cultures when hepatic drug clearance was studied (Chan et al., 2013). Before the 3D model can be widely used the model should be validated and characterized. For example, the quantitative expression of drug metabolizing enzymes and transport proteins should be known to be able to scale the *in vitro* data into *in vivo*.

9.2 THE ROLE OF HYDROGELS IN THE DEVELOPMENT OF ORGANOTYPIC LIVER CELL CULTURES

Soft biomaterial hydrogels have been exploited in cultures because the conventional glass and polystyrene are proposed to be too stiff for organotypic cell culturing. The hard surfaces favor a proliferative rather than differentiated phenotype (Wells, 2008). Instead, hydrogels mimic the stiffness of the tissue better than the conventional materials (Wichterle and Lim, 1960), and can offer both the mechanophysical and biochemical cues towards the physiological microenvironment (Prestwich et al., 2007). Collagen hydrogel has played a remarkable role in the liver cultures since the 1970s (Michalopoulos and Pitot, 1975). Collagen sandwich conformation, especially, has succeeded in remodeling liver cell cultures towards an organotypic-style (Dunn et al., 1991), and function as the only *in vitro* model for hepatobiliary transport (Brouwer et al., 2013). However, the sandwich culture expresses a limited number of cell-cell contacts, rather low cell density, a limited lifespan, and a dependency on primary hepatocytes; therefore, improved true organotypic liver cell culture models are needed.

The good results achieved with collagens have encouraged the work with hydrogels. The 3D cultures are formed by entrapping the cells in the hydrogel or by culturing the cells on the surface of non-adhesive hydrogels. The advantages of hydrogel entrapment over the other spheroid forming techniques (e.g., bioreactor, suspension, hanging drop) is that the cells are in a soft environment, including fiber networks similar to the *in vivo* ECM. In hydrogels, the cell receives both biochemical and physical signals of the ECM and is not forced to form spheroids, thereby differing from the other spheroid techniques that force the cells to aggregate.

A few papers show that the 3D hydrogel environment can induce the hepatocytes to form cell aggregates with a polarized architecture and extended/improved functions (Glicklis et al., 2000; Semino et al., 2003; Prestwich et al., 2007; Ramaiahgari et al., 2014). Our 3D hydrogel cultures with HepaRG cells (I, III) are the first ones with this cell line and demonstrate that the hydrogel technique suits the HepaRG culture as well as the 3D agitation, hanging drop, and bioreactor approaches (Darnell et al., 2009; Darnell et al., 2011; Leite et al., 2012; Gunness et al., 2013). The study with HepG2 (II) showed for the first time that this cell line benefits from the RAD16-I peptide hydrogel.

Despite the potential advantages, the 3D hydrogel cultures are not the most popular cell culture initiative (Fig. 4). The reasons might be that it requires experienced technique in the initiation and analysis of the cultures. In addition, the biomaterials might be expensive and the analysis methods must be modified to suit hydrogel cell cultures. Another concern is the variation in the spheroid size, and the restricted diffusion of nutrients, oxygen, and metabolites (such as bile acids). In addition, stability of the gel in long-term cultures is an issue. The challenges listed above must be solved

before extensive application of hydrogel cell cultures in drug testing, especially in HTS and HCS formats.

9.3 NANOFIBRILLAR CELLULOSE (NFC) HYDROGEL AS A CELL CULTURING AGENT

Cellulose draws attention because it is the most abundant polymer in the world; thus, its availability is limitless. Plant-derived NFC started to receive attention in the beginning of the twenty-first century when it could be produced in a cost-effective way (Siro and Plackett, 2010). To date, plant NFC has been utilized as a reinforcement agent in composites, and as a thickener or stabilizer in suspensions and emulsions (Klemm et al., 2011). In addition, a few studies have shown its potential as a drug delivery system (Kolakovic et al., 2012a; Kolakovic et al., 2012b; Valo et al., 2013) and as a pharmaceutical excipient for tablet manufacturing (Kolakovic et al., 2011).

Recently, interest has grown in applying plant-derived NFC as a reinforcing agent for tissue engineering composites (Borges et al., 2011; Cherian et al., 2011; Eyholzer et al., 2011; Mathew et al., 2012a; Mathew et al., 2012b). However, plant NFC hydrogel has never been studied as a cell culturing matrix, and this thesis presents the first publication whereby the hydrogel of plant NFC is utilized as cell culturing material (I, III).

In our first paper, we showed that the fiber network and rheological properties of native NFC showed similarities to natural ECM (I). In addition, the diffusion properties of macromolecules resembled the diffusion coefficient in the natural ECM (I), and encouraged studying NFC hydrogel as such without any additives. The results of this thesis revealed the applicability of NFC in 3D cell culturing (I). Furthermore, the results showed its capability to support morphological and functional differentiation of HepaRG liver cells (I, III), although NFC does not present biological adhesion signals that might be necessary for cell maintenance and differentiation. Hereby, the results of studies I and III confirm the recent recognition that physical cues are important determinants of cell growth, cellular functions, and tissue assembly (Discher et al., 2005; Brandl et al., 2007; Lozoya et al., 2011). Actually, it has been clearly demonstrated that hepatocytes can survive in a 3D biomaterial environment without matrix-based biological signals as long as the environment supports 3D cell-cell contacts. These kinds of materials have been: PEG hydrogels, polystyrene scaffolds, and pHEMA coated surfaces (Underhill et al., 2007a; Schutte et al., 2011; Acikgöz et al., 2013).

The possibility of adding biological adhesion signals either by physical or chemical binding, however, raises the usability of the NFC hydrogel. Simply physical mixing of ECM proteins (e.g. collagen, laminin, or fibronectin) may be successful because the NFC hydrogel slows down the diffusion of

molecules greater than 70 kDa in size (I, Fig. 6A). Thus, even though the ECM proteins are not covalently linked to the hydrogel, they may be retained. If covalent bonding of biological cues is nevertheless desired, the numerous hydroxyl groups of NFC offer attractive targets for chemical modification (Klemm et al., 2011). The fact that NFC resembles the already successful polysaccharide polymers, HA, alginate, and chitosan (Drury and Mooney, 2003), adds to the interest of the NFC hydrogel.

As an animal-free and fully characterized material, the NFC hydrogel does not express batch-to-batch variability, thereby providing favorable characteristics for tissue engineering and drug testing approaches. However, mammalian cells are not capable of degrading the cellulose. This feature may impede the utility of the NFC hydrogel in tissue engineering applications, but the stability of the cellulose polymer may be actually preferential in *in vitro* use enabling long-term cultures. The other advantageous characteristic of the NFC hydrogel is the transparency that allows convenient monitoring of cell processes by traditional optical techniques (I). But then, NFC hydrogel filters, refracts, and scatters the light, thereby preventing the full depth imaging of the culture and impairing the image quality. The light scattering, refraction, and attenuation are typical for all 3D hydrogels (Tibbitt and Anseth, 2009).

NFC hydrogel is a preformed reversible colloidal gel; thus, no gelification or use of toxic photoinitiators or crosslinking agents are needed. Instead, the cells are mixed into the hydrogel (I). This is possible because the internal structure of the hydrogel allows reversible “gelation”. The viscosity of the gel decreases while shear stress, such as by regular pipetting, is introduced.

The preformed fibrillar network is also a challenge. When cell suspension is mixed into the NFC hydrogel by standard pipetting, the heterogeneous culture may be generated. The risk for heterogeneous culture increases while the NFC concentration increases, meaning that the cell suspension is easier to mix evenly with the 0.5% hydrogel than with the 1% hydrogel. The cell suspension, as well as the growth medium, easily forms cavities in the hydrogel, leading to uneven distribution of the cells and the gel (unpublished data). This flocculation phenomena of the NFC hydrogel has been demonstrated with a combination of photography and rheological measurements (Karppinen et al., 2012). The problem relating to the flocculation is that the uneven gel structure may promote the heterogeneous cultures.

The complete mixture of cell suspension into the hydrogel may require more than 10 Pa shear stress since this power is needed to decrease the viscosity of 1% NFC hydrogel (I). In fact, the manufacturer (UPM-The Biofore Company) recommends the use of a vortex mixer; however, this produces too high of a speed for the living cells and is not an applied mixing technique in the standard cell culturing.

The third major challenge of NFC hydrogel is that it tends to adhere to the standard disposable polystyrene pipette tips, used in the cell culturing,

probably due to the numerous hydroxyl groups of NFC. In addition, NFC hydrogel is a weak gel (I) in practice, meaning that it requires the support of well walls to maintain the cell culture. This characteristic may prevent the wide applicability in HTS assays.

To conclude, NFC hydrogel has several positive features as a 3D cell culture matrix. Therefore, it is a very good base material for the development of physiologically relevant tissue models. For practical ease of use, some of its properties still require optimization.

9.4 LIVER CELL MODELS AS PREDICTIVE TOOLS FOR PHARMACOKINETICS AND TOXICITY IN HUMANS

Liver cell models are important in the early stages of drug discovery due to the central role the liver plays in drug metabolism, excretion, and toxicity (EMA, 2008; Mandenius et al., 2011). Human pharmacokinetics and toxicity are predicted using a combination of *in vivo* animal, *in vitro* human, and *in silico* computational models. The current models are not anyhow satisfactory and they are primarily designed to study phase I drug metabolism. Some pharmacokinetic parameters, such as the inhibition constant for drug-drug interaction (Ki), can be obtained with primary hepatocytes. However, the high phenotypical variability and the underprediction of *in vivo* clearance, limit the use of data from primary hepatocytes (Hallifax et al., 2010).

The study of vectorial transport has revealed its challenges (Giacomini et al., 2010; US FDA, 2011; EMA, 2012; Hillgren et al., 2013). Hepatic influx transporters can be studied using conventional hepatocyte cultures in suspensions or monolayers, but the study of hepatobiliary efflux transport does not succeed due to the dislocation of the transporters in the conventional hepatocyte cultures (Hillgren et al., 2013). The efflux transport is generally investigated with the membrane vesicles, which, however, have shown to result in false positives (Pedersen et al., 2013). Fortunately, sandwich cultured hepatocytes can be utilized in the prediction of *in vivo* biliary excretion at least for some chemicals (Abe et al., 2009; Swift et al., 2010; Pedersen et al., 2013).

Primary hepatocytes or hepatoma cells are suitable for studying the hepatotoxic potential of the compounds. These models provide information of toxicity mechanisms and can predict *in vivo* acute hepatotoxicity (Cheng et al., 2011; Hrach et al., 2011). However, toxicity cannot be studied accurately, because the conventional 2D cell models are altered and do not react to chemicals with the same sensitivity as the liver tissue (Dash et al., 2012; LeCluyse et al., 2012). Long-term studies are also impossible due to the short lifespan or unstable phenotype of the conventional 2D cultured cells.

This thesis together with other recent initiatives has proven that novel cell culture techniques allow for the formation of 3D structures toward organotypic tissue models (I-III) (Kim et al., 2012; Godoy et al., 2013). The

improved architecture (II) and the functionality of liver cells (II, III) suggest that 3D culture technology may provide a better system for studies on drug metabolism, vectorial transport, and toxicity. These kinds of organotypic cell models have already been shown to allow the prediction of *in vivo* clearance (Darnell et al., 2011), more accurate assessment of acute toxicity (Gunness et al., 2013), and long-term toxicity testing with repeated dosing (Messner et al., 2013).

In the future, the 3D *in vitro* tissue models may become more reproducible and predictive test systems than the gold standard of primary hepatocytes. Organotypic 3D models may replace or at least accompany the current simplified drug screening models. At best, an organotypic 3D *in vitro* liver model would reduce and replace the use of laboratory animals and improve the prediction of drug effects in humans. However, there is still a long way to go. Before its wide use, the 3D liver models should be standardized, validated, and approved by the regulatory authorities.

10 SUMMARY AND CONCLUSIONS

This thesis introduces new 3D human liver cell cultures that resemble the structures and functions of human liver tissue. The main conclusions are summarized in the following paragraphs:

1) The wood-derived NFC hydrogel was shown to suit the 3D cell culture. The viscoelastic properties of NFC hydrogel allowed encapsulation of cells into the preformed hydrogel and conventional gelification of the hydrogel initiators was not needed. NFC hydrogels were compatible with the cells, allowing for cell proliferation and aggregation.

2) The hydrogels of NFC, peptide nanofibers, and HG promoted the formation of 3D liver cell cultures. Human hepatoma cells, HepG2, and HepaRG, organized in 3D aggregates in which cells expressed polygonal morphology, differing from the flat and elongated phenotype of the 2D cultures, and thereby representing the *in vivo* counterparts. This study shows for the first time that 3D culturing in hydrogel can improve the polarity of HepG2 and HepaRG cells (II, III). The peptide nanofiber hydrogel improved the cytoarchitecture and liver-specific functions of HepG2 cells (I, II), whereas NFC and HG hydrogels induced the properties of HepaRG cells (I, III).

3) Most of the analytical methods were modified and optimized to suit 3D cultures of hydrogels. Especially, the staining, viability analysis, and the microscopy required revision to the standard protocols.

4) 3D hydrogel cultures were capable of remodeling the characteristics of human hepatic cell lines, HepG2 and HepaRG. The spheroid structure of HepG2 and HepaRG cells resembled the liver architecture, with functional bile canaliculi-like structures and correct localization of drug efflux transporters, MDR1 and MRP2. In addition, NFC hydrogel promoted CYP3A4 metabolism and canalicular efflux transport of HepaRG cells.

5) The study performed with HepaRG liver progenitors and HepaRG hepatocyte-like cells revealed the importance of the differentiation stage of hydrogel entrapped cells. NFC and HG hydrogels promoted the differentiation of liver progenitor cells more than the standard 2D culture environment. However, the hydrogels failed to maintain the phenotype of differentiated liver cells when compared to the 2D culture.

Taken together, these results deepen our current knowledge of 3D liver model development. Particularly, we compared the effect of different hydrogels to each other, which is generally not done, and showed the possibility of improving the drug metabolism and transport capability of human liver cell lines with 3D hydrogels.

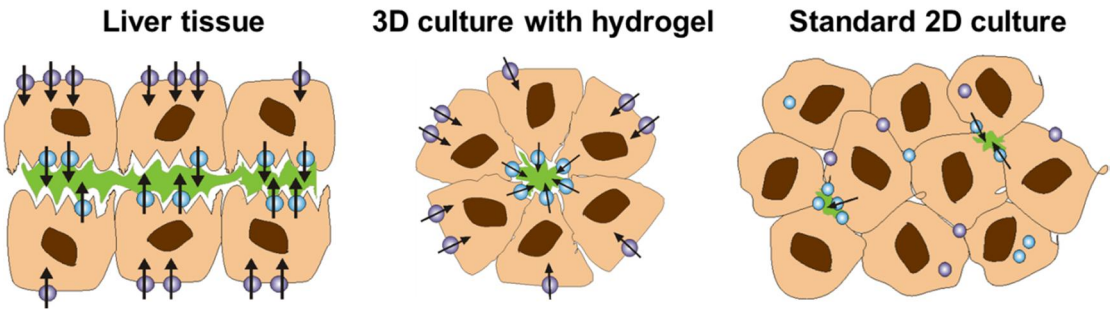

Figure 8 Only highly polarized hepatocytes express vectorial transport from sinusoidal membrane to the bile canaliculi. Here we present new 3D liver cell cultures that differ from the standard 2D liver cell cultures by improved polarity and vectorial transport. The 3D spheroid cultures have potential applicability in drug metabolism, transport and toxicity studies hereby facilitating the drug discovery.

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