An alternative approach based on microfluidics to study drug metabolism and toxicity using liver and intestinal tissue

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The research presented in this thesis was financially supported by ZonMw (program Alternatives to Animal Experiments, project number 114000074) and was performed at the University of Groningen in the Pharmacokinetics, Toxicology and Targeting Group and in the Pharmaceutical Analysis Group, which are both members of the Graduate School GUIDE.

The printing of this thesis was financially supported by:
Groningen University Institute for Drug Exploration (GUIDE)
Rijksuniversiteit Groningen
ZonMw

Paranimfen: Laurens-Jan Jellema
Marjolijn Merema

ISBN printed version: 978-90-367-4597-0
ISBN electronic version: 978-90-367-4598-7

Cover design: Annelies van Midwoud

Printing: ProefschriftMaken.nl

RIJKSUNIVERSITEIT GRONINGEN

An Alternative Approach Based on Microfluidics to Study Drug Metabolism and Toxicity Using Liver and Intestinal Tissue

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
vrijdag 26 november 2010
om 14.45 uur

door

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geboren op 18 juli 1983
te Hoogeveen

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Introduction: Scope of the Thesis

Introduction

Over a few thousand medicines are currently under development. In preclinical testing, those new candidates are first tested on animal and human cells (*in vitro*) and in living animals (*in vivo*). By extrapolating the information obtained *in vitro* to the *in vivo* situation, a prediction is made of the pharmacokinetics, pharmacodynamics and toxicity of a medicine in the human body. If the drug successfully passes the preclinical phase, that is, it exhibits promising therapeutic effects with no accompanying toxicity, it is tested in humans in clinical trials. Despite all the preclinical testing, however, a substantial number of potential new medicines entering clinical trials still fail due to unforeseen toxicity, an undesirable pharmacokinetic profile or lack of effect.[1] As alluded to above, prediction of the effects of xenobiotics on biological pathways in the human body is based on our knowledge of the biological pathway in animals such as rats, mice, dogs or monkeys. This prediction has often proven to be unreliable due to important species differences in cell biology. In particular, drug metabolism in humans and animals is often very different.[2] To avoid interspecies extrapolation, human cells and tissue is also used in preclinical testing. However, human tissue is scarce and validation of *in vitro* models to fully predict the *in vivo* situation has not been accomplished yet. Therefore, there is an incessant drive for developing new *in vitro* systems that are more predictive for man *in vivo*. Moreover, these *in vitro* systems can contribute to minimizing the use of experimental animals.

The goal of this thesis was to contribute to a better prediction of drug metabolism and toxicity in man with a concomitant reduction in the use of experimental animals. The research focused on the development of an improved *in vitro* system to investigate drug metabolism and toxicity in rat and human liver and intestine.

Precision-cut tissue slices (PCTS)

The metabolism of xenobiotics, including medicines, occurs largely in the liver.[3] The liver has a complex architecture consisting of several cell types (Figure 1). The parenchymal cells are hepatocytes, which represents 60-65% of all cells.[4] Of the remaining 35-40% of the cells, Kupffer, endothelial and stellate cells are the most abundant. As most of the enzymes involved in drug metabolism are expressed in the hepatocytes, most *in vitro* metabolism studies are performed using freshly isolated hepatocytes cultured as monoculture. However, hepatocytes alone do not represent the full spectrum of all liver functions. For example, Kupffer cells play a role in mediating hepatic toxicity,[6] and stellate cells are involved in the development of fibroses.[7] Moreover, interaction between hepatocytes and other cell types is important in the physiology and pathology of the liver. Precision-cut liver slices (PCLS) have been shown to be a powerful research tool because they contain all cell types in their natural environment to incorporate those multicellular interactions. PCLS are pieces of intact

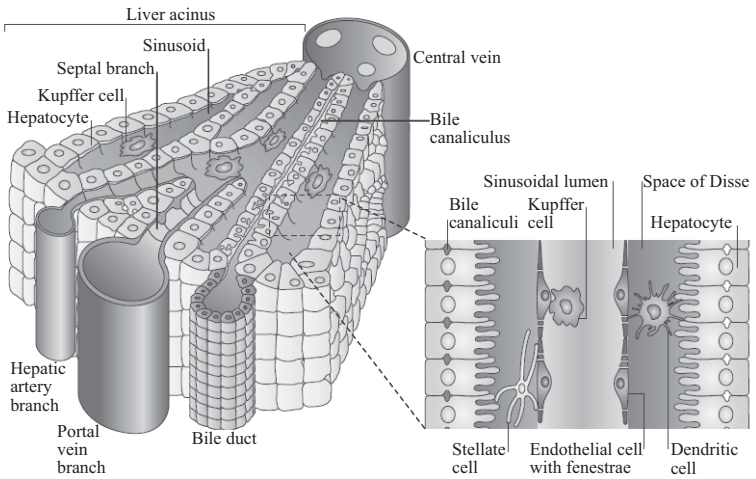

Figure 1. Schematic view of the liver architecture (figure reprinted with permission from Adams and Eksteen[5]).

organ, cut into slices with a very reproducible thickness. The first example of the use of liver slices was in 1923 by Otto Warburg.[8] However, at that time the slices were made manually, and were therefore too thick to exhibit good reproducibility in function and viability. *In vivo*, most cells do not survive when they are more than a few hundred micrometers from the nearest capillary.[9, 10] In the liver, every cell is adjacent to a capillary. Therefore, it is important to prepare thin slices of only a few hundred micrometers thickness, which is very difficult to do manually. Nowadays, slices can be made using specially designed apparatus such as the Krumdieck slicer or the Vitron slicer.[11] Both slicers are able to produce slices of 100 µm or more in a reproducible manner. The preparation is rather simple, and starts with the excision of the liver from the animal or human body. A hollow drill bit is then utilized to prepare cylindrical pieces of tissue (cores) with diameters ranging from 3 to 8 mm. These cores are placed in the tissue slicers, which produce thin precision-cut slices in ice-cold buffer (see Figure 2). The optimal thickness of liver slices generally lies between 100 and 250 µm.[13] In slices thicker than 250 µm, the diffusion of oxygen and nutrients toward the inner cells is limited and necrosis in the inner cell-layers of the slice occurs during incubation.[14] Precision-cut liver slices were successfully used to study many liver-specific processes, for example the metabolic pathways and rates of metabolism of xenobiotics[15], xenobiotic-induced toxicity[16], mechanism of fibrogenesis[17], or gene transfection[18]. The production of PCLS is straight-forward, and it is possible to prepare slices from all kind of animals and from human material, allowing the study of interspecies differences.[2, 19] Human material can be obtained from redundant pieces of liver after surgical resection, which is considered waste material. Many slices can be prepared from one small piece of tissue (up to 200-250 slices can be obtained per

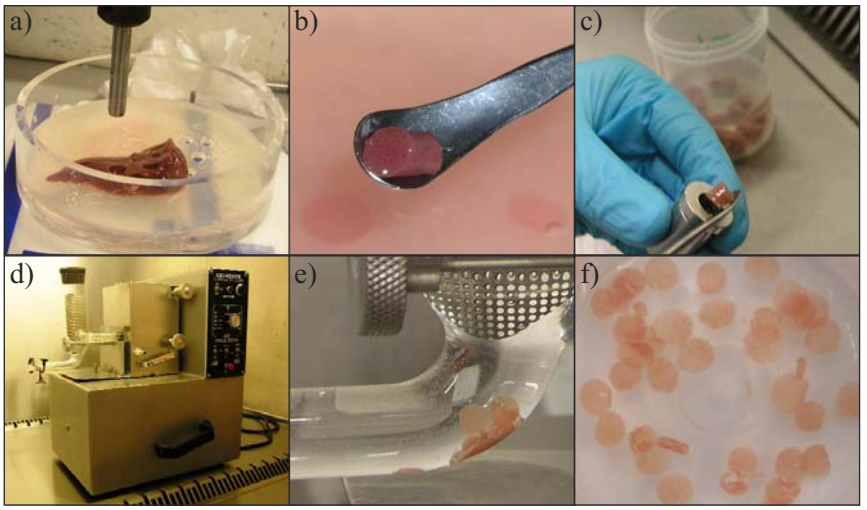

Figure 2. Preparation of liver slices. Liver cores are prepared from the intact organ using a hollow drill bit (a-b) and placed in the tissue slicer (c-d). In the tissue slicer, precision-cut slices are produced in ice-cold buffer (e-f). Photographs reprinted with permission from De Graaf et al.[12]

10 gram tissue), and treatment of slices from different sources can be performed in a similar manner. Metabolism and toxicity of xenobiotics in rat PCLS has already been proven to correlate well with the rat *in vivo*.[15, 20]

Since the liver is not the only organ involved in the metabolism of xenobiotics, slices from other extra-hepatic organs have also been used to predict *in vivo* drug metabolism. Precision-cut tissue slices (PCTS) have been prepared from intestine[21, 22], kidney[15], lung[15], brain[23], heart[24], prostate[25], spleen[26] and tumors[27]. Most tissue can be sliced in a manner similar to liver slices. If necessary, the organ is first embedded in agarose and then sliced (for lung and intestinal slices).

