The native liver as inspiration to create superior in vitro hepatic models

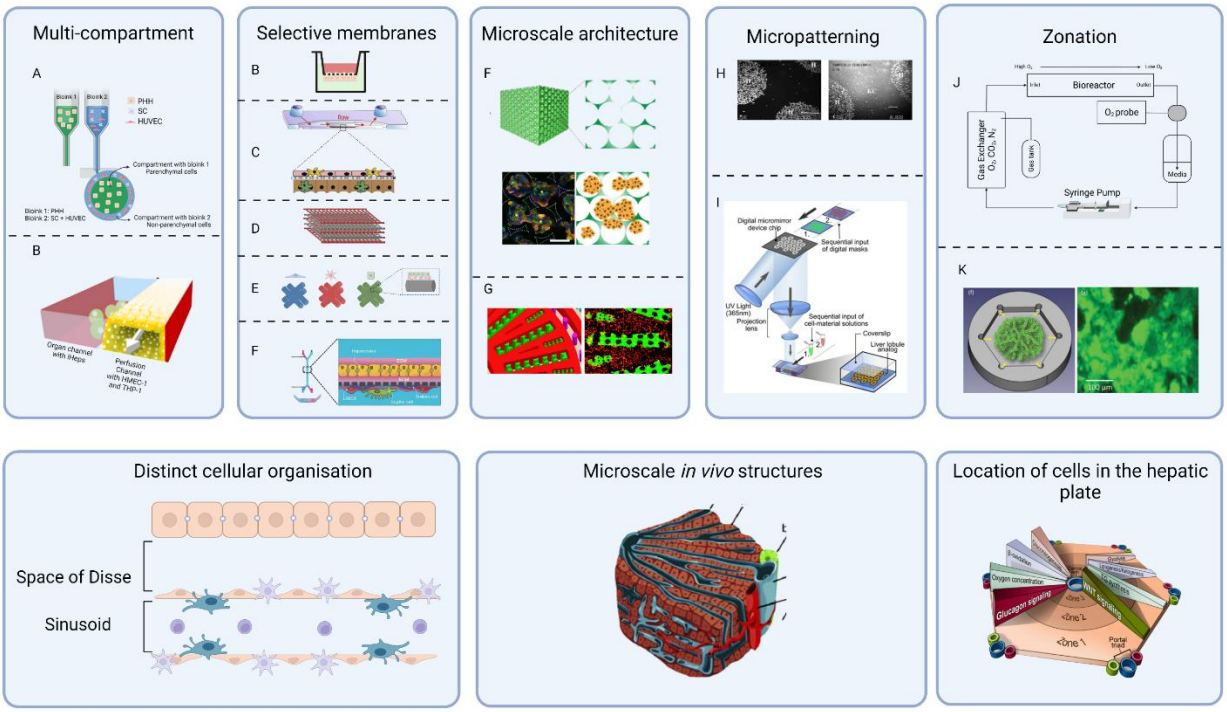
N. Carpentier[1], L. Urbani[2,3], P. Dubruel[1] , S. Van Vlierberghe[1]

1) Polymer Chemistry & Biomaterials Group, Centre of Macromolecular Chemistry, Ghent University, Ghent, Belgium.
2) The Roger Williams Institute of Hepatology, Foundation for Liver Research, London SE5 9NT, UK
3) Faculty of Life Sciences and Medicine, King's College London, London, UK

Abstract

Drug induced liver injury (DILI) is one of the major reasons of drug withdrawal during the different phases of drug development. The later in the drug development a drug is discovered to be toxic, the higher the economical as well as the ethical impact will be. *In vitro* models for early detection of drug liver toxicity are under constant development, however until today a superior model of the liver is still lacking. Ideally, a highly reliable model should be established to maintain the different hepatic cell functionalities to the greatest extent possible, during a period of time long enough to allow for tracking of the toxicity of compounds. In the case of DILI, toxicity can appear even after months of exposure. To obtain this goal, *an in vitro* model should be developed that mimics the *in vivo* liver environment, function and response to external stimuli. The different approaches for the development of liver models currently used in the field of tissue engineering will be described in this review. Combining different technologies, leading to optimal materials, cells and 3D-constructs will ultimately lead to an ideal superior model that fully recapitulates the liver.

Graphical abstract



1. Introduction

Drug-induced liver injury (DILI) is one of the major reasons for drug withdrawal during the different phases of drug development.[1] Drug development is a very time- and cost-intensive process and goes along with economical as well as ethical constraints. The average estimated cost of each drug compound reaching the market is $ 2.6 billion and the current time-to-market takes on average 10 years.[2,3]

There exists an enormous need for superior drug toxicity screening assays to guarantee a more selective transition of novel drugs throughout the different phases encompassing the drug development process. The time point at which drug toxicity is detected, is crucial and determines the economic impact. To date, from the 1000 compounds released from industry, only 30 proceed towards the clinical trials and in a final stage, only 1 drug reaches the market.[4]

With more efficient and reliable drug toxicity screening systems, the costs, time-to-market and the amount of compounds tested on experimental animals could be significantly reduced.[5,6]

DILI not only poses a huge problem for the pharmaceutical industry, but also for human health. Depending on the specific drug compound and its metabolic pathways, DILI can encompass liver disorders including steatosis, cholestasis, fibrosis, cirrhosis, liver cancer and acute liver failure.[7,8]

To improve the efficiency of the toxicity screening procedure, *in vitro* models that recreates the liver microenvironment and function to study toxicity of new drug compounds are in development and some are already commercially available. The most relevant are summarized in Table 1. Although research and validation studies are currently ongoing, a model of superior performance is still lacking. The ultimate goal is to mimic the *in vivo* liver environment, or a specific part of it, to the greatest extent possible.[9]

A plethora of 2D- and 3D-models have already been developed and have been described in literature, all attempting, to some extent, to maintain functional hepatocytes for months to be able to perform long-term toxicity screening, especially for drugs that elicit immune-mediated reactions that only may manifest weeks after administering the drug.[10] Cells used for the generation of such models are of various origin. Primary human hepatocytes (PHH) are the gold standard[11,12,13], but even in 3D-culture conditions they are very difficult to culture for longer than one month while maintaining their functionality. Furthermore, their availability is rather limited. Primary rat hepatocytes on the other hand, are much more abundant[14], but have the disadvantage of interspecies differences leading to an alternative xenobiotic biotransformation.[15] A third resource is represented by human cell lines. These are also largely available and since they usually are immortalized, they are easier to handle and to maintain in culture.[16] Different cell lines show distinct metabolic function, which can be similar or differ significantly from primary human hepatocytes. For this reason, hepatocyte-like cell lines can be selected dependently of the model to be established.[17] Another cell source comprises stem cells, which can be embryonic[18], adult[19] or induced pluripotent[20]. After expansion, these cells are differentiated into hepatocyte-like cells using different growth factors and media combinations. A summary of the different cell types is provided in

Table 2.

Table 1: Overview of the major commercial in vitro liver models with associated properties

Model name	Company	Model type	Cell type	Analyses/Analytes	Specifications	Reference
3D InSight™ Human Liver Microtissue	Insphero	Spheroid via hanging drop	• Primary human hepatocytes (PHH) • Kupffer cells • LSEC	• Viability • α-GST • HMGB1 • miR-122 expression	• Functional during 5 weeks • Multi-donor	[21,22]
HepaRG spheroid model	Cyprotex	Spheroid via ultra-low adhesion (ULA) plate or hanging drop	• HepaRG	• minimal effective concentration (MEC) • half-maximal activity concentration (AC_{50}) • Spheroid count • Spheroid size • DNA structure • Mitochondrial mass • Mitochondrial membrane potential • Glutathione (GSH) content • Reactive oxygen species (ROS) • Adenosine triphosphate (ATP)	• Functional during 14 days • Cost effective due to ULA plate	[23]
HEPATOPAC model	BioIVT	Micropatterned – hepatocytes surrounded by stromal cells.	• PHH • Stromal cells	• ROS • Cell density • Nuclear intensity • GSH • Mitochondrial potential • Albumin • Urea • ATP	• Functional during 28 days • High-throughput screening format	[24]
TRANSPORTER CERTIFIED™	BioIVT	Sandwich culture	• PHH	• ATP • Phase II activity • Biliary excretion • qRT-PCR (FXR target genes, FGF19, and OSTβ) • Lactate dehydrogenase (LDH) production	• Functional during 5 days • Cholestatic DILI model	[25]
Biomimesys® hydroscaffold	Biomimesys	Crosslinked RGDS and galactosamine- functionalized hyaluronic acid with bioactive ECM compounds (collagen and Fibronectin) combined with adipic acid dihydrazide	• PHH/HepG2	• Viability • Immunostaining • Growth • Gene expression • Cytochrome P450 (CYP) activity • Bile canaliculi formation • Albumin • Urea	• Functional during 21 days • Mimics in vivo stiffness	[26]
Hμrel® microliver	HμREL® corporation	Self-assembling co-cultures of primary cryopreserved hepatocytes with stromal cells	• PHH • Stromal cells	• Morphology (real-time cell analyzer) • Mitochondrial competency (cell-titer blue) • Mitochondrial viability (cell-titer glo) • CYP activity • Liquid chromatography–mass spectrometry (LCMS) • Albumin	• Functional during 4 weeks • Used for NAFLD, steatosis, NASH, fibrosis	[27]
Human Liver-Chip	Emulate	Porous membrane coated with ECM compounds (rat tail collagen I and bovine Fibronectin). At the opposite sides of the membrane, hepatocytes embedded in Matrigel and liver sinusoidal endothelial cells (LSEC) are seeded.	• PHH • LSEC • Kupffer cells	• Immunofluorescent imaging (AdipoRed) • DAPI staining • Smooth muscle actin (SMA) • Morphology • Albumin • Urea • Triglyceride (TG) • alanine aminotransferase (ALT) • Gene expression	• Functional during 14 days • Flow conditions • Used for hepatocellular injury, Cholestasis, Steatosis, Kupffer cell depletion	[28,29]
OrganoPlate®	MIMETAS	Organoids in matrix from Matrigel and rat tail collagen I	• PHH/iPSC/HepG2 • Kupffer • Bile duct • LSEC	• Immunostaining • Barrier integrity • Transport • Viability • LCMS • qPCR	• Functional during 15 days • High throughput • Formation of bile canaliculi • Pump-free perfusion • Microfluidics • Membrane free co-culture • Cells can interact and migrate	[30]
SynTox	SynVivo	Multi-compartment microfluidic chip	• Hepatocytes • LSEC	• Optical and fluorescent imaging • Chemical assays • Toxicity assays • Biomarker analysis	• Drug diffusion • Drug toxicity • Real-time optical monitoring • Multi-compartment architecture • Microfluidic	[31,29]

PhysioMimix™ Organ-on-Chip	CN-Bio	Microfluidic Organ on chip 3D-scaffold	• PHH • Kupffer cells	• albumin • urea • ALT/AST • LDH release • CYP3A4 activity • Viability •	• Functional up to 4 weeks • Acute and chronic • Immune cells available • Multi-organ	[32]

Table 2: Overview of potential sources of hepatocytes for in vitro liver models and associated advantages/disadvantages.

Cell type	Advantage	Disadvantage	References
Primary human hepatocytes	• Gold standard[13] • Representing population[33]	• Dedifferentiation[34] • Availability[33] • Invasive • Time-consuming isolation[35] • Limited proliferation[33]	36,37
HepG2	• Highly proliferative[33] • Easy to handle[38] • Cost effective[39] • Availability[38] • Stable phenotype[38]	• Lack of important metabolizing enzymes[40] • Only 1 donor[33]	41,42,43,44
HepaRG	• Expression of important enzymes comparable to PHH[45,46] • Stable phenotype[47] • Availability[48]	• Expensive[45] • Only 1 donor[33] • Difficult to maintain in culture	49,50
iPSC	• Availability[51] • Personalized[51] • Representing population[52] • Proliferative[33]	• Time-consuming[52] • Complex and expensive differentiation protocol[53] • Maintain fetal features[52] • Genetic aberrations[54]	55,56
Mouse/rat hepatocytes	• Availability[57] • Cost effective[57]	• Life ending[58] • Time-consuming isolation[58] • Interspecies difference[15]	59,60,57
Adult stem cells (ASC)	• Bipotent[61] • Highly proliferative[54] • Genetically stable[54]	• Expensive differentiation protocol[54] • Invasive[62] • Availability[63]	54,64
Embryonic stem cells	• Highly proliferative[65]	• Availability[63] • Ethics[66] • Genetic aberrations[54] • Time consuming[67] • Complex and expensive differentiation protocol[67]	68,69,70

A more advanced approach involves the generation of co-cultures. The liver contains more than only hepatocytes and during liver metabolism, more cell types are involved (see section 2. Liver anatomy). Commonly used cells in co-cultures include hepatic stellate cells, liver sinusoidal endothelial cells and Kupffer cells. Depending on the type of DILI to be studied, cells can be selected accordingly. For example, if a type of DILI is investigated in which inflammation is important, the application of Kupffer

cells is essential[71]. Whereas for a drug that induces fibrosis, co-cultures that include stellate cells should be considered.[72,73 ,74,75,76]

2D in vitro liver models mainly consist of one layer of hepatocytes, optionally in co-culture with one of the aforementioned cells (stellate cells, Kupffer cells and LSEC) seeded on the bottom of a well plate which is sometimes coated with a bioactive layer. Frequently used are extracellular matrix components such as Fibronectin and collagen.[36,77]

Besides the simplicity of the 2D models, they do not meet the requirements with regards to maintaining the functionality of hepatocytes. In this respect, 3D models are superior over 2D models. In fact, hepatocytes are highly polarized cells that require their 3D environment to remain functional throughout the time span of the toxicity assay.[78]

The research area of 3D models is expanding at a rapid pace. 3D models usually consist of cells cultured in 3D conditions, in the presence[79] or absence[80] of a supporting material. An example of a 3D model without supporting material is a spheroid. It consists of clustered hepatocytes with optionally other cells in co-culture. The close cell-cell contact significantly improves the functionality of the cells compared to a 2D-cell culture.[36,81] The main disadvantage is the lack of shape and size control and the occurrence of core necrosis due to insufficient diffusion of nutrients through the spheroids.[82] Scaffolds can be used in combination with spheroids as well, but this is rather to provide a spatial organization and defined geometry than for cell attachment.[83]

Models with supporting material mainly consist of cells in combination with hydrogels. Hydrogels are generally considered the most interesting material type in this regard.[9] They are water-soluble biomolecules or synthetic polymers that are crosslinked to form a network. This network does not dissolve in water, but can comprise large amounts of water within its network structure while maintaining its supportive mechanical properties. This aqueous environment is ideal for cell growth.[84]

The hydrogel stiffness is also very important.[9,85] On the one hand, the scaffolds need to provide structural stability to maintain its shape, but on the other hand, they need to be soft enough for their application in liver tissue engineering (being a soft tissue). From previous research, it is known that hepatocytes are highly influenced by the stiffness of the matrix they are cultured on.[86,77] Consequently, to develop reliable in vitro models, the materials used should be characterized by a similar stiffness as that of natural liver tissue (i.e. 1 – 5 kPa) [87], or should be adaptable to mimic specific changes to the liver stiffness typical of pathological states.

As an additional advantage, hydrogels can be selected that very closely resemble the extracellular matrix (ECM) of the native liver. The liver ECM comprises core proteins, glycoproteins, proteoglycans and glycosaminoglycans.[88] Research revealed that models constituting such natural ECM compounds are superior materials to cultivate hepatocytes.[89,90]

The most widely used protein in this regard is gelatin. Gelatin is derived from collagen, being the major compound of the ECM throughout the whole body.[91,92,93] Additionally, gelatin is less immunogenic than collagen[94] and has superior processing capabilities due to its gelation properties and upper critical solution temperature (UCST) behavior.[95]

The preferred polysaccharides in order to mimic the native ECM are glycosaminoglycans (GAG), because these are of great structural and functional importance in the native ECM and are present incorporated in proteoglycans.[96,97,88] The GAGs present in the liver are (in increasing order of occurrence): hyaluronic acid, dermatan sulphate, chondroitin sulphate and heparan sulphate.[88] In a fibrotic liver, the GAG content is elevated with more than 5-fold.[97]

Models which include supporting materials are the sandwich culture and the 3D-scaffold models. In the sandwich culture model, hepatocytes are present between two layers of ECM-mimicking material,[98] which is usually collagen or Matrigel.[99,100] The 3D-scaffold model consists of hepatocytes supported by a scaffold material processed into a 3D shape. This 3D-shape is created by a controlled emulsification, lyophilization, solvent casting, salt leaching or gas foaming process.[101] More advanced methods include photolithography, different 3D-printing techniques and electrospinning.[9,102]

Since specific ECM compounds can be very expensive, there is an option to produce scaffolds from more cost-efficient polymers and afterwards applying a bio-active coating to enhance cell adhesion and spreading.[103] Often used coating compounds in this regard include laminin, Fibronectin and collagen.[104,105,106] Alternative strategies described in literature include the application of decellularized native ECM (dECM) compounds used as biomaterial ink for scaffold fabrication/printing or the decellularization and subsequent recellularization of natural liver tissue matrix. Although these strategies show significant improvement of hepatocyte function and generation of complex multicellular models[107,108], they are not suitable to realize high throughput toxicity screening models.[90,109]

All previously mentioned models lack sufficient resemblance with the *in vivo* liver microenvironment (except for the dECM),[9] which leads to systems that are not able to maintain hepatocyte viability and functionality for longer periods of time. Current models described in literature are able to maintain hepatocytes functional up to one month[110,80,111,112,113] which is not sufficient for long-term toxicity screenings.[114]

Increasing the *in vivo* - *in vitro* resemblance of hepatic models could render them superior over the liver models currently reported in the state-of-the-art. In this review, we provide an in depth overview of existing in vitro models, specifically focusing on the specific strategies and processing methods used to mimic the native liver with its composition of the ECM, microenvironment compartments and relevant cell types to the greatest extent possible in order to establish a model with long-term functionality. For this reason, this review is not a simple list of existing in vitro models of DILI, but aims to describe the most advanced and multidisciplinary approaches (encompassing materials science and engineering approaches) to mimic the liver architecture in the rapidly progressing field of liver tissue engineering, with the hope to inspire scientists working on the development of advanced *in vitro* hepatic models.

2. Liver anatomy

When mimicking liver tissue *in vitro*, the liver morphology and cellular composition should be considered. To generate an *in vitro* model, cell-cell and cell-ECM interactions should stimulate cellular events and phenotypes similar to those occurring *in vivo*.[115] Ideally, depending on the disease that needs to be modelled, different liver compartments and features should be mimicked.[116,117,118,119]

Together with the main cell type (i.e. hepatocyte), the stellate cells (SC), LSEC, the Kupffer cells (KC), Natural killer cells (NKC) and the cholangiocytes make up the liver tissue and function. The properties of the different liver cells are summarized in Table 3.

Table 3: different cell types with related function and their location.

Cell type	Function	Location	References
Hepatocytes	• Metabolism • Detoxification • Protein synthesis	Hepatic cords in the hepatic plate	[120,121]
Stellate cells	• Role in liver physiology and pathology • Storage of fat and vitamin A	In the space of Disse between the hepatocytes and the LSEC	[122,123,124]

	• Quiescent state in healthy liver • Activated upon injured liver to produce excess ECM		
Sinusoidal endothelial cells	• Fenestrated enabling fluid exchange • Selective barrier between sinusoid and hepatocytes	Surrounding the liver sinusoid	125,122
Kupffer cells	• Macrophages • Innate immune system • First contact with blood from portal vein • Metabolism	In the sinusoid adhering to the LSEC	126
Natural killer/Pit cells	• Innate immune system • Natural killer cells • Anti-tumor capacity • Recognizing target cells by their surface receptors	In the liver sinusoid	127,128
Cholangiocytes	• Modify the concentration and composition of the bile • Enabling continuous bile flow	Along the biliary tree	129

The liver is organized into hexagonally shaped hepatic lobules, with the central vein in the center and the portal triad at each corner (see Figure 1) [130]. In the hepatic plate, blood flows from the hepatic artery and the portal vein to the central vein through the sinusoids.[125] The hepatic artery provides oxygen-rich blood and the portal vein provides deoxygenated and nutrient-rich blood originating from the intestines.[122] Both blood flows are mixed and the composition of the blood changes continuously in its concentration of, amongst other, oxygen[131,132], ammonia, glucose and some hormones[133] such as insulin and glucagon throughout its path through the hepatic lobule due to the metabolic activity of the hepatocytes, leading to zonation. Hence, the specific function and phenotype of the hepatocytes depends on the blood composition they are exposed to and by consequence to their specific location[134] (called zone) in the hepatic lobule.[135] These zones are defined by the distance of the hepatocytes to the center of the hepatic plate.[136] Hepatocytes in the different zones differ in several enzymes, translocators, receptors and subcellular structures. These phenotypic variations lead to a difference in functional capacity, but also to a variation of produced ECM compounds throughout the different zones. At the periportal zone, the ECM mainly consists of the structural proteins collagen IV and V and the adhesion protein laminin. At the perivenous zone, however, the structural proteins are mainly collagen I, III and VI, while the adhesion protein is Fibronectin.[137,138]

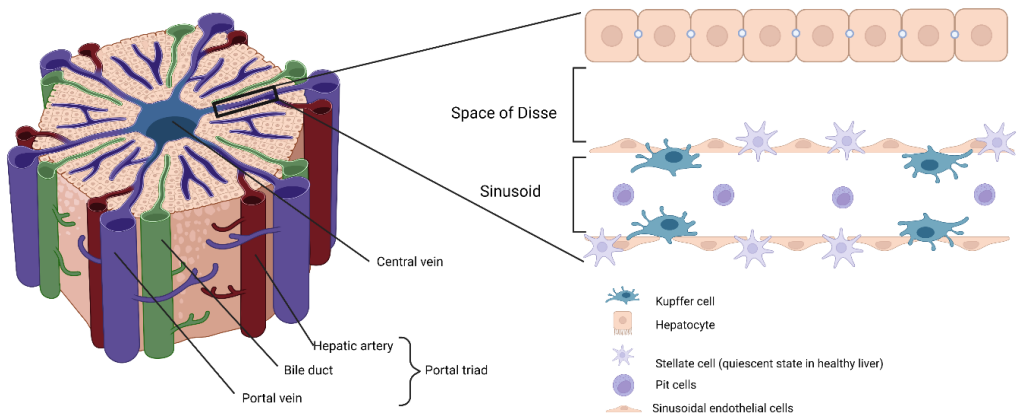


Figure 1: Liver anatomy and location of the different cell types present in the liver.

3. Advanced *in vitro* models

3.1 The workflow of developing *in vitro* models.

As stated earlier in the introduction, 3D-liver models often consist of liver cells in combination with a supporting material, typically referred to as scaffolds. To develop an ideal model the most relevant cell types in combination with superior scaffolds should be considered. Generally, the optimal strategy is to tune the physico-chemical properties including the mechanical properties and the chemical composition as well as the microscale architecture of these scaffolds in such a way that they resemble the *in vivo* microenvironment of the liver to the greatest extent possible. This should ensure long-term viability and functionality of the cultured liver cells *in vitro*, allowing for long-term studies. However, to date, no models have been identified that can support primary hepatocytes for longer than one month.[110,112]

To develop a superior model enabling longer hepatocyte cultivation, the *in vitro* culture systems should be more sophisticated, by resembling the overall liver microenvironment to a greater extent beyond mimicking the composition of the ECM.

In time, models are being optimized continuously based on materials used, selected cell types and the construct architecture. The different aspects that should be taken into account when developing a complex liver model are highlighted in this section by giving an historical overview of the models available.

Most of the models and examples mentioned in the upcoming section are characterized by physiologically relevant sizes. However, a lot of interest is currently also going towards miniaturized tissues grown inside microfluidic chips, also known as organ-on-chip devices. In this respect, most of the physiologically sized relevant models can also be integrated into microfluidic systems thereby establishing livers-on-chip combining the advantages of tissue engineering and microfabrication, including a higher control over the microenvironment and the possibility for direct observation of cell and tissue behaviour. Additional examples of these organ-on-chip analogues are covered in different sections.[139] (i.e. section '3.2 Multi-compartment models' at page 9, '3.3 Selective membranes' at page 10, '3.5 Micropatterning' at page 13 and '3.6 Zonation' at page 16.)

3.2 Multi-compartment models

Hepatocytes and other hepatic cells (stellate, Kupffer and LSEC) fulfil a specific role and are important to support the functionality and viability of each other. They have also various functions in drug metabolism, making them considerably important in the overall mechanism of DILI.[140,141] Co-cultures that were considered in the early 2D and 3D liver models included only hepatocytes with other non-parenchymal cells randomly distributed and without compartmentalization. An example is the model of Li et al.[142] in which a suspension of primary human hepatocytes and human Kupffer cells were developed and were able to detect the toxicity of 100 drug compounds with a known DILI status with more sensitivity than a conventional monoculture layer. Another example includes the model of Baze[143] et al consisting of a spheroid with PHH, human KC, LSEC and stellate cells in co-culture. The latter spheroid contained cells with superior functionality in comparison with a 3D monoculture spheroid of PHH, which remained functional during 14 days.

A superior approach involves cell seeding in multi-compartments rather than resulting in a random distribution. In the native liver, cells are present in specific locations and are sometimes separated from each other by ECM or other components[144]. Having hepatic cells in distinct compartments *in vitro* would resemble the liver architecture to a greater extent.

Nguyen *et al.*[145] described scaffolds developed by Organovo in a two-compartment planar geometry via continuous deposition microextrusion of encapsulated PHH, stellate and human umbilical endothelial cells (HUVEC) in a Novogel® 2.0 hydrogel (see Figure 2A). The non-parenchymal cells were printed at the border of each compartment and the PHHs were printed to fill the compartments, both embedded in a gel. The cells remained functional for 28 days. The model was validated by exposure of the model to the drug compounds trovafloxacin and levofloxacin. Trovafloxacin is known to induce hepatotoxicity, but this toxicity could not be detected through the use of 2D-culture toxicity assays. Levofloxacin is structurally similar to trovafloxacin, although this compound is non-toxic. The model was treated during 7 days with the compounds. As anticipated, trovafloxacin induced significant decreases in both albumin and ATP indicating hepatotoxicity of the compound. Levofloxacin however did only show a decrease in albumin at the highest dose tested (100 µM), while the ATP levels remained unaffected. Although both compounds are structurally similar, the 3D-model was appropriate to serve as model to detect DILI and to make a distinction in toxicity between two structurally similar compounds.

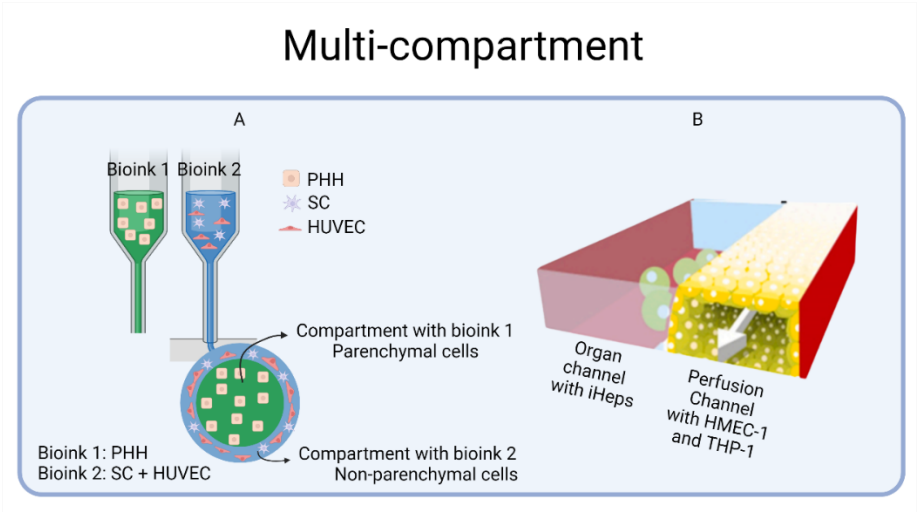


Figure 2: (A) A representation of the application of a multi-compartment by the use of different bio-inks as developed by Nguyen et al.[145] *and (B) the use of this approach in a liver-on-chip device by Bircsak et al.*[30]

Since stellate cells play a key role in the development of liver fibrosis, the above-described model can be of special interest to test compounds that induce fibrogenesis. Norona *et al.*[124] exploited the Organovo model to predict the toxicity of methotrexate (MTX) and thioacetamide (TAA), both known to induce fibrosis. Exposure of the model to these compounds enabled the detection of liver fibrosis characterized by patterns of collagen deposition similar to those observed in clinical samples obtained from patients with liver fibrosis.

Bircsak et al[30] integrated this approach in a liver-on-chip system (see Figure 2B) using an OrganoPlate 2-lane. In one lane (i.e. the organ channel), a mixture of iPSC-induced human hepatocytes (iHep) embedded in collagen I was injected, while in the other channel, endothelial-like and Kupffer-like cells were seeded, being directly in contact with iHep/collagen (organ channel) without physical barrier[146] mimicking the liver sinusoid. The cells remained viable and functional (as reflected by albumin and urea levels) during 15 days of culture. CYP3A4 activity increased pointing towards further maturation of iHep. The automated set-up of the OrganoPlate renders it suitable towards high throughput screening.

Using distinct compartments to separate different cell types however exhibits some limitations. It is known that cells tend to migrate and interact with other cells leading to movement of cell types into other compartments. This leads to poor control over the cellular distribution in the 3D-constructs.[147,148]

As a result, the compartmentalization of the different cells in the model will gradually be lost. This problem can be tackled through the application of a selective membrane separating the different cell types, while nutrition, waste and signaling compounds can cross the membranes.

3.3 Selective membranes

Another strategy to separate different compartments within the same model is the application of selective membranes. More specifically, one or more membranes can be used to seed the different cell types and as such, act as separative cell layers and cellular compartments.

This approach is often used to separate hepatocytes and LSEC to recreate the structure of liver sinusoids, where the membrane acts as the space of Disse.[149,148,150]

Kang *et al*.[148] described a sinusoid model using primary rat hepatocytes and LSEC. Cells were seeded onto opposite sides of a collagen-coated membrane in a transwell (see Figure 3A). The model maintained cell viability, morphology and functionality (as reflected by urea synthesis and CYP activity) during 39 days of culture, which is longer than most models described in literature. Later, they converted this into a humanized model using PHH and bovine aortic endothelial cells. The model was integrated in a chip to be used in combination with a flow set-up. The viability and morphology of the cells was maintained during 26 days, while PHH seeded without endothelial cells lost their morphology within a week.[151]

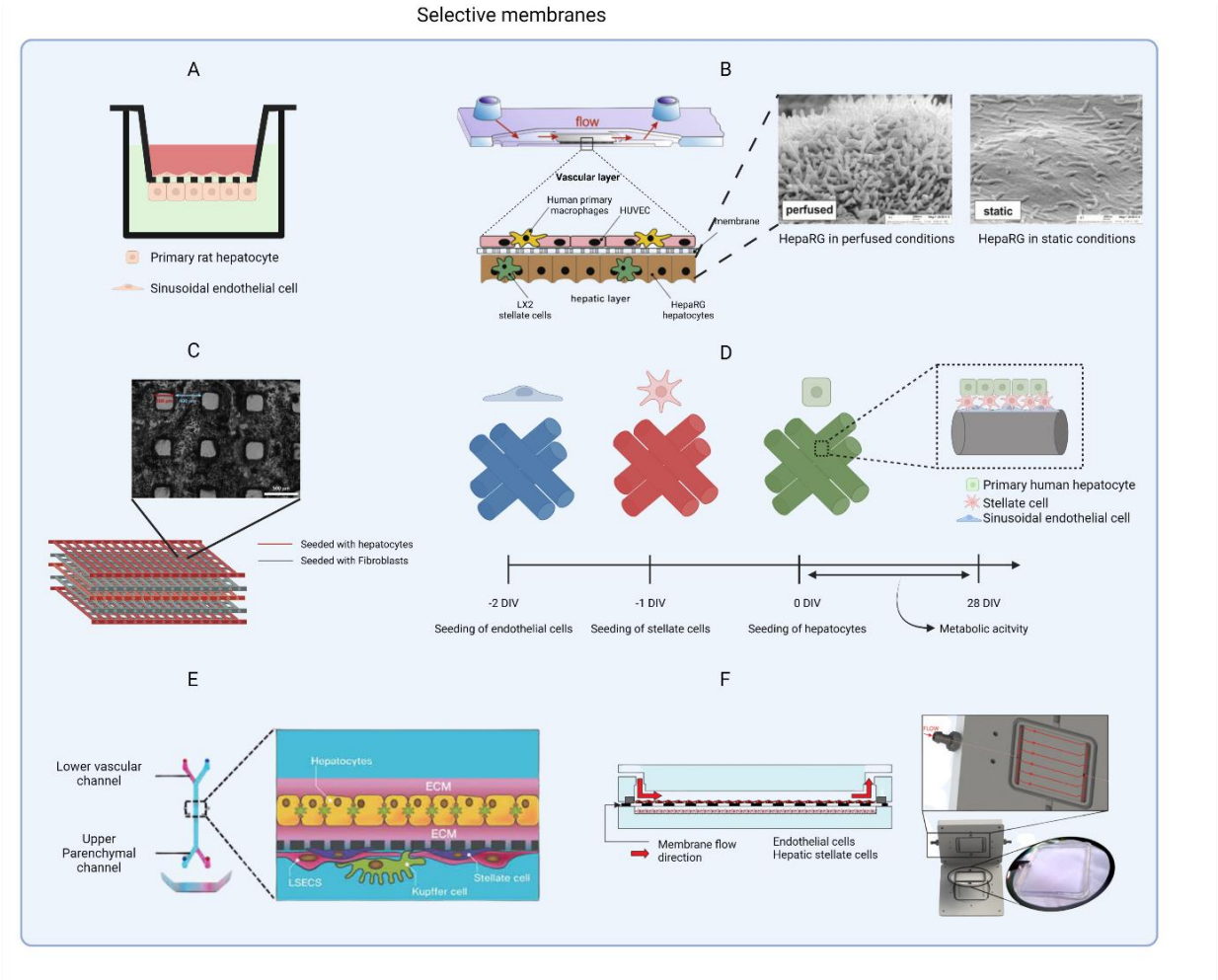


Figure 3: (A) Schematic representation of the model of Kang et al.[148] to mimic the liver sinusoid. (B) Schematic overview of the MOTiF biochip set-up and corresponding cell seeding approach by Rennert et al.[152] (C) Schematic overview of the 3D-co-culture model of De Maria et al.[153] by stacking alternately seeded PLGA grids seeded with hepatocytes and fibroblasts. (D) The

developing process of the hollow fiber membranes developed by Ahmed et al.[154] *to establish a co-culture of SEC, SC and PHH. (E) The liver-on-chip multi-compartment approach by Jang et al.*[155] *(F) The liver-on-chip device of Illa et al.*[156]

Applying a flow system resembling the dynamic nutrient and oxygen uptake in the space of Disse has been evidenced to contribute to *in vivo* mimicry. For example, Rennert *et al*.[152] utilized the Multiorgan Tissue Flow (MOTiF) biochip in combination with a 3D-liver organoid. This biochip was comprised of staggered seeded vascular and hepatic cell layers as shown in Figure 3B. For the vascular layer, HUVEC cells were mixed with monocytes and seeded on top of a 12.2 µm thick PET membrane with a pore diameter of 8 µm. The monocytes were then differentiated towards macrophages, resembling the Kupffer cells. For the hepatic cell layer, a mixture of HepaRG and LX-2 (i.e. a hepatic stellate cell line) cells were seeded on the opposite side of the membrane. Structural as well as functional preservation of the hepatocytes was observed. In Figure 3B, the scanning electron microscopy (SEM) image clearly shows that the HepaRG cells are structurally superior in the perfused culture compared to in a static control culture. The cells exhibit a high plasticity with increased microvilli formation at the cell surface. Furthermore detection of ASGPR-1, ZO-1 and MRP-2 confirmed bile secretion activity and observation of urea and albumin proved the metabolic activity of the hepatocytes during the four days of culture.

Selective membranes can also be applied for 3D-stacking after seeding cells in separate layers. More specifically, different cell types are seeded onto different membranes, which are subsequently stacked onto each other to create a 3D-scaffold (see Figure 3C) in which the cells are distributed in a controlled manner. In this regard, De Maria *et al* developed scaffolds by stacking poly(lactic-co-glycolic acid) (PLGA) layers onto each other that were alternately seeded with hepatocytes and fibroblasts.[153]

Selective membranes are not always applied to separate cell layers. They can also facilitate the selective mass transfer of molecules towards and away from the cell compartments without causing shear stress. This can be useful in other systems, such as bioreactors, where the fluid stream can cause these high shear stresses. An example of this application is the model described by Ahmed *et al.*[154] in which a co-culture of LSEC, stellate cells and PHH was seeded onto Fibronectin-coated polyethersulfone hollow fiber membranes. The cell layers were seeded on top of each other with an interval of 24 hours at the outer surface of the membranes. Two layers of hollow fiber membranes were cross-assembled into a bioreactor (see Figure 3D). The cells reorganized themselves into a complex cytoarchitecture with the presence of tube-like structures formed by LSEC. This model was able to give rise to the production of urea, albumin and diazepine biotransformation products during 28 days of culture.

Jang et al[155] developed a liver-on-chip device using the multi-compartment approach. Primary human hepatocytes were seeded in a sandwich culture on top of a porous membrane that separates two parallel microchannels. At the opposite side of the membrane, human LSEC, Kupffer and stellate cells were seeded (see Figure 3E). Liver toxicity related to hepatocellular injury, steatosis, cholestasis and fibrosis could be phenotypically detected when treated with tool compounds.

Another liver-on-chip model mimicking the liver sinusoid that makes use of a membrane to separate cellular compounds was established by Illa et al[156]. It consists of two transparent plates with a porous polytetrafluoroethylene microporous membrane in between (see Figure 3F). The aim was to serve as mimic for the liver sinusoid by seeding primary human umbilical vein endothelial cells on top of the gelatin-coated membrane. On the other side of the membrane, activated stellate cells (LX-2) were seeded. The flow is provided through channels above the membrane, hence over the endothelial cell layer. In this way, the continuous shear stress of the blood flow on the endothelial cells is mimicked. An improved morphology of the endothelial cells and an improved phenotype of the stellate cells was reported. Ortega-Ribera[157] et al applied this set-up to seed hepatocytes and LSEC on the opposite side of the membrane, again with continuous shear-stress over the LSEC layer. The PHH maintained

morphology and high urea, albumin and CYP3A4 activity, HNF4alfa and ATP-binding cassette (ABC) transporters transporter after 7 days. This points towards a delayed hepatocyte dedifferentiation. The model responded better to acute treatment of drugs (i.e. troglitazone, diclofenac and acetaminophen) with known hepatotoxicity as compared to conventional static culture platforms.

When using these multi-compartment models, the overall tissue structure still does not fully recapitulate the native liver lobule. However, the presence and organization of multiple cell types within the compartmentalized structure of the 3D liver tissues likely play a significant role in preserving liver-specific functions.[145]

3.4 Models encompassing microscale architecture

Having distinct compartments for the different hepatic cell types is a valuable approach when establishing a co-culture. However, this approach still faces the limitation of a non-reliable architecture. More specifically, the cell organization does not exhibit the same architecture nor compartmentalization as the cells exhibit *in vivo*. A model that respects the microscale architecture of the liver ensures cell-cell and cell-matrix contacts similar to the *in vivo* situation and hence, is more representative for native liver tissue.[158,159]

Several models have been described in literature resembling the 3D-microscale architecture to a greater extent. Seng *et al*.[160] described a model constituting 3D hexagonally arrayed lobular human liver tissue resembling the hepatic plate architecture, developed by creating an inverse crystal colloid (ICC) hydrogel scaffold. This was established by first creating a sacrificial construct of polystyrene beads as schematically presented in Figure 4A. The inverse scaffold was created with a solution of polyethylene glycol diacrylate (PEGDA) and acrylated polyethylene glycol-N-hydroxysuccinimide in the presence of Irgacure 2959 to enable UV-induced crosslinking. The model exhibited advanced key features of freshly isolated human fetal total liver cells that were preserved up to 5 months in culture, being the longest period reported in literature so far.[160] In more recent work, Seng et al[56] additionally explored the application of iPSC-derived hepatocytes in this model. After seeding iPSC-derived hepatic progenitor cells, they were differentiated towards hepatocyte-like cells. The resulting organoids were matching better with adult tissue, as compared to 2D and 3D controls, with respect to morphology, gene expression, protein secretion, drug metabolism and viral infection. A representative picture of these organoids in the ICC scaffold is shown in Figure 4A.

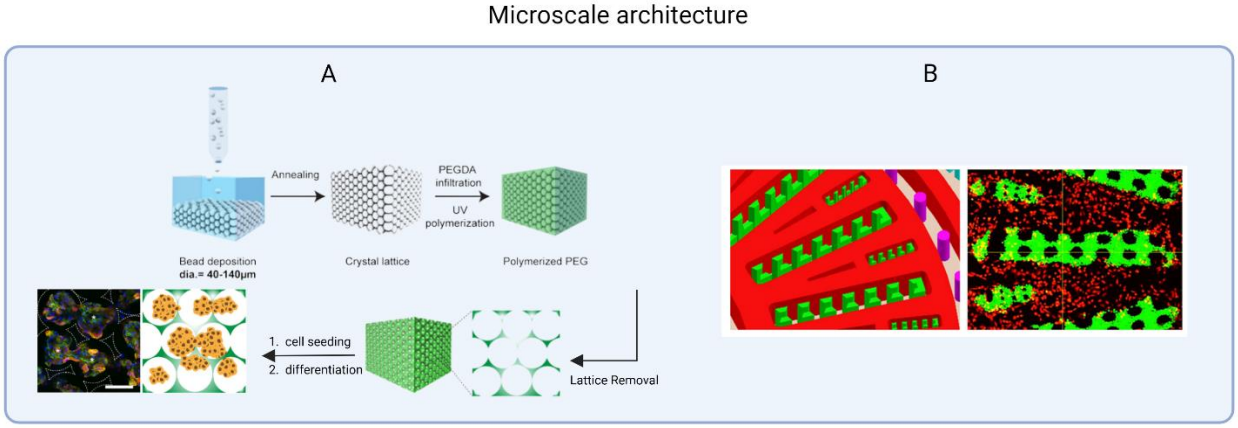


Figure 4: (A) Overview of the ICC development process of Seng et al.[56] providing a long-term HepG2 culture. (B) Design of the microtissue mimicking the liver lobule architecture developed by Zhao et al.[161] with the corresponding cell imaging after seeding of the co-culture. HepG2 cells and HAECs were respectively pre-stained in green and red with cell tracker dyes.

Zhao *et al*.[161] developed a 3D liver lobule-like microtissue with biomimetic morphological architecture. The model, shown in Figure 4B, resembles the sinusoidal-like structure and neighboring cells exhibited

a cord-like arrangement. The construct was designed by AutoCAD and fabricated with PDMS using multilayer soft lithography. The device consisted of a radial micro-pattern of multiple pillar arrays and a pneumatic microvalve system. The device was coated with collagen and seeded with HepG2 and a human aortic endothelial cell line (HAEC). Although the culture was only performed during 4 days, the morphology, activity of CYP-1A1/2 and UGT were highly preserved.

3.5 Micropatterning

Micropatterning is a technique to better control cell shape, position, spreading and multiphase architecture of tissue at different scales and complexities.[162,163] Since the liver is a very complex organ, this technique can ensure the replication of the native liver architecture at cellular as well as tissue level. Different approaches are available depending on the application, but they all result in a surface with areas of different adhesiveness towards certain cells.[163]

Micropatterning of cells can be achieved by modifying a substrate with biomaterials to create micropatterns[164], by using deep elastomeric microchannels[165] or elastomeric PDMS stencils[166], by applying a laser[167], by plasma ablation or through direct cell printing at specific locations[168]. Some examples will be discussed in the upcoming sections.

Applying a PDMS stencil is one method to realize micro-patterning. Yekaterina *et al*.[169] applied this method visualized in Figure 5A to establish a co-culture between Kupffer cells and hepatocytes. A negative photoresist pattern was applied onto a glass wafer using photolithography. Furthermore, liquid PDMS was spincoated onto the wafer, baked and peeled-off. The stencils provided 2 mm diameter islands of hepatocytes with 2 mm space in between for Kupffer cells (KC). For cell seeding, the wells were coated with collagen and the stencil was placed in the wells. Hepatocytes were seeded on top of the stencil. After removal of the stencil, KCs were seeded, which attached to the free space in between the hepatocyte islands.

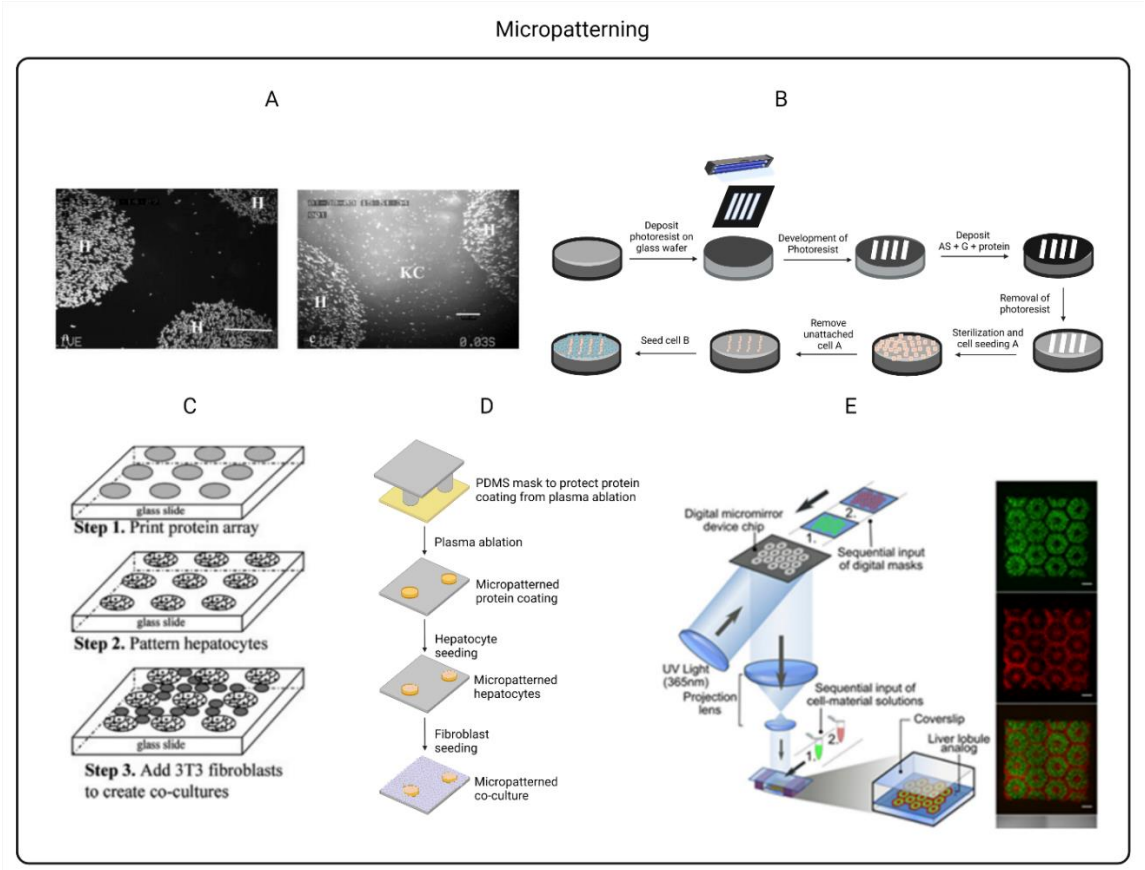


Figure 5: (A) Resulting cell image of the PDMS-stencil based micropatterned model developed by Yekaterina et al.[169] A co-culture of hepatocytes and Kupffer cells was established. (B) Schematic overview[170] of the UV-based micropatterning process using a mask of Bhatia et al.[171] Cell type A corresponds to primary rat hepatocytes, and cell type B to fibroblasts. (C) Schematic representation of micropatterning using protein contact-printed microarrays. This method was used by Lee et al.[172] and Revzin et al.[173] (D) Schematic overview of the plasma ablation technique using a physical mask by Lin et al.[174] (E) DLP-based 3D-bioprinting as micropatterning technique used by Ma et al.[175] to establish a co-culture of hiPSC-derived hepatic cells, HUVEC and mesenchymal cells.

A second method developed by Bhatia[170] et al exploiting borosilicate glass wafers was also used by Yekaterina et al[176]. The wafers were first cleaned with Piranha solution, rinsed, dried with nitrogen and baked. A positive photoresist was spincoated over the wafer, soft baked and exposed to UV light through a mask. Subsequently, the wafers were baked hard. To ensure cell attachment, the wafers were coated with glutaraldehyde-crosslinked rat tail collagen I. After removing all photoresist in an ultrasonic machine, a patterned protein coating remained. The wafers were placed in a well plate and hepatocytes were seeded on top. After 2 hours of incubation, unattached cells were washed away. After 24 hours, KCs were seeded and attached onto the free space on the glass wafers. The different steps of this method are shown in Figure 5B.

Interestingly, the outcome of both methods revealed that over time, the KCs migrated towards the hepatocyte islands until an ideal heterotypic cell-cell contact was established. This significantly improved the function of the hepatocytes as evidenced by an increased urea and albumin production. Also the ability for clearing ammonia was maintained during the culture period of 10 days.

This second method (Figure 5B) was applied before by Bhatia[171] et al to establish a co-culture of primary rat hepatocytes and fibroblasts. They compared a random co-culture of hepatocytes and fibroblasts with a co-culture established using micropatterning. They concluded that micropatterning provided superior control over the homo- and heterotypic cell-cell contacts as compared to a random co-culture resulting in a steady state urea production and an increasing albumin excretion over the

culture period of 11 days. The latter technique was also applied for a co-culture of primary human hepatocytes with mouse embryonic fibroblasts and could sustain the hepatocytes for 4 – 6 weeks in culture.[177] Finally, Ware et al[178] extended this model to a triculture using primary hepatocytes, mouse embryonic fibroblasts and LSEC. The hepatic and endothelial phenotype remained stable for 3 weeks.

Another method includes printing of protein microarrays onto a glass substrate[172,173]. The glass slides were cleaned with Piranha solution, rinsed and dried under nitrogen. Subsequently, the glass slides were oxygen plasma-treated and placed into a solution of 3-(acryloxypropyl)-trichlorosilane in order to perform silanization of the glass. Next, the slides were rinsed and dried and protein microarrays were contact-printed using a microarray spotter. HepG2 cells were first seeded onto the microarrays followed by a rinsing step. After incubation, the glass was exposed to a fibroblast suspension to create a HepG2-fibroblast micropatterned co-culture. This process is explained in Figure 5C. Preservation of cell functionality was evidenced by a preservation of albumin and urea synthesis over a course of two weeks.

Lin et al[174] made use of the plasma ablation technique (see Figure 5D) to micropattern islands of PHH surrounded by fibroblasts. Using this technique, a PDMS stamp is applied as a physical mask to selectively ablate a protein (herein collagen) coated glass or polystyrene substrate. The PDMS stamp is brought into contact with the protein coated substrate and an oxygen plasma is applied. Only the protein coating that is in contact with the stamp remains after plasma treatment. Next, PHH were seeded and attached onto the collagen islands. After a washing step, 3T3-J2 murine embryonic fibroblasts were seeded. These cells adhered to the regions between the collagen islands, hence surrounding the hepatocytes. Similar to the previously mentioned micropatterning methods, the co-culture outperformed a hepatocyte monoculture and the cells remained viable and functional (based on CYP450 activity) during 4 weeks of culture.

Ferrari et al[179] applied the micropatterning technique with microfluidics establishing a liver-on-chip model in combination with colon cancer cells containing a connected liver chamber and a colon cancer chamber in order to check to what extent the prodrug Tegafur (UFT) can kill colon cancer cells after metabolization (into 5-fluorouracil (5FU)) by the liver. A collagen-coated glass slide was micropatterned (i.e. separated collagen islands) using the plasma ablation technique. The glass slide was bonded to a microfluidic layer in order to establish the microfluidic chip. HepG2 cells were seeded onto the collagen islands and NIH-3T3 (murine embryonic fibroblasts) were surrounding them in the liver chamber. A colon cancer cell line (HCT-116) was seeded in the cancer chamber. The cells in the liver compartment showed excellent viability up to one week. The outcome of the experiment confirmed the hypothesis. When 5FU was directly added to the colon cancer cells, a direct decrease in cell viability was observed. However, when UFT was administered through the liver chamber before the cancer chamber, a delayed decrease in colon cancer cell viability was observed. The latter indicated that HepG2 successfully converted the prodrug UFT into the 5FU metabolite being toxic for the colon cancer cells.

DLP-based 3D-bioprinting has been exploited as a micropatterning technique to resemble the pattern and cell composition of the liver by *Ma et al*.[175] Two hydrogels were created encapsulating different liver cell types, including a gelatin methacryloyl (GelMA) layer containing human induced pluripotent stem cell (hiPSC)-derived hepatic cells and a glycidyl methacrylate-hyaluronic acid (GMHA) layer containing HUVEC and mesenchymal cells. Complimentary hexagonally shaped patterns were sequentially created in these hydrogel layers using Digital Light Processing (DLP) based 3D-printing. This resulted in a 3D-model consisting of an array of liver lobule structures characterized by physiological dimensions (see Figure 5E). Interestingly, the cells maintained intrinsic hexagonal structures and reorganized themselves within the construct leading to expression of E-cadherin and

albumin evidencing a favorable cell-cell contact. Analysis of the markers albumin, Transthyretine (TTR), hepatocyte nuclear factor 4alfa (HNF4a) and different CYP's suggested a gradual maturation of the cells into the haptic lineage. After day 7 post-printing, the hiPSC-HPC can be considered matured and are able to respond positively to rifampicin in the same extent as reported before for primary hepatocytes. Furthermore, the model shows both phenotypic and functional enhancements in the hiPSC-HPCs over weeks of *in vitro* culture.

3.6 Zonation

In the liver models described so far, all hepatocytes are considered functionally identical. However, in the native liver, all hepatocytes within the same hepatic plate are functionally slightly different depending on the zone to which they belong to, as defined by the distance to the center of the hepatic plate (i.e. zonation, see section 2. Liver Anatomy).

A perfused flat plate bioreactor, as schematically presented in Figure 6A, was described to create a controlled oxygenated medium flow[180]. The bioreactor was loaded with a collagen-coated plate (glass or PDMS) seeded with a co-culture of primary hepatocytes (human or rat) and fibroblasts or stellate cells. The medium was controlled and equilibrated with a mixture of O_2, CO_2 and N_2 at the inlet and the oxygen content was measured at the outlet. The system was stabilized during 5 days and drug exposure was performed between day 5 and 7.

The outcome of the model[180] showed heterogeneity in CYP2B and CYP3A enzymes along the different zones of the hepatocytes and a similar acetaminophen (APAP) cell death pattern as in *vivo*. Indeed, the medium that reaches the first zone of hepatocytes was still oxygen-rich, diminishing the CYP activity. The more distant zones were gradually exposed to medium with a lower oxygen level, enhancing the CYP activity and therefore, increasing the formation of the toxic APAP metabolite (N-acetyl-p-benzoquinone imine) NAPQI. Various similar models described in literature reported on a zone-dependent expression of the genes studied.[181]

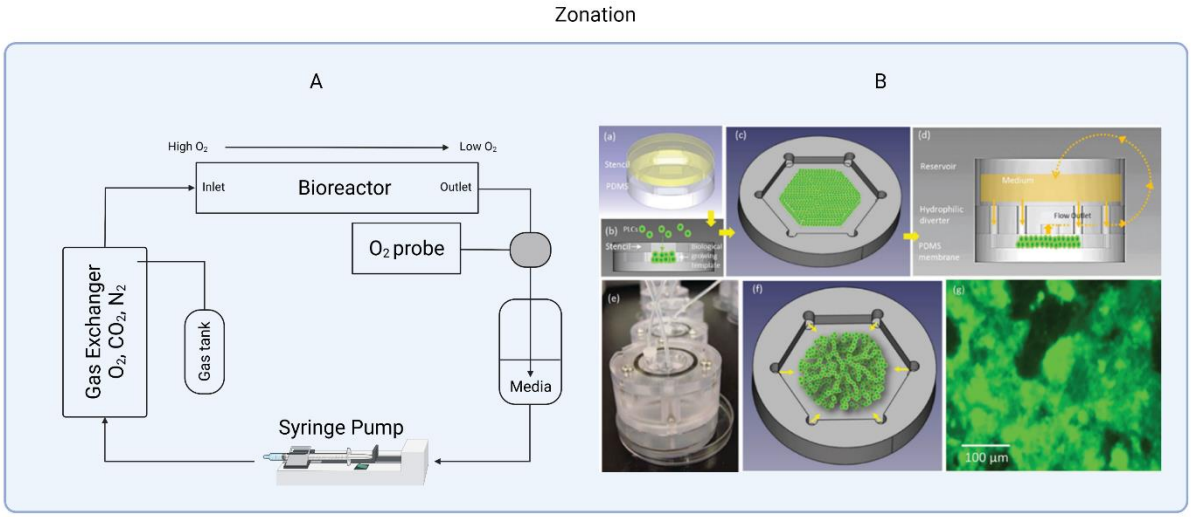


Figure 6: (A) Experimental bioreactor set-up to induce zonation in the hepatic model described by Allen et al.[180] (B) The approach of Weng et al.[182] to mimic the hexagonally shaped hepatic plate to investigate the occurrence of zonation.

Besides the gradual differences in functionality, morphological changes have been described as well. A co-culture of primary rat hepatocytes and stellate cells was seeded via a micro-patterned hexagonally shaped PDMS membrane. The medium flowed radially from 6 inlets, each at a corner of the hexagon, into the chamber and was collected at the center of the hexagon resembling the flow from the portal to the central vein in a hepatic plate. The flow removed the unhealthy cells and

connected clusters with space in between were formed, which closely resembled the sinusoids present in the hepatic plates as visualized in Figure 6B.[182] Albumin and urea were maintained during 14 days and significantly higher than the control cultured in a Petri dish. The metabolic capability as well as the metabolic dynamics were maintained during 14 days, which was evidenced by the response in cell behavior after exposing the model to drugs. Rifampin and ketoconzazole are respectively a CYP inducer and inhibitor. Indeed, 12 hours after drug administration, the metabolic activity increased in the rifampin and decreased in the ketoconzazole group. When administering the hepatotoxicant APAP, desensitization occurred in the 2D-control. However, in the perfused model, regional heterogeneity of APAP-induced hepatotoxicity was observed. Interestingly, on day 7, more cell damage occurred in zone 2, as compared to in zone 1 - the latter being the first cells that come into contact with APAP. This can be explained by the fact that the medium still contains a lot of oxygen when flowing through zone 1. Oxygen reduces the CYP activity, leading to less production of the toxic NAPQI metabolite. When the flow reaches zone 2, less oxygen is present to reduce CYP activity, hence leading to higher formation of NAPQI and increased cell death.

Models that regulate the oxygen concentration have also been reported. The reactors typically consist of multiple compartments resembling the different zones in the hepatic plate and are exposed to an oxygen gradient resembling physiological conditions.[181,183]

As mentioned before, the composition of the blood changes continuously when flowing through the hepatic lobule. The models described so far only took into account the change in oxygen concentration throughout the hepatic lobules. However, other compounds present in the blood change in concentration as well. Examples of important molecules are various metabolic modulators such as hormones and enzymatic inducers. A variation of the concentration of the latter compounds throughout the hepatic lobule, results in a gradient in nitrogen and carbohydrate production, but also in xenobiotic metabolism.[132] For example, a gradient in the insulin/glucagon balance (i.e. hormones) results in a gradient in carbohydrate, glucose and urea production of the hepatocytes.[184,185] Another example is when a gradient is induced in the CYP1A enzyme production using 3-methylcholanthrene (3MC) (i.e. inducer), this gives rise to a gradient in cell viability after administration of APAP. [186,187]

Monitoring of the cell culture conditions in reactor-based models are challenging. Conventional tools such as optical measurements, collecting supernatants or cellular samples are not always straightforward and time-consuming to use, especially during an ongoing experiment. In- and output of the reactors can easily be monitored[108], but does not always provide accurate information on what happens in the cell culture area. Examples of sensors integrated in the cell culture area without disturbing functionality has also already been described.[188] Moya et al[189] described for the first time the integration of an inkjet printed amperometric oxygen sensor (consisting of a work and counter electrode) in the thin and porous membrane of the ExoLiver (*vide supra*) liver-on-chip-system enabling real-time dissolved oxygen monitoring being the most important parameter in terms of zonation. The oxygen gradient along the hepatocyte culture could be monitored at each point where an electrode was present. An oxygen gradient along the PHH of 32.5 % was measured.

3.7 Liver organoids

Liver organoids are discussed in this separate section since they can be considered an emerging technology within liver tissue engineering, although they don't require advanced processing techniques in order to create an environment mimicking the native liver as they are self-organizing into 3D hollow cyst-like structures.[190] Liver organoids are defined as 3D-structures derived from stem cells, progenitor or differentiated cells that self-organize through cell-cell and cell-matrix interactions recapitulating aspects of the native liver tissue, including hepatic cells' functions. These structures are considered mini-versions of the liver.[191] One of the main bioengineering questions in organoid research

is situated around the development of new matrix materials to support organoid culture and the optimization of the culture conditions in order to obtain fully matured hepatocyte-like cells.[192] The matrix development can be subdivided into two main aspects, being the chemical composition and the mechanical properties. It is known that the proliferation and differentiation of organoids towards hepatocyte-like cells are highly subjected to mechanical cues.[193] Also the chemical composition can promote proliferation and differentiation of organoids via biological cues, although several studies currently focus on the development of chemically defined materials[194,195] to replace the standard used murine originated tumor matrices derived from Engelbreth-Holm-Swarm (EHS) sarcoma, such as Matrigel.[196] The application of organoids in in vitro drug testing can take into account the population diversity, or even personalized drug testing with a patient's own cells.

Mun et al[69] established a model consisting of mature human hepatic organoids derived from human pluripotent stem cells to evaluate drugs that induce steatosis. The organoids exhibited toxic responses to clinically relevant concentrations of APAP and two drugs that have been withdrawn from the market (troglitazone, trovafloxacin) due to hepatotoxicity related to steatosis. A high-throughput model for liver cholestasis (i.e. impairment of bile-flow) was established by Shinozawa et al[197] who developed human pluripotent stem cell-derived liver organoids from 10 different patients in a reproducible manner with the formation of bile canaliculi-like structures. They were able to predict the toxicity of 238 marketed drugs with known cholestatic toxicity with a specificity of 88.9%. These organoid cultures were also subjected to continuous perfusion with culture media via a microfluidic device[198]. This enhanced the liver-specific functions such as albumin secretion and urea synthesis and exhibited an increased sensitivity towards hepatotoxicity. This could be further extended towards coupling with other microfluidic devices containing different organoids towards multi-organ-on chip models. A combination of heart and liver was described by Yin et al.[199], in which the cardiac toxicity triggered by the antidepressant drug clomipramine was assessed after being metabolized by the liver using a co-culture of liver and heart organoids separated by a porous membrane. Albumin and urea were maintained in the liver organoids, while the beating function of the heart organoids was preserved before exposure to the compound. After the system was exposed to the drugs, the function of the heart organoids was impaired showing the toxicity of the compound after being metabolized by the liver.

Since organoids are currently being cultured using the ECM-dome method, there is only little control on the microenvironment, distribution, reproducibility, as well as organoid size, density and mass. To overcome these problems, Xu et al[200] developed a PEG-based micropatterned cell-adhesion substrate to culture individual liver organoids in a controlled way in order to promote the application of organoids for high-throughput DILI testing resulting in an in vitro model representative for the fetal liver. Jiang et al[201] developed another micropatterned organoid model using microfabricated hexagonal closely packed cavity arrays, with a single organoid in each cavity. In both micropatterned models, human hiPSC were differentiated towards liver organoids. Interestingly, in the resulting organoids, hepatocytes as well as non-parenchymal markers were expressed. In these studies, exposure to APAP induced liver toxicity, and in one case, fibrosis was also induced.[201] These systems could be further improved by increasing complexity via the introduction of microfluidics enabling dynamic culturing conditions. The application of a co-culture (cfr. Section 3.5: Micropatterning) could further improve maturation and sensitivity towards the detection of DILI for drugs with different toxicity pathways.

The feasibility to create complex and customizable architectures combined with human adult liver organoids has also been investigated using biofabrication techniques such as extrusion-based 3D-printing[202] and volumetric bioprinting.[64] The extrusion-based constructs showed a comparable APAP

toxicity to non-printed controls. Volumetric bioprinting allows for developing perfusable complex constructs enabling the integration into organoid-on-chip models.

4. Application potential of DILI models with increasing complexity

In the current review, an overview is provided regarding the different approaches to develop superior hepatic models in order to serve detection and mechanistic insight in DILI. Hepatocytes are highly influenced by the environment they are cultured in, including the chemical composition, the surrounding cell types and the microscale architecture mimicking the in vivo cell-cell and cell-ECM contacts.[9] In this regard, it is clear that hepatocytes will exhibit a more in vivo-like phenotype, and thus more reliably respond to drug compounds, when an in vivo-like complexity is recreated.[26] This leads to an improved toxicity assessment resulting from in vitro models according to the following order: 2D-monoculture[203] – 2D-co-culture[204] (e.g. KC, stellate cells, LSEC) – 3D-culture[205] – 3D-co-culture[206] with an increasing sensitivity for drug compounds as evidenced for reference drug compounds, such as APAP. Furthermore, the greater the native liver architecture is mimicked, the better the hepatotoxicity is predicted.[207]

A very important feature of in vitro DILI models involves their long-term cell viability and functionality, which is only preserved during several hours in case of conventional 2D-cultures resulting in a limited presence of phase I and II metabolic enzymes.[37] Within this short term, it is not possible to track the toxicity of drug compounds that lead to enzyme induction, immune-mediated toxicity (i.e. idiosyncratic DILI - iDILI) or compounds with a low clearance rate.[140,208] Hence, the latter would not be evidenced in 2D-cultures, rendering them inadequate for hepatotoxicity prediction.

3D cultured cells exhibit an improved viability as well as functionality enabling longer-term toxicity testing in experiments that with current technologies can last for up to one month. This makes the basic 3D-models more suitable DILI predictors, although only for a limited number of compounds. A study by Bell et al[100] comparing a 2D- and 3D-culture of PHH as model to evaluate the toxicity of APAP, bosentan, diclofenac, fialuridine, troglitazone and pioglitazone revealed that a 3D-culture was more sensitive in the detection of DILI than 2D-cultures. However, pioglitazone was not identified as hepatotoxicant in neither models, whereas this was detectable in a perfusable liver microtissue constituted by PHH and KC co-cultures[209]. This example evidences the need for more advanced 3D-models in order to increase sensitivity towards hepatotoxicity in vitro. Small molecules with straightforward metabolic pathways (e.g. direct cytotoxicity by the accumulation of reactive oxygen species (ROS))[210] can be assessed in basic 3D-models. More advanced models encompassing co-cultures of hepatic cells are needed for compounds that elicit toxicity as evidenced from a complex interplay between multiple cell types, for example molecular therapeutics such as gene therapy agents (e.g. siRNA).[211] The application of non-parenchymal liver cells in co-culture encompasses both a supporting function, to improve the phenotype of hepatocytes, but is also involved in eliciting DILI. This was evidenced through research from Li et al[142] during which drug compounds were tested in a 3D-model consisting of PHH which was compared to a 3D-co-culture of PHH with KC. More specifically, an increased sensitivity was revealed for trovafloxacin, paroxetine, indomethacin and sulindac when KC were included. Interestingly, tolcapone induced more cell death in the PHH in comparison to the co-culture, suggesting the protective role of KC in the DILI mechanism of this compound. Also in a 3D-co-culture of primary hepatocytes and stellate cells, the co-culture was more sensitive towards APAP and isoniazid toxicity compared to the hepatocyte mono-culture.[212] The latter can be explained by stellate cells' activation upon treatment with a hepatotoxin leading to the production of excessive ECM compounds.[213]

Since these findings are relatable to clinical data observed in vivo, this evidences that increasing the complexity of in vitro models towards superior mimicry with the liver in vivo does give rise to superior models for DILI prediction.

Compounds that are only toxic after multiple doses or that only show toxicity after being metabolized require better performing models encompassing cells that preserve a long-term stable phenotype. An example of this is the integrated biomimetic array chip containing a collagen-based 3D primary hepatocyte culture from Xiao et al[214]. This model allowed for drug treatment at day 7 and day 14 resulting in an increased toxicity for 15 compounds tested after the second dose, evidencing delayed toxicity. Furthermore, some compounds elicit toxicity dependent on the zone in the hepatic lobule, therefore a model including hepatic zonation (see 3.6 Zonation), is required to detect the toxicity of these compounds.

As evidenced in this paragraph, for every type of toxicity in assessing DILI, a different approach can be applied (see different sections in '3. Advanced models'). However, for drugs in development, there is no preliminary foundation to rely on, hence very little is known about the toxicity and the toxicity assessment has to start from the very basics. A model that 'fits all' still needs to be developed, but would have an enormous impact on the efficiency of drug induced liver injury assessment.

5. Conclusions and outlook

The ultimate goal when developing a superior *in vitro* liver model to test drug toxicity during the early phases of drug development, is to engineer a 3D-construct consisting of all relevant cell types present in the liver that can preserve its viability and functionality as long as possible. In this way a reliable model can be deployed in the drug development process even before the compounds are tested on animals. A fixed time frame for the cells to stay functional is difficult to estimate, since the manifestation of drug toxicity is highly drug dependent. Especially for immune-mediated idiosyncratic drugs, this toxicity is only revealed months after administering the drug.

Resembling the *in vivo* environment of the liver is clearly a key aspect in the development of a superior *in vitro* model. Hepatocytes require their *in vivo* 3D environment to guarantee similar cell-cell and cell-matrix interactions in order to maintain their functionality *in vitro*. This environment includes a similar architecture as well as similar chemical composition as the *in vivo* liver ECM. Decellularized ECM scaffolds represent the ideal microenvironment, but pose limitations when considered for high-throughput screening. Nevertheless, ECM scaffolds are interesting for matrix characterization, creating knowledge to be used as input for the development of engineered scaffolds, including information regarding the stiffness, chemical composition, microscale architecture and the presence of bioactive compounds.[215,216] The enormous progress that has been made in the last decade in the field of 3D-printing, micropatterning and hydrogel development really makes it possible to tune these properties to the desired targets. Combining the knowledge in these different fields will be a crucial aspect in the development of the future hepatic models.

Besides the cell-ECM interactions, cell-cell crosstalk is also a key aspect to preserve functionality of the cells leading to reliable detection of the hepatotoxicity of drug compounds. Models currently encompass limited variety of cell types. To fully recapitulate the liver function, all cell types present in the liver should be represented in the model. Primary cells are the gold standard, since they show the ultimate phenotype that needs to be reached *in vitro*, but their lack of availability remains a major drawback in tissue engineering approaches to liver modeling. Stem cells have been described for many years as very promising alternatives and are, depending on the source, more abundant. However, methods to differentiate them into mature hepatocytes still need optimization. Stem cells have also

the advantage of fulfilling the personalized medicine principle, with the potential of developing patient-specific models to study drug toxicity or efficacy.

Preservation of long-term viability in combination with functionality of hepatocytes still remains a major challenge when developing an in vitro liver model. Especially some key metabolic enzymes are crucial when developing a model for drug-induced liver injury.

Primary hepatocytes are the gold standard, but do not remain functional when placed in culture over a long time period.[217] Cell lines however are immortalized and highly proliferative, but do not express enzymes to the same extent as primary hepatocytes.[218] For example, the HepG2 cell line is very limited in its expression of drug-metabolizing enzymes and transporters, especially those involved in phase I metabolism such as the CYPs.[40] Some initial research has already exploited gene modifications to combine the immortality of cell lines with the overexpression of certain genes that encode for important metabolizing enzymes in order to mimic the CYP540 metabolizing features.

The most abundant metabolizing CYPs are CYP2C9 and CYP3A4. Via a lentiviral expression vector, CYP2C19[219] and CYP3A4[220] overexpressing HepG2 clones were generated. The function of these enzymes was assessed in a metabolism-dependent cytotoxicity experiment using various xenobiotics. The HepG2 clones exhibited a higher sensitivity to the xenobiotics compared to the parental HepG2 cells. Furthermore, some of the important metabolites were only detected in the clones. This approach is very interesting to further investigate in combination with the more advanced in vitro models discussed in this review.

Another very important and little explored aspect in in vitro liver models is the integration of the immune system.[221] When considering the bigger picture *in vivo*, immune cells should not be neglected. Two types of immune cells can be distinguished, including the intrahepatic innate immune cells and the adaptive immune system. The innate immune system consists of the Kupffer cells, the liver sinusoidal endothelial cells, the dendritic cells, the natural killer (NK) cells and the natural killer T (NKT) cells. It represents the initial fast immune response and has by consequence its main contribution regulating liver injury, fibrosis, and regeneration.[222] This immune response can either evade or increase tissue damage under the influence of the pathways initiated by xenobiotics.[223]

The adaptive immune system, consisting mainly of T cells and natural killer T cells generally comes in place with a latency and upon exposed to repeating dose of the xenobiotic. This can manifest between 1 – 8 weeks, but examples of 12 months are known as well.[224] Furthermore, the immune response is person-dependent and complex, while the underlying mechanisms are mainly unclear rendering it very difficult to predict. Due to the high vascularization of the liver, circulating leukocytes can also be recruited upon activation of relevant signaling pathways.[221] However, substantial research remains needed since reliable models are still lacking.

An important next step is to first and better understand the interactions ongoing between immune cells and other liver cells. Current models focus on studying immune mediated DILI (im-DILI) by treating hepatocytes with im-DILI associated cytokines or conditioned media from immune cells instead of directly adding immune cells to the culture.[221]

On the other hand, immune cells have already been treated by direct addition of hepatotoxic compounds or through the addition of danger-associated molecular patterns (DAMP) released by apoptotic or necrotic hepatocytes after drug injury. Nevertheless, little is known about the direct interaction between immune cells and hepatocytes.[225]

Kupffer cells represent the 80-90% of the macrophages in the human body and are the main immune cells studied in more complex in vitro liver models to date.[226] The expansion and the inclusion of other

immune cell types remain to be optimized and a new dimension in architectural design should be included in order to be able to mimic the sequence of immune events. However, it is clear that much more research is required to predict immune mediated DILI in a reliable way, which will be inevitable in the future, when aiming to create superior *in vitro* models.[221]

Besides from the construct of the model itself and the cell type, the environment is also very important. *In vivo,* the liver is perfused by the blood stream, which is very poorly mimicked using static culture conditions. A dynamic culture using a bioreactor represents this blood flow with much more reliability. Especially the occurrence of zonation is mimicked more reliably in this way, which has turned-out to have an impact on the metabolism of some drug compounds, and by consequence on their toxicity.[227]

Developing superior liver models remains the ultimate goal, and the development and use of appropriate analytical techniques are also inevitably a key aspect of this challenge. In static cultures, this encompasses the detection and quantification of analytes at distinct time points[41], while in dynamic cultures, parameters such as the composition, pressure, temperature, and flow rates of fluids at the inlet and outlet should be controlled and analyzed.[152] However, the analyses are mainly performed off-line[228] and at specific end points, meaning that gradual changes or intermediate or short living analytes may not be detected.[229] More advanced in situ, longitudinal and real-time monitoring should elevate our understanding on what happens in the cell culture at a higher level. This understanding will lead to superior and more specific treatments for DILI and associated diagnostics.[230] Ideally, the sensors should be incorporated in the culture systems without disturbing the cells.[188] These sensors should allow for the detection of diagnostic enzymes in serum such as alanine transaminase, aspartate aminotransferase, bilirubin, glutathione S-transferase and other DILI indicators such as reactive nitrogen, reactive oxygen or reactive sulfur species in a sensitive, simple and specific way.[229] Also, multi-detectors are being researched to explore DILI mechanisms given the complexity of the physical environment. Sensors exist that make use of fluorescence[231], circular dichroism[230], photo-acoustics[230] and optics[232] or a combination thereof.

The closer we get to mimicking the *in vivo* hepatic microenvironment, the better the phenotype and viability of the hepatocytes is maintained and the more reliable the outcome of the *in vitro* experiment will be. However, comparison of the different models described in literature is not straightforward. As described in this review, a plethora of hepatocyte-based *in vitro* systems have been developed for toxicity testing over the past years. The main problem is that a wide variety of parameters are being exploited to assess the performance of the *in vitro* models. Some researchers only report on the viability when evaluating their models, while others report on the functionality characterized by gene expression, protein expression, morphology or toxicity.

There clearly exists a need for more transparency and a defined set of criteria for proper benchmarking. Vinken et al.[233] have published a proposal for a possible validation method of *in vitro* models based on several important parameters accompanied with suggested analysis to characterize the performance of the developed model. This validation encompasses a combination of requirements regarding cell viability, morphology, functionality and the ability to reproduce the human *in vivo* intrinsic drug-induced liver injury.

Besides the need for a proper comparison between the different models, future research should lead to innovative combinations of the different approaches in order to strive towards a model that fully resembles the native liver in all aspects, hence paving the way towards more reliable *in vitro* liver models.

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