Two incubation systems are currently used for PCTS, namely the dynamic organ culture system (DOC), and flask or well plate systems in which the slices are continuously submerged in culture medium.[13] In the DOC, the slice is placed on an insert and the slices are intermittently exposed to medium and air. In continuously submerged incubation systems, the slices are always floating freely in the medium and therefore not attached to a surface. Each incubation system has its own specific advantages and disadvantages, as described by others.[14] In both systems, the medium is only refreshed every 24 hours, which implies that the slices are exposed to a continuously changing environment. Incubation will result in accumulation of waste products and metabolites formed by the tissue slices, and depletion of nutrients. The accumulation of waste products might negatively affect liver function. The depletion of nutrients is prevented by using a rather large volume of medium (a few milliliters).

However, this large volume dilutes the metabolites formed by the slices, making it difficult to measure metabolite formation early during incubation due to the low concentration of metabolites. Another disadvantage of both of these incubation systems is the lack of ability to measure interorgan interactions. As mentioned above, more organs are involved in the metabolism of xenobiotics, and interorgan interactions do occur in the human body. For example, a liver-derived metabolite of naphthalene causes lung toxicity[28], and a liver-derived metabolite of acetaminophen causes kidney toxicity[29]. Such organ interactions can in principle be studied by co-incubation of slices from two organs in these conventional incubation systems; however, mutual interactions may take place, making it difficult to conclude which organ was responsible for the observed effect. Introducing a medium flow to perifuse the slices may be a solution for the non-steady state conditions in conventional systems, and for studying interorgan interactions by directing the medium formed by one organ slice to a second organ slice. This flow will also result in a constant incubation environment without an accumulation of products. However, when incorporating flow, it is important to use low volumes and flow rates of incubation medium to avoid dilution of the metabolites. This miniaturization and introduction of continuous flow with minimal metabolite dilution can be accomplished by making use of microfluidic technologies.

Microfluidics

Research in the field of microfluidics involves the development of miniaturized devices (microchips) and methods to control, manipulate, and analyze flows in sub-millimeter dimensions.[30] It is a multidisciplinary field encompassing chemistry, physics, engineering, microtechnology and biotechnology. The basis for the fabrication processes of most microfluidic devices is photolithography, a set of techniques originally developed to integrate semiconductor structures on microelectronic chips.[31] While microelectronic chips have integrated metallic and semiconductor pathways to transport electrons, however, microfluidic chips contain interconnected microchannel networks to transport minute (pL to μL) quantities of liquids. Microfluidic chips have been developed for various purposes, like real-time PCR[32], immunoassays[33], whole blood sample preparations[34], two-dimensional chromatographic separations[35], and to culture cells under flow conditions[36]. It is the creation of networks of interconnecting channels that makes the field of microfluidics so powerful.[31]

Photolithography is the most-used set of techniques to develop microfluidic devices, and exploits a photosensitive polymer, so-called photoresist, to pattern chip surfaces. Two types of photoresist exist, positive and negative photoresist. When positive photoresist is exposed to UV light, the exposed region becomes soluble and the unexposed region is polymerized. In the case of negative photoresist, it is the

other way around: the exposed region becomes polymerized and the unexposed area is soluble in appropriate solutions. Commonly, glass or silicon wafers are coated with a thin layer of photoresist. Subsequently, a latent image is formed in the resist by exposing it to ultraviolet light through a photomask with opaque and transparent regions containing the channel layout of the microchip. After patterning, the unpolymerized photoresist is removed by rinsing with an appropriate solvent, leaving the desired pattern behind on the wafer. Subsequently, the glass or silicon can be selectively dissolved with an appropriate etching solution like hydrofluoric acid (wet etching) or with e.g. reactive ion etching (dry etching), to form the structures in the substrate. However, silicon and glass are rather expensive, and silicon is opaque, which means it cannot be used for optical measurements in the UV or visible range.[30] Another approach is to fabricate chips in polymers, as shown in Figure 3, using a process known as soft lithography. Instead of using the wafer material to fabricate the chip, the wafer is now used as mold with a positive relief of the channel structure. This mold can be re-used multiple times. In this procedure an elastomer like polydimethylsiloxane (PDMS), a silicone rubber, is poured over the mold, and allowed to polymerize. In this case, the thickness of the original photosensitive layer determines the depth of the channel. After polymerization, the elastomer with structure can be gently peeled off the wafer (Figure 3). The open channel structure in the elastomer can be closed by placing another piece of elastomer or glass on top of it, to obtain a closed microchip. The bonding of these two pieces occurs by chemical interaction without the need of glue. The microfluidic-based chips presented in this thesis, except those presented in Chapter 8, are produced using soft-lithography and PDMS.

In general, microfluidic devices offer many advantages over conventional systems. Because chip volumes are minute, only small amounts of the tested substrates are needed, an important consideration during early drug development when large quantities of the new compounds are not yet available. Other benefits of reduced volumes include less waste and lower reagents costs compared to conventional systems, and safer use of radioactive or toxic compounds. The channels to transport liquids are small (sub-millimeter cross-sections), and therefore it is possible to incorporate multiple channels onto one chip to perform multiple reactions in parallel. This allows high-throughput analysis in a compact device. The analysis and response times are also much faster compared to conventional systems, due in part to the short diffusion distances. The control of fluid flows and composition on microchips is very precise, facilitating improved process control. An important issue in microfluidic devices is the surface-to-volume ratio, which is much larger compared to conventional systems. The effect of surface roughness and composition becomes dominant compared to conventional systems. This can be an advantage when interaction with the channel wall is preferred, for example for chromatographic separations. However, for many applications this is a drawback, resulting in adsorption of compounds onto

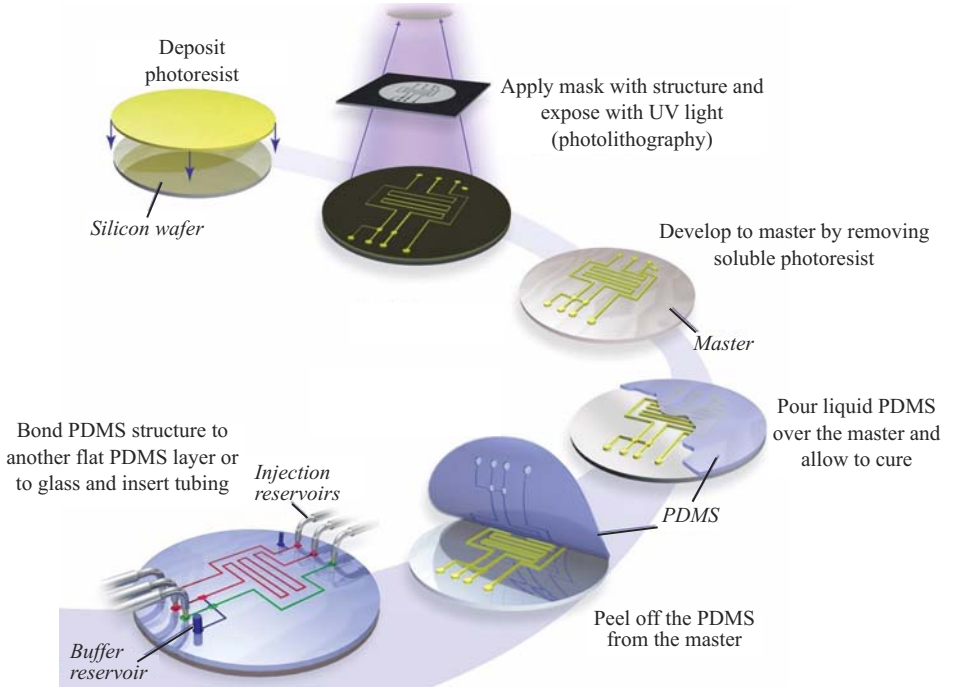

Figure 3. Fabrication of a polydimethylsiloxane device using soft photolithography (Illustration reprinted with permission from Sebastiaan Donders, Bionieuws 10-03-2006).

the channel walls.

With microfluidic technology, many new devices and applications have been and continue to be developed for various applications, in order to improve on conventional systems or to open up new applications.

Aim of the thesis

The research in this thesis was focused on the development of an improved *in vitro* system to investigate drug metabolism and toxicity in rat and human liver and intestine, with the goal to contribute to a better prediction of drug metabolism and toxicity in man with concomitant reduction in the use of experimental animals.

The first objective was to develop a microfluidic device in which precision-cut liver slices could be incubated under continuous flow conditions. By combining microfluidics with PCTS, an incubation system was realized to study the function of tissue with an intact cellular architecture incubated in a controllable and stable microenvironment. Due to the continuous flow, steady-state conditions are created, and accumulation of waste products and metabolites, and depletion of nutrients, will be avoided. The requirements for this microfluidic device were biocompatibility of

the material used and the maintenance of constant oxygen concentration, pH, medium composition, and temperature conditions over time. Various cell types have been integrated on a chip, however, no examples were available for the incorporation of PCLS in microfluidic devices. **Chapter 2** gives an overview of the current literature about available *in vitro* systems based on microfluidic technology which have been developed to represent liver metabolism. In this chapter also the incorporation of cells from two different organs is described to be able to measure interorgan interactions.

An extensive description of the final prototype biochip made of polydimethylsiloxane for the incubation of precision-cut rat liver slices is given in **Chapter 3**. Maintenance of the viability of the slices for 24 hours was possible, and short-term (3 h) metabolism of a model compound was assessed and verified with that obtained in the well plate system. In **Chapter 4**, the biochip set-up was further extended with an on-line analysis system for quantitative assessment of metabolites. The continuous flow of medium allows the samples of the outflow to be injected directly onto an on-line HPLC column. Such on-line analysis is very beneficial for the detection of unstable metabolites, which remain undetectable in conventional systems due to decomposition during incubation. The microfluidic system enables the measurement of time-dependent drug metabolism effects, and on-line inhibition and induction studies can be performed using a limited number of slices, which is especially beneficial for tests employing scarce tissue like human material. In **Chapter 5**, we succeeded in prolonging the viability of the slices to 72 h by embedding slices in hydrogel (Matrigel).

Since one of the goals of this research was to contribute to the reduction in the use of experimental animals during preclinical studies, one study was performed with human tissue integrated in the biochip. The successful integration of human liver slices in the developed microfluidic device is presented in **Chapter 6**. Their viability and metabolic competence was confirmed over 24 h of incubation.

Furthermore, the integration of rat intestinal slices in the biochip was accomplished, as demonstrated in **Chapter 7**. In addition, rat intestinal-liver interaction was studied by sequentially perifusing an intestinal and liver slice. The first *in vitro* data demonstrating interorgan interaction between intestinal and liver slices are also presented in **Chapter 7**.

Soft lithography techniques enable rapid prototyping of PDMS devices. PDMS is a very suitable material for chip production; however, its hydrophobic character is an important drawback. Due to its porous structure and hydrophobicity, hydrophobic compounds can absorb into and adsorb onto PDMS.[37] Because of the easy production process, the first prototypes were made of PDMS. However, we investigated as a final step whether plastics such as polycarbonate and polystyrene could be used as alternative materials. **Chapter 8** describes the fabrication of devices in several plastics and how to modify the surface of these materials to minimize adsorption. The

biocompatibility of the plastics was also tested by culturing HepG2 cells in the various devices made.

Finally, a summary, discussion and the future perspectives of the developed microfluidic-based incubation system are presented in **Chapter 9**.

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2

Microfluidic Devices for *In Vitro* Studies on Liver Drug Metabolism and Toxicity

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Elisabeth Verpoorte
Geny M.M. Groothuis

Submitted

Abstract

Microfluidic technologies enable the fabrication of advanced *in vitro* systems incorporating liver tissue or cells to perform metabolism and toxicity studies for drugs and other xenobiotics. The use of microfluidics provides the possibility to utilize a flow of medium, thereby creating a well-controlled microenvironment. The general goals of most *in vitro* systems in drug research are to optimally mimic the *in vivo* situation, and to minimize the number of animals required for preclinical studies. Moreover, they may contribute to a reduced attrition rate of drugs at a late stage of the drug development process; this is especially true if human tissue or cells are used. A number of factors are important in achieving good *in vivo* predictability in microfluidic systems, of which the biological system itself (tissue or cells) and the incubation conditions are the most important. The last couple of years has seen various microfluidic-based *in vitro* systems being developed to incorporate many different cells and/or tissues. In this review, microfluidics-based *in vitro* systems realized to study liver metabolism and toxicity are summarized and discussed with respect to their applications, advantages, and limitations. The biological basis of these systems as well as the incubation conditions are evaluated. Precise control of the cell or tissue microenvironment is a key advantage of using microfluidic technologies, and the benefits of exposing the cells to medium flow are demonstrated. Special attention is also paid to the incorporation of multiple cell types or tissues into a microfluidic device for the investigation of interorgan interactions, which are difficult if not impossible to study in conventional systems.

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1. Introduction

In vitro methods are widely used to investigate kinetic, metabolic, and toxicity (ADME-Tox) profiles early in the drug development process. Only new chemical entities (NCE) with appropriate ADME-Tox characteristics will enter *in vivo* animal studies and clinical trials. However, an average of only 10% of the drugs entering Phase I clinical trials is ultimately approved for the market.[1] One of the main reasons for this high rate of withdrawal is unforeseen toxicity.[2] Compared to preclinical tests, the costs of performing clinical tests are very high.[1, 3] For this reason, there is a demand to develop new *in vitro* systems which better predict toxicity *in vivo*. Since the liver plays the major role in the biotransformation of drugs, and is an important target organ for drug-induced toxicity, most of the *in vitro* systems incorporate liver cells or tissue. Various *in vitro* models are currently available to study liver metabolism and/or toxicity of NCE's, of which microsomes (a subcellular fraction), cell lines, primary cells, or intact tissue preparations are the most widely used. The differences between these *in vitro* models are substantial, and they all have their own advantages and disadvantages, as indicated in Tables 1 and 2 and described in other reviews.[4-8] In each study, the choice of the *in vitro* system used is mainly dependent on the type of information that is being sought. However, intact cells expressing physiological levels of drug metabolizing enzymes are clearly indispensable for a proper prediction of metabolism and toxicity.

Table 1. In vitro models used in the development of new drugs and discussed in this review, with their ability to mimic the in vivo situation.

	Cell free	**Subcellular fractions**	**Cell lines**	**Primary cells**	**Intact tissue**
In vivo resemblance					
Simplicity					
Ease of application					
Physiological integrity					

One of the causes for the limited predictive power of *in vitro* tests is the use of cells or tissues of animal rather than human origin. A second contributing factor is the impaired functioning of cells in established culture formats. Conventionally, cell-based *in vitro* experiments are performed in a multi-well plate format or in culture flasks. This well-established methodology, including the more sophisticated sandwich culture system, has been optimized over the past few decades, and has proven useful for the prediction of ADME-Tox profiles in humans for many drugs. However, it remains difficult to retain the full functionality of these cell cultures, as the expression of drug-metabolizing enzymes and transporters continuously decrease over time. An important issue with these systems is that the microenvironment during cell culture

is very different compared to the native environment, and is in addition continuously changing during culture. In well plates and culture flasks, the hepatocytes (parenchymal cells of the liver) are normally cultured in two-dimensional monolayers with an excess of medium over the cells, leading to an unphysiological medium-to-cell ratio. Moreover, they lack interaction with non-parenchymal cells (e.g. Kupffer cells and stellate cells) and are not exposed to the important shear-mediated signaling mechanisms induced by flow.[9-11]

New *in vitro* systems have therefore been developed based on microfluidic technologies, with the aim to provide cells with a microenvironment which resembles the *in vivo* situation more closely.[12-14] By making use of microfluidics, the applied medium flow can be controlled very carefully, thereby mimicking blood flow and vessels. Moreover, the composition of the medium can easily be altered during an experiment, and more physiological medium-to-cell ratios can be achieved as a result of small system volumes. Metabolites can be detected on-line at higher concentrations than in well plates, where metabolites produced early on in an experiment are substantially diluted. Metabolite-containing medium can also be directed to other cells integrated elsewhere on the chip. Even the complex architecture of an organ can be achieved by combining microfluidics with tissue engineering.[15, 16]

The number of publications using the keywords "*in vitro*" and "microfluidics" has increased tremendously over the last couple of years, according to the ISI Web of Knowledge (see Figure 1). This indicates the extensive growth in the number of new *in vitro* systems based on microfluidic technologies. Examples include microdevices in which cells were cultivated and incubated in micropatterns to recreate cell-cell interactions found *in vivo*.[17] In-line detection of metabolites on-chip has also been demonstrated.[18] Various cell types have been integrated on chip, and novel microscale systems have been developed with integrated concentration gradient generators to expose cells to various concentrations of drugs.[19-21]

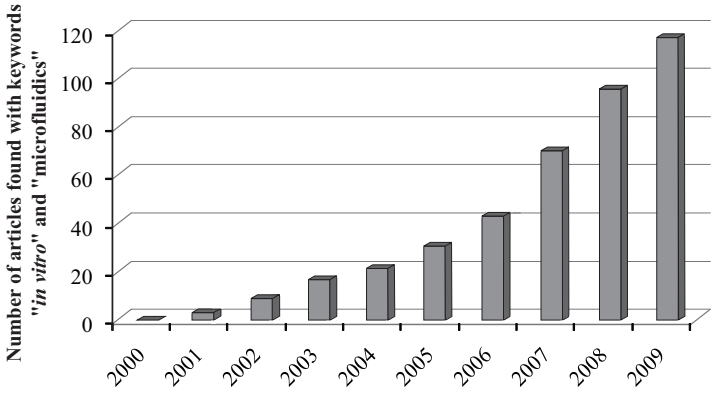

Figure 1. Number of publications found per year using the keywords "in vitro" and "microfluidics" from 2000-2009, according to ISI Web of Knowledge.

This paper reviews the studies in which microfluidic devices are employed for liver metabolism and toxicology of drugs. The incorporation of microsomes, cell lines, primary cells, and intact tissue on chip is described. In addition, the application of cell-free systems to predict biotransformation is discussed. Biotransformation of NCE occurs also in non-hepatic tissue, like the intestine, lung, kidney and skin[4, 22] and the role of these tissues in drug metabolism and toxicity cannot be neglected. It is therefore important to develop *in vitro* systems which allow the incorporation of these types of tissue as well, to more accurately mimic the *in vivo* situation. In this review, special attention is paid to the co-culture of non-hepatic tissue/cells together with hepatic tissue/cells, to be able to investigate interorgan interactions or to improve metabolic activity. Important requirements of a well-developed microfluidic *in vitro* system include the biocompatibility of the device and a tight control over the oxygen concentration, pH, medium composition and temperature during culture. The fabrication of the device itself should also be low-cost, in order to make the device disposable as in the case of culture flasks and well plates, to prevent contamination and infections.

2. Cell-free systems

The major phase I reactions are oxidation, reduction and hydrolysis. These reactions modify the structure of xenobiotics to make them suitable for phase II conjugation with glucuronic acid, sulfate or other polar moieties. To get a full overview of the phase I metabolites formed by the liver, intact cells or subcellular fractions such as microsomes or S9 fractions should be used (see Section 3). However, it is also possible to mimic the phase I oxidative reactions without the need of cells or subcellular fractions. Several researchers have reproduced the oxidation of xenobiotics by making use of electrochemistry.[23-25] Chemical can be oxidized by applying a potential difference between electrodes which is more positive than the reduction potential of the xenobiotic. By coupling this type of electrochemical reaction to a mass spectrometer, the product formation and depletion of the mother compound can be measured directly on-line. Getek *et al.* even demonstrated the formation of phase II glutathione conjugates of acetaminophen by electrochemically oxidizing this compound first and then mixing it with glutathione.[25]

Recently, the application of electrochemical reactions for drug metabolism studies was incorporated on chip.[26] Odijk *et al.* developed a glass microfluidic device with a channel of only 9.6 nL containing a palladium reference electrode and platinum working and counter electrodes for the on-line oxidation of amodiaquine. They revealed a metabolic pathway for amodiaquine dissolved in ammonium formate (pH adjusted to 7.4) which was similar to conventional cell systems. The ability to mimic oxidative pathways of new drug entities on chip with electrochemical methods is useful for producing large quantities of metabolites for further toxicity studies or

providing a quick and clean screening method for combinatorial chemistry.[27]

However, electrochemical approaches are not suitable for the prediction of metabolic profiles of drugs formed *in vivo*, as the profile of oxidation products is not the same as *in vivo*.[28] In addition, only oxidation products are formed, which generally do not completely represent the full palette of metabolites produced *in vivo* or in cells *in vitro*.

3. Subcellular fractions

Subcellular fractions are still the most widely used *in vitro* tool to predict liver metabolism, since they are easy to handle and among the best characterized *in vitro* systems for drug metabolism research. Liver microsomes, liver cytosol fractions, or S9 fractions, which contain both microsomes and cytosol, are routinely used. Of these three, the liver microsomes are the most extensively used fraction. Liver microsomes are fragments of the endoplasmatic reticulum obtained by differential centrifugation after homogenization of the tissue. Microsomes thus contain mainly cytochrome P450 (CYP) enzymes, uridine diphosphate glucuronosyltransferase (UGT) and methyl transferases. Cytosolic fractions contain the soluble phase II enzymes, N-acetyl transferases, sulfotransferases (SULT) and glutathione-S-transferases (GST). The microsomal CYP enzymes use NAPDH as cofactor and thus a NADPH-generating system or NADPH solution is needed to ensure the proper activity of the CYP enzymes, as well as cofactors and activation by a detergent for phase II metabolism. However, the addition of cofactors results in unphysiological concentrations. Another disadvantage of subcellular fractions is the rapid decline in activity over time. Despite these limitations, subcellular fractions are very useful for screening in the early phase of drug development, due to their ease of use and availability as they can be stored frozen.

One of the first examples of the incorporation of liver microsomes for drug metabolism onto a microfluidic chip was performed by Benetton *et al.* in 2003.[29] A mixing chip with a Y-junction containing a porous monolithic solid-phase extraction column was developed. Microsomes were mixed and incubated on chip with substrates and co-substrates such as NADPH. Subsequently, the reaction mixture was flushed onto the extraction column to preconcentrate and desalt the sample. The mixture was then eluted from the column and the metabolites were detected on-line with a mass spectrometer. The coupling of the chip with a mass spectrometer is attractive, as the mass spectra give immediate insight into the metabolites that are formed. In this example, the microsomes were infused into the chip together with substrate at a 1:1 flow rate ratio until the channel was filled, at which point the flow was stopped for mixing. The microsomes were then allowed to react with the substrate in the channel for 30 min. The pH and oxygen concentration were not controlled during an experiment other than by using a pH buffer. Tris is often used as pH buffer

in microsome studies, as was the case here. It should be noted, then, that Tris inhibits monamine oxidases (MAO) in rat liver microsomes.[30] MAOs catalyze the oxidation of monoamines, and are also involved in the metabolism of a small number of xenobiotics.

Zguris *et al.* embedded human liver microsomes in a poly(ethylene) glycol (PEG) hydrogel.[31] The CYP activity of microsomes entrapped in hydrogel was comparable with microsomes in solution.[32] A few advantages of entrapping microsomes in a hydrogel include the ease with which products are separated from the microsomes, the increased storage stability, and their reusability for other tests.[33] In the study described by Zguris *et al.*[31], the pH and oxygen levels were not monitored or controlled during the incubation.

Recently, Ma *et al.* developed a sol-gel bioreactor for metabolism studies with human liver microsomes.[18] Instead of using PEG, the sol-gel was based on tetramethoxysilane (TMOS) to encapsulate the liver microsomes. Both the incubation of microsomes with substrates and on-chip separation and detection of metabolites were described for this device. Drugs and metabolites were injected into microchannels, separated by electrophoresis, and detected with UV absorbance detection. The device itself was made by sealing a from quartz chip with a and polydimethylsiloxane (PDMS) chip. During incubation, the device was placed in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C. PDMS is gas permeable and the reservoirs were open, so that the incubation environment (pH, oxygen, and temperature) was stable over time. However, the sample mixtures were introduced electrokinetically, and it is unknown if applying an electric field over the microsomes influences the metabolism. Unfortunately, the applied voltage was not mentioned, but normally this is around a few hundred volts for sample injection on microchips.

To date, only examples of the incorporation of microsomes onto microfluidic devices have been published. No examples of devices using cytosolic or S9 fractions have been reported. The microsomes can easily be embedded in a sol-gel, while maintaining a CYP activity which is comparable to microsomes in solution. Therefore, microsomes are very useful for continuous-flow incubation and analysis on chip, or on-line mass spectrometry for drug screening in the early phase of drug development. Unfortunately, the metabolites formed do not fully represent all the metabolites produced by the liver, since only the enzymes in the endoplasmatic reticulum are present. However, this approach will give insight into the metabolites formed by the CYP enzyme complex and, when adding uridine diphosphate glucuronic acid (UDPGA) as cofactor and a detergent for activation, also the glucuronide metabolites. The general advantages of microfluidic devices incorporation microsomes are the possibility of coupling with a variety of downstream detection devices for drug metabolites and performing multiple experiments on one device.

4. Cell lines

A cell line is a permanently established cell culture, where the cells will proliferate indefinitely when given proper fresh medium and enough space. In contrast to cell cultures, cell lines are due to mutations not restricted to a limited number of cell divisions (Hayflick's limit),[34] and therefore become immortalized. Liver cell lines in conventional systems are a very popular *in vitro* model to study liver function and mechanisms of toxicity. However, they are unsuitable for drug metabolism and toxicity prediction, due to the fact that cell lines do not contain all the metabolic enzyme families, and the enzymes that are present are not at their physiological levels. One important advantage of human cell lines is that they can be used to generate data relevant to humans. Moreover, they are easy to handle and replace the use of animals. Disadvantages are the dependence of gene expression on passage number, unstable cell lines, and dedifferentiated cells whose phenotype no longer resembles that of the cell *in vivo*. An often overlooked pitfall is the occurrence of cross-contamination with other cell types, which is a general problem for all cell lines. It is estimated that 15-20% of cells are misidentified or contaminated with another cell line.[35, 36]

4.1 Liver cell lines

The most commonly used and best characterized human liver cell line is the hepatocellular carcinoma or HepG2 cell line. However, compared to isolated hepatocytes, the overall CYP activity is low.[37] Another frequently used cell line is the HepG2/C3A, a clonal derivative of the HepG2, which was selected for its improved differentiated hepatocyte phenotype. Both cell lines have been cultured on chip (e.g. Viravaidya *et al.*[38], Carraro *et al.*[39], Zhang *et al.*[40]). Recently, a metabolically more competent cell line was generated (HepaRG); however, no reports have been published to date on studies incorporating HepaRG cells in microfluidic devices.[41]

The first integration of HepG2 cells into a microfluidic device was performed in 2003 by LeClerc *et al.*, who showed that the cells functioned properly (as judged by glucose consumption and albumin production) for at least 12 days.[42] However, HepG2 cells are also viable in conventional systems for periods longer than 12 days.[43] HepG2 cells in microfluidics have mainly been used for toxicity studies.[18, 21, 44] The cells were treated with various concentrations of different compounds (e.g. acetaminophen, daunorubicin, idarubicin, Triton X-100) and viability was determined by optical imaging using live/dead staining.

Two advantages of using microfluidics are the possibility of multiple incubations on one chip and the ease with which concentration gradients can be generated on chip. This is especially useful for high content screening of cells. Ye *et al.* developed a PDMS microfluidic device with eight identical structures comprising integrated gradient generators for toxicity tests on HepG2 cells (see Figure 2).[21] The gradient generator was based on the principle published in 2001 by Dertinger *et al.*[45] Two

inlets were integrated on chip, one for medium and one for drug plus medium. These two liquids were combined into a wide channel and then split multiple times to generate solution mixtures having different concentration ratios of the two initial liquids. The HepG2 cells located in cell culture chambers downstream were exposed to varying concentrations of drugs as a result, and their response was directly observed with a microscope. The eight uniform structures mean that eight compounds can be tested on chip at the same time. The PDMS device was placed in a humidified incubator set at 37°C with 95% air and 5% CO_2. Oxygen and pH levels were not monitored; however, it is likely that a stable microenvironment was established.

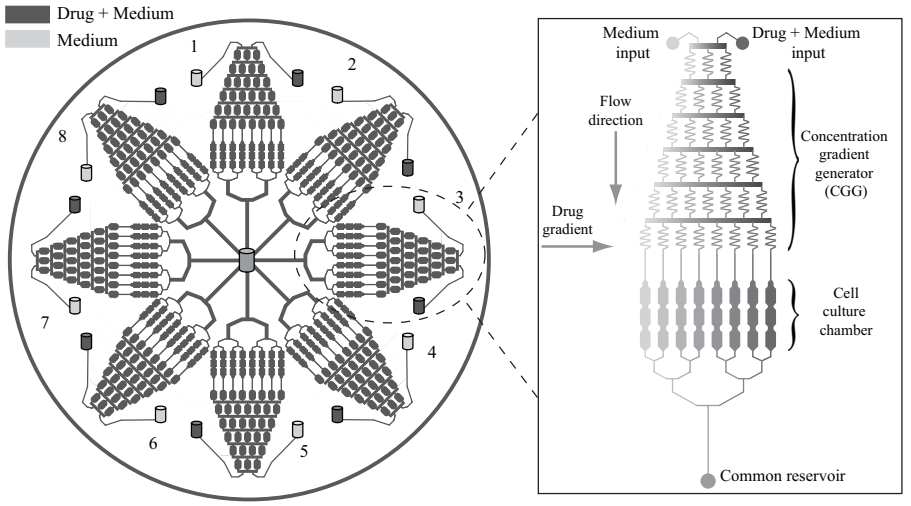

Figure 2. Schematic view of the chip developed by Ye et al. with eight identical structures with integrated gradient generators (reprinted with permission from Ye et al.[21]).

As mentioned above, HepG2 cells have mainly been used for measuring toxicity and are rarely applied in metabolism studies, which is probably due to their low metabolic enzyme activity. However, it is precisely this low enzyme activity that also make HepG2 cells less suitable for toxicity prediction. When the expression of drug metabolic enzymes is low, not only the biotransformation of drugs can differ with respect to the *in vivo* situation, but the toxicity profile could be affected as well, as shown previously.[43] However, cell lines are very useful for investigating molecular pathways in toxicity and for testing new devices and/or techniques due to their robustness. Sung *et al.*, for example, developed an optical detection system for *in situ* real-time monitoring of CYP activity.[46] Their system consisted of a green light-emitting diode (LED) for excitation of the compound, resorufin (metabolite of ethoxyresorufin), present in the detection chamber integrated on chip, and a photodiode for detection. HepG2/C3A cells were cultivated in Matrigel on this chip[46], as it

has been reported that cells maintain their functions better when cultivated in hydrogels like Matrigel, due to this matrix representing a more physiological microenvironment.[47] CYP1A1 and CYP1A2 activity were assessed using ethoxyresorufin. An improved metabolic activity was obtained compared to conventional monolayer culture. However, this improvement is, as mentioned above, most likely due to the Matrigel and not the microchip, since improved metabolic activity and differentiation is also observed in conventional systems when cells are embedded in Matrigel.[47] A continuous flow was applied in the device described by Sung *et al.*, which ensured that the cells were exposed to medium with constant composition, approaching steady-state conditions. This device might be very useful for real-time monitoring of CYP activities if applied to primary cell culture as well.

Since hepatocytes *in vivo* are all located along sinusoids, Carraro *et al.* developed a PDMS device where they mimicked the human hepatic microvascular bed.[39] HepG2/C3A cells were viable over the 10-day period tested, and also able to form phase I and phase II metabolites during this time. Moreover, they also showed that the incorporation of primary cells was possible. These cells remained viable for at least 7 days, but to the best of our knowledge, drug metabolism was not tested. Analogous to the *in vivo* situation, the hepatocytes were not directly exposed to medium flow, with the exchange of medium components taking place by diffusion through a polycarbonate membrane with a pore size of 0.4 μm. As the chambers with cells were only 100 μm deep, the diffusion distance was somewhat longer compared than *in vivo*, but short enough to presume that cells were sufficiently exposed to oxygen and substrates by diffusion.

4.2 Co-incubation of different cell lines

The existence of interorgan interactions *in vivo* has been known for a long period of time. Since blood flows sequentially through several organs, the products formed by different organs might affect other organs, causing toxicity or detoxification, depending on the situation. An example of such an interorgan effect is a liver-derived metabolite of naphthalene which causes lung toxicity.[48] Similarly, kidney toxicity can occur upon exposure to a metabolite of acetaminophen formed in the liver.[49] To investigate these interorgan interactions, various *in vitro* systems have been proposed, incorporating cell lines from different organs. The most elaborate of these systems is the micro cell culture analog (μCCA) developed by Shuler and co-workers (see Figure 3a).[50] Their first published design in 2004[51], made from silicon, consisted of three chambers incorporating cell lines representing lung cells (L2 cells), fat cells (3T3-L1 adipocytes) and liver cells (HepG2/C3A cells), and a fourth compartment representing other tissues (see Figure 3a). In the μCCA, care was taken to achieve *in vivo*-like residence times in the different compartments.[52] The toxicity of the liver-derived metabolite of naphthalene on lung tissue was probed with this particular chip. Since

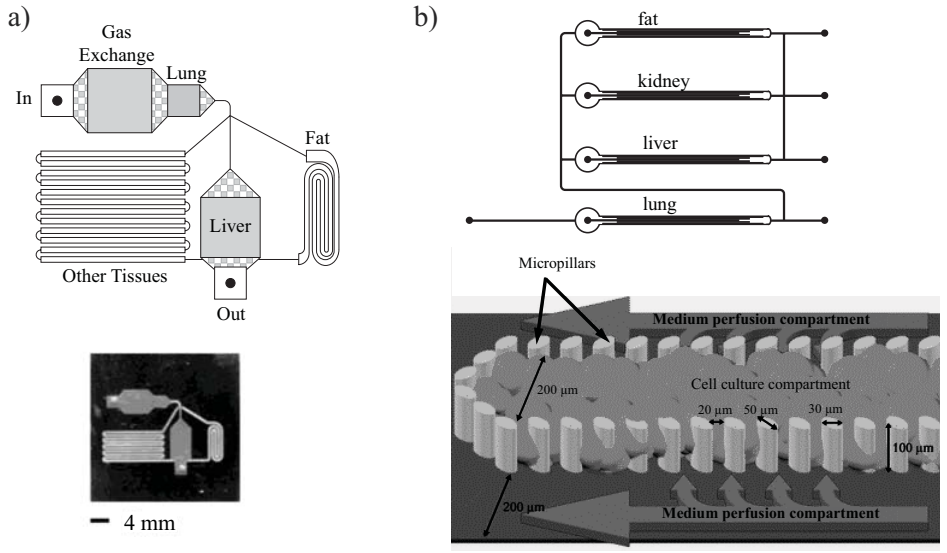

Figure 3. (a) A schematic diagram (above) and photograph (below) of the silicon chip for the µCCA developed in the Shuler lab, with the medium stream passing directly through the culture compartments (reprinted with permission from Viravaidya et al.[38]). (b) Schematic diagrams of the 3D-µFCCS developed in the Yu lab with two medium perfusion streams flowing parallel to the culture compartment (upper figure reprinted with permission from Zhang et al.[54]; lower figure reprinted with permission from Toh et al.[73]).

then, chips consisting of liver, colon, bone marrow, fat, lung and/or tumor cell lines have been developed.[14, 50, 52, 53] A continuous medium flow ensured that a stable microenvironment was established, and cells were contacted directly by the liquid stream to minimize possible diffusion problems. However, a disadvantage is that the chips have been mainly developed in silicon, which is rather expensive.

In 2009, Zhang *et al.* published a similar PDMS chip into which fat, kidney, liver and lung cells could be integrated, called the 3D microfluidic cell culture system (3D-µFCCS) (Figure 3b).[54] The 3D-µFCCS has an array of micropillars surrounding a central cell culture chamber. Two medium streams flow around the outside of this array, separated from the compartment where the cells are integrated, which implies that medium exchange into the cell compartment only occurs by diffusion (Figure 3b, lower half). The fact that the cell culture in this device is not in direct contact with these solution flows distinguishes it from the µCCA described above. Another difference between the two devices is that two-way valves were integrated off-line into the flow control system of the 3D-µFCCS, to be able to isolate compartments from one another in terms of solution contact. This means that the biological pathway of drugs in individual tissues, as well as interorgan effects, can be assessed with this

device. The fabrication of the device in PDMS is much cheaper than in silicon. Due to the gas permeability of PDMS, it is also easier to control and regulate the oxygen and pH levels of the medium during an experiment. However, PDMS has a hydrophobic character and can therefore adsorb or even absorb hydrophobic drugs and/or metabolites.[55]

The incorporation of cells from cell lines in microfluidic devices is ideal to test newly developed devices, due to the ease of handling and robustness of the cells themselves. They are also very useful for testing the potential toxicity of compounds and examining possible interorgan effects. However, cell lines are less useful for hepatic metabolism studies and for the prediction of the toxicity *in vivo*, due to the lack of normal expression levels of important metabolic enzymes and transporters in cell lines in general.

5. Primary cells

It is generally accepted that freshly isolated primary hepatocytes are a better *in vitro* model to predict *in vivo* drug metabolism than cell lines.[56, 57] This is because these differentiated cells contain all metabolizing enzymes and transporter proteins, and express them at their natural levels. They can therefore be successfully applied to predict hepatic metabolism quantitatively and qualitatively. Primary hepatocytes are isolated from intact liver tissue by collagenase perfusion.[58, 59] This process digests the connective tissue, and hepatocytes can be purified by low-speed centrifugation. With this technique, it is possible to isolate hepatocytes from animal and human livers, while maintaining their metabolic activity. Although primary hepatocytes initially do contain metabolic enzymes at their physiological concentrations, the CYP-mediated metabolism decreases upon culturing. This can be prevented in part by culturing the cells in Matrigel, supplying the medium with inducers, or as co-cultures with non-parenchymal cells.[60-65]

5.1 Primary hepatocytes

The culture of primary hepatocytes in microfluidic devices has been reported by several researchers.[9, 10, 66-70] Lee *et al.* nicely showed the potential of using microfluidics in the development of new *in vitro* systems by fabricating a low-cost PDMS device which resembles the liver anatomy (Figure 4).[67] The device included an artificial liver sinusoid complete with a physical barrier mimicking the natural endothelial barrier layer. Primary rat and human hepatocytes were maintained viable for over 7 days. Unfortunately, no metabolism or toxicity studies performed with this particular device have been reported yet.

Griffith and co-workers designed a device to culture primary hepatocytes under flow conditions in a three-dimensional format by using microfluidic techniques, and

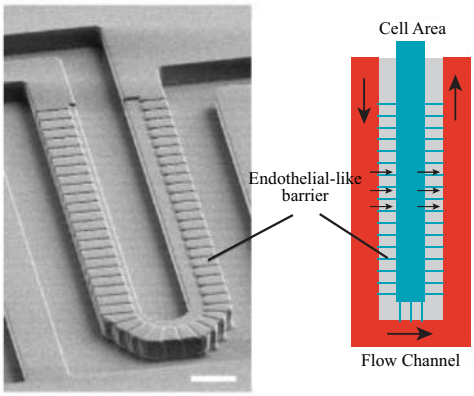

Figure 4. Photograph (left) and schematic view (right) of the device which resembles a liver sinusoid, including the endothelial barrier layer, developed by Lee et al. Cells were cultured in the Cell Area. Medium flowed around the outside of the endothelial-like barrier, with a portion crossing the endothelial barrier to the cells (reprinted with permission from Lee et al.[67]).

tested the hepatocytes for metabolic stability.[71] Deep reactive-ion etching was used to construct three-dimensional culture scaffolds in silicon, in which primary hepatocytes were subsequently cultured (Figure 5). Cells cultured for 2 weeks in the bioreactor retained basal mRNA expression of most of the CYP enzymes, transcription factors, phase II drug metabolizing genes and nuclear receptors.[9] This indicated that in this three-dimensional culture with continuous flow, near-physiological levels of some important liver-specific functions of hepatocytes could be maintained. Recently, the Griffith group have further improved their device for higher throughput and developed a perfused multiwell plate with an integrated pneumatic micropump and a fluidic capacitor to achieve a pulseless flow (Figure 5).[72] The cells in this device were shown to be viable using dye staining and retained their capacity for albumin synthesis, although no attempt was made to determine if the synthesis rate was at physiological levels. The devices in this study were placed in a humidified incubator to control oxygen and pH under a continuous flow (250 µL/min), and adequate oxygenation was achieved. Cells were also cultured in 3 mL of medium, which for microfluidic systems is a large volume.

Chao *et al.* showed that it was also possible to incorporate cryopreserved human hepatocytes in the micro cell culture analogue (µCCA) developed in the Shuler group, as mentioned above.[69] Only one of the three chambers of the polystyrene biochip was collagen-coated and used to culture hepatocytes. This device mimicked the *in vivo* situation with *in vivo*-like residence times and stable environmental conditions due to the continuous flow. The clearance of six substrates was tested with this particular device. For one of the substrates, the µCCA better predicted the *in vivo* clearance

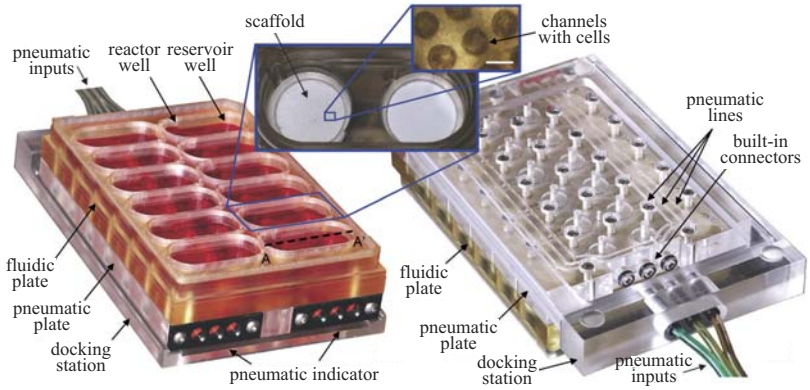

Figure 5. The perfused multiwell plate with an integrated pneumatic micropump to perfuse primary hepatocytes which were cultured in the culture scaffold. This system was developed by Griffith and co-workers (reprinted with permission from Domansky et al.[72]).

than the 96-well plate. For two other substrates the opposite was true, and the 96-well plate method predicted the *in vivo* situation better. However, all values were considered within acceptable ranges, being not more than 5-fold different from the *in vivo* situation.

The group of Yu has not only integrated cells from cell lines in their developed 3D microfluidic cell culture system (3D-μFCCS), as mentioned above, but also primary cells and gradient generators.[68, 73, 74] This device was used for the determination of the potential of drugs to inhibit metabolism, by assessing the IC50 values (the concentration at which 50% of the enzyme is inhibited) of five compounds. These values correlated well with the *in vivo* data. These researchers cultured the cells for 72 h, but found a decrease in basal phase I metabolism enzyme activity and stable phase II activity. However, Zhang *et al.* showed that when hepatocytes in their 3D-μFCCS were exposed to transforming growth factor beta 1 (TGF-β1), their functionality was better maintained.[68] Phase I and II metabolism was more stable over a period of 7 days compared to primary hepatocytes without TGF-β1. TGF-β1 is not only secreted by hepatocytes in the liver, but also by Kupffer cells, stellate cells, and endothelial cells.[75] It is a multifunctional polypeptide that regulates cell growth and differentiation, and immune functions.[76] The role of TGF-β1 is one example which underlines the importance of the non-parenchymal cells for optimal liver function. Incubations in the low-cost PDMS device reported by Zhang *et al.*[68] were performed by placing the device on a hot plate. Temperature regulation may have been an issue in this case, as a temperature gradient could be generated in the incubation channel when heating from only one side. Since the environment was not humidified, evaporation of medium through the PDMS may also have occurred, due to the low

volumes used.

Instead of incubating hepatocytes with TGF-β1, liver-specific functions can also be improved in three-dimensional scaffolds when hepatocytes are embedded in hydrogels like collagen[66, 77, 78] or Matrigel.[77, 79, 80, 81] One should be aware that nutrients and oxygen gradients can occur in gels, since the penetration of gases and nutrients in the matrices are diffusion-dependent.[82, 83] Since both the presence of TGF-β1 and extracellular matrix, like Matrigel, appeared important to maintain liver function, it is believed that cell-cell interaction and the extracellular matrix both play an essential role during *in vitro* cultures of hepatocytes.

5.2 Co-incubation of hepatocytes with other cell types

There are, unfortunately, no examples available to date of microfluidic devices incorporating primary hepatocytes and other primary cells to study interorgan interactions. However, cocultures with fibroblasts or other non-parenchymal cells were developed in the 1980's to maintain hepatocyte viability and support the performance of many liver-specific functions.[84, 85] Primary hepatocytes have also been cocultured on chip[86] with 3T3-J2 fibroblasts cells, which is a very potent cell line to improve liver function.[86-89]

Domansky *et al.* successfully established cocultures of primary hepatocytes with a preparation of liver sinusoidal endothelial cells (LSEC), which also contained stellate and Kupffer cells.[72] During culture, LSEC retained their differentiation marker SE-1; however, the metabolic functioning of the hepatocyte culture still has to be demonstrated.

Khetani and Bhatia showed the retention of CYP activity in a coculture of primary hepatocytes with 3T3-J2 cells.[89] PDMS stencils were used to pattern collagen spots in polystyrene microwells, to which primary hepatocytes adhere. 3T3 cells were subsequently cultured in the remaining bare areas surrounding the hepatocytes. Cells were cultured in a multiwell format, and the plates were placed in an incubator to control pH, temperature, and oxygen level. Liver-specific functions were better maintained for over 20 days in this coculture than in monocultures of pure hepatocytes. Almost no albumin secretion and urea synthesis was detected in the monoculture after 20 days, whereas substantial amounts of albumin and urea were found in the coculture. The genes for phase I and II metabolism at day 42 were almost comparable with freshly isolated hepatocytes, while gene expression in the monoculture was much lower at day 7. These results were obtained without applying medium flow; the medium, 300 μL per well, was refreshed only once every 24 h during experiments. This questions the notion that flow is beneficial for maintaining viable and active hepatocytes, as suggested by many researchers.[10, 11, 90-92] However, with the high cell-to-volume ratio, which is usually the case in microfluidic devices, it is important to refresh medium more often compared to conventional systems or the

coculture system presented by Khetani and Bhatia, to prevent a shortage of nutrients. When cells are cultured in a gel, flow might be beneficial to overcome possible mass-transfer problems. It is therefore advisable to apply a low flow in microfluidic devices in any case, with higher flows being required in some situations.

In vitro systems incorporating primary hepatocytes are a good model to study drug metabolism and toxicity, in view of the fact that these cells correlate well with the *in vivo* situation. Another advantage of primary hepatocytes is the possibility for cryopreservation of the cells,[93] though the hepatocytes have reduced viability and metabolic function after thawing compared with freshly isolated hepatocytes.[94] The disadvantage of the use of pure hepatocyte cultures is that a liver consists of other cell types besides hepatocytes, such as Kupffer, stellate, dendritic and endothelial cells, for example. Although hepatocytes represent 60%-65% of all cells present in the liver and 80% of total cell volume,[95] the other non-parenchymal cells are also important in toxicity studies[96] and it has been suggested that metabolic co-operation exists between these cells.[97, 98] Normally the activity of CYP enzymes present in hepatocytes decreases upon culturing. However, by coculturing cells with non-parenchymal cells like fibroblasts in a microscale architecture, this activity can be maintained for a few weeks.[89] The role of non-parenchymal cells in metabolism and toxicity studies thus cannot be neglected.

6. Intact tissue

Primary hepatocytes are isolated from intact tissue, as mentioned above, and after isolation cocultured with non-parenchymal cells to recreate the complex architecture of the liver. Instead of using isolated cells or cell fractions, however, it is also possible to take intact tissue for *in vitro* assessment of liver metabolism and toxicity. Intact tissue systems have the advantage that cell-cell contacts remain intact, and that all cell types, including their enzymes, cofactors, and transporters, are present at their physiological levels. They thus closely resemble the complex architecture of the organ, with its sinusoids and endothelial barrier layers. Intact liver tissue can be obtained from animals, and from humans as surgical waste after liver surgery or transplantation. There are two possibilities for the incorporation of intact liver tissue into microfluidic devices, namely liver biopsies and precision-cut liver slices. Liver biopsies can be made by cutting liver tissue to the desirable size by hand or by using a biopsy punch, resulting in samples of variable size. Precision-cut tissue slices can be prepared by using a Krumdieck or a Brendel-Vitron tissue slicer.[99] Both slicers allow the production of precision-cut tissue slices of reproducible thicknesses ranging from 100 µm and up. Care must be taken that the thickness of the slices is small enough to allow nutrients and oxygen to penetrate to the inner cells, which occurs by diffusion. Analogous to primary hepatocytes, the activity of metabolic enzymes also

declines in slices upon culturing, although at a slower rate than in hepatocytes cultured alone.[100, 101] The incorporation of biopsies into microfluidic devices has been shown;[102] however, until now no examples of the incorporation of precision-cut liver slices into microfluidic devices has been shown.

6.1 Biopsies

In 2008 Hattersley *et al.* published the development of a microfluidic chip made of glass for the incorporation of liver biopsies.[102] The device consisted of a Y-shaped channel (two inlets and one outlet) with 3-mm-diameter tissue chamber for inserting the biopsy. The tissue chamber was located on top of the microfluidic channel and resembled a chimney-like structure. As a result, the tissue was not placed directly in the liquid stream, implying that nutrients could be delivered to the tissue by diffusion only. This could be considered a limitation of the device, as tissue biopsies of 4 mm^3 were used and the diffusion length was rather long. The cells furthest away from the fresh medium flow in the microfluidic channel were at a distance of more than 1 mm from the medium and were probably exposed to a lower concentration of nutrients compared to the cells exposed directly to fresh medium.[103] *In vivo* hepatocytes are located less than a few micrometers from the bloodstream, and most cells do not survive when they are more than a few hundred micrometers from the nearest capillary.[104-106] The pH and oxygen were regulated by placing the chip in an incubator. An adapter filled with PDMS was placed on top of the device, through which gaseous exchange with the surroundings could occur. Hattersley *et al.* showed that with their device it was technically possible to measure lactate dehydrogenase and DNA release in the outlet fractions and assess the morphology of the cells after an experiment. This system might be useful for addressing toxicological questions; however, unfortunately no metabolism studies were performed and the tested device was not verified with respect to conventional systems.

6.2 Precision-cut liver slices (PCLS)

Until now there are no examples available of the incorporation of precision-cut liver slices (PCLS) in a device made with microfluidic technologies. However, in 1996 a miniaturized system was published where liver slices were fixed with a plasma clot onto a microscope slide, and were continuously perfused with medium.[107] This device was used to assess the regional distribution of cytochrome P450 activities in PCLS by using fluorescence confocal laser cytometry. However, only one side of the slice was exposed to fresh medium, which may have resulted in a lack of nutrient diffusion to the cells at the bottom of the slice (attached to the glass with the plasma clot). In addition, this system did not allow quantification of enzyme activities.

Another example of a miniaturized system made from polycarbonate was used by Khong *et al.* to perfuse thick (0.3-1 mm thick) PCLS.[108] To facilitate diffusion, the

tissue slice was placed directly in the liquid stream and seven needles (300 μm OD) were inserted directly in the tissue slice to deliver the medium. Stable CYP1A activity and even an increase in UGT activity in the perfusion system were reported for a period of 3 days, indicating that this device could be useful for induction and inhibition studies.

6.3 Co-incubation of liver and other tissue slices

As mentioned above, interorgan interactions do occur in the human body which can involve toxicity.[48, 49] To date, there have only been a few systems available which consider the co-incubation of fresh tissue from several organs to mimic these interorgan interactions. Studies were performed in which precision-cut tissue slices of liver and kidney were placed in the same well, and cooperation of the two organs with respect to biotransformation was elegantly shown.[109] However, mutual interaction can take place with this system, and it would be difficult to conclude which organ was responsible for the observed effects.

Li *et al.* developed a well-plate system with slices from different organs placed in individual wells, which were interconnected by overlying medium in a wells-within-a-well concept.[110] Here too, mutual interactions between slices can take place which cannot be attributed to a specific organ. A drawback of this concept is the large volume of medium required, which might result in the dilution of metabolites to an extent that they cannot effectively be detected anymore. Microfluidics, which enables the sequential perfusion of the various compartments, might be a good technique to solve these problems.

For the *in vitro* models described, the use of intact tissue is the best representation of the *in vivo* situation. All cell types are still present, and in their natural tissue-matrix configuration. Nutrient delivery to the inner cells of the tissue occurs mainly by diffusion. Therefore, the dimensions of the tissue sample should be small enough to allow for diffusion of substrates and oxygen to the center of the tissue. By using precision-cut tissue slices, the thickness can be varied from 100 to 250 μm with good reproducibility. The routine use of precision-cut tissue slices is still hampered by the fact that the cryopreservation of tissue slices with undiminished activity has not been optimized yet,[101] and the viability of fresh slices is relatively short (up to 5-7 days).[111-113] However, the use of intact tissue is the preferred *in vitro* model described to date for the prediction of multicellular processes. Sequential perfusion of chambers containing different organ slices in a microfluidic device to study interorgan interactions will be a powerful tool to elucidate unknown mechanisms involved in toxicity and metabolism.

7. Conclusion

The use of microfluidics in cell and tissue incubation systems offers many advantages for applications in metabolism and toxicology studies. Microfabrication allows the fabrication of chips with precisely defined structures to create controllable and reproducible microenvironments, and integrated microfluidic components, such as pumps[114, 115], valves[114], detection systems[116], and concentration gradient generators.[19, 20] They enable high-throughput analysis[117] with *in-situ*[46] detection in flows. Since microfluidic chips are mainly made out of transparent biocompatible materials like glass, elastomers (PDMS) and/or plastics, the devices can easily be placed under a microscope for imaging purposes. The fabrication costs are also low. Tight control of oxygen concentration, pH and medium composition can be achieved through the introduction of flow. This flow is necessary in microfluidic devices to prevent a shortage of nutrients, due to the low volume-to-cell ratio. Care should be taken to prevent adsorption of compounds onto device materials. In microfluidics, high surface-to-volume ratios are the norm, and therefore adsorption might be more prevalent than in conventional *in vitro* systems. PDMS, for example, has a hydrophobic character, and hydrophobic metabolites or substrates tend to adsorb onto this material.[55] However, glass and plastics might also adsorb compounds present in the medium or formed by the cells.

To measure liver drug metabolism and toxicity in microfluidic devices, the use of primary hepatocytes and precision-cut tissue slices reflect the *in vivo* situation relatively well. The disadvantage in both systems is the decline in enzyme activity during culturing. This can be minimized by co-culturing primary hepatocytes with fibroblasts in micropatterns,[89] or by adding growth factors or other signaling molecules to the medium.[68] A disadvantage of primary hepatocytes compared to PCLS is the loss of normal liver integrity and the absence of non-parenchymal cells, which may be important when toxicity is of interest.

The use of human cell lines in microfluidic devices has great potential for testing newly developed systems, but is not suitable for complete mimicking of *in vivo* liver metabolism. This could change if new cell lines were developed containing all enzymes and transporters at their physiological levels. Such a cell line would reduce the use of animal material in *in vitro* systems. Subcellular fractions are very effective for high-throughput screening tests. Nevertheless, these fractions alone are not a good model to predict *in vivo* metabolism and are unsuitable for toxicity testing. Finally, cell-free analysis using electrochemical approaches is interesting for getting a first impression of what kind of metabolites might be formed through oxidation in the liver. However, many of the oxidation products formed are not representative of the metabolites formed *in vivo*. Therefore this technique is mainly useful to synthesize metabolites in high quantities.

Additional interesting advantages of microfluidics are the low volume-to-cell ratio

Table 2. Advantages and disadvantages of different microfluidic in vitro approaches for metabolic studies

In vitro model	*Advantages*	*Disadvantages*	*Examples*
Cell free	- No animal usage - Quick screening	- Only phase I oxidation reactions - Unsuitable for toxicity testing - Not representative/predictive for *in vivo* situation	*26*
Subcellular fractions	- Low costs - Simplicity - Well-characterized	- Cofactors not present at physiological levels - Not all metabolic enzymes are present - Not suitable for toxicity testing	*18, 29, 31*
Cell lines	- Easy to culture - Relatively stable enzyme concentrations - Useful to study molecular mechanisms of toxicity	- Absence or low expression of phase I and some of the phase II drug metabolizing enzymes and transporters	*31, 51, 52, 54, 65*
Primary cells	- Viability up to 4 weeks - All enzymes and transporters initially present - Good prediction of *in vivo* drug metabolism	- Absence of non-parenchymal cells - Interindividual variation for human material - Decrease in metabolic capacity and transporter expression upon culturing	*9, 67, 69, 70, 73*
Tissue	- All cells, metabolizing enzymes and cofactors present in their physiological matrix - Good prediction of *in vivo* drug metabolism	- Short viability time period (5 days) - Decrease in metabolic capacity upon culturing - Interindividual variation for human material	*102*

and the precise control of medium flow and composition. Medium can be directed to different compartments containing different cell types. Due to the low volumes employed, metabolite concentrations are not diluted to any great extent and remain relatively high. Systems incorporating cells from various tissues enable the study of interorgan interaction studies.[38, 54]

Despite the progress in this field, *in vitro* models at this stage are still unable to replace *in vivo* screening completely. However, by better mimicking the *in vivo* situation using microfluidic technologies, the number of animals needed in the preclinincal phase for drug development could be greatly reduced while prediction could be improved simultaneously. The coupling of compartments containing different tissues is a promising route to new possibilities for the study of interorgan interactions in the field of drug metabolism, regulation of gene expression and toxicity. The goal of this thesis is to develop a microfluidic device incorporating precision-cut tissue slices, with an ultimate goal to measure interorgan interactions. It is likely that such microfluidic *in vitro* systems will become increasingly important for improving our insight into the metabolism and toxicity profiles of newly developed drugs.

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3

Microfluidic Biochip for the Perifusion of Precision-Cut Rat Liver Slices for Metabolism and Toxicology Studies

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Biotechnology and Bioengineering 2010:105(1) 184-194

Abstract

Early detection of kinetic, metabolic, and toxicity (ADME-Tox) profiles for new drug candidates is of crucial importance during drug development. This article describes a novel *in vitro* system for the incubation of precision-cut liver slices (PCLS) under flow conditions, based on a polydimethylsiloxane (PDMS) device containing 25-µL microchambers for integration of the slices. The microdevice is coupled to a perifusion system, which enables a constant delivery of nutrients and oxygen and a continuous removal of waste products. Both a highly controlled incubation environment and high metabolite detection sensitivity could be achieved using microfluidics. Liver slices were viable for at least 24 h in the microdevice. The compound, 7-ethoxycoumarin (7-EC), was chosen to test metabolism, since its metabolism includes both phase I and phase II metabolism and when tested in the conventional well plate system, correlates well with the *in vivo* situation. The metabolic rate of 7-EC was found to be 214 ± 5 pmol/min/mg protein in the microdevice, comparable to well plates, and was constant over time for at least 3 hours. This perifusion system better mimics the *in vivo* situation, and has the potential to significantly contribute to drug metabolism and toxicology studies of novel chemical entities.

Introduction

In vitro methods are widely used to predict the pharmacokinetics and toxicity of drugs for risk assessment. Such methods contribute to a reduction in costs and animal use, and to a better prediction of the human *in vivo* situation when human tissue or cells are applied. As a result, the attrition rate for new drugs due to pharmacokinetic problems has declined greatly during the past 10 years.[1] However, there is an incessant drive for developing new *in vitro* systems, as the currently applied systems are not fully predictive of the *in vivo* situation. Primary human hepatocytes are widely available nowadays, though it remains difficult to maintain metabolic capacity in cultures of these cells beyond 24 h.[2] New approaches in culturing techniques such as that recently presented by Khetani and Bhatia[3] do offer possible solutions to this issue. These researchers developed a microscale multiwell co-culture system of human hepatocytes with fibroblasts in which phenotypic functions were maintained for several weeks in micropatterned co-cultures. However, primary hepatocytes cultured alone cannot be a model for those toxicity phenomena that are mediated *via* multicellular processes, for instance when Kupffer cells play a role in mediating hepatic toxicity.[4] Likewise, stellate cells are key players in the development of liver fibrosis.[5] Precision-cut liver slices (PCLS) represent an established alternative, since they closely resemble the intact architecture of the organ, with all cell types present and in their natural tissue-matrix configuration. Consequently, they are an appropriate tool for the prediction of multicellular processes. However, PCLS also lose metabolic capacity during culturing, albeit at a slower rate than hepatocytes alone.[6] Continuous perfusion culture of liver slices with medium has been demonstrated to slow the rate of loss of metabolic activity and maintain protein expression, as demonstrated by Khong *et al*.[7] and Schumacher *et al*.[8] respectively. Khong *et al*. perfused thick liver slices using an intra-tissue approach, using a microneedle array inserted into the slice to deliver medium through the tissue. Schumacher *et al*. adopted a different approach, choosing instead to study slices in a flow-through device which was continuously perfused with fresh medium. We thus hypothesize that both the depletion of substrates, as mentioned by Schumacher *et al*.,[8] and the accumulation of waste products, as occurs in the medium of conventional cell cultures, may contribute to the observed decrease.

We therefore set out to develop a new *in vitro* system with continuous medium flow for absorption, distribution, metabolism, excretion, and toxicity studies (ADME-Tox) in liver slices. By making use of microfluidic technologies, both the delivery and removal of soluble factors becomes possible, so that precise control of the slice microenvironment can be achieved. Additional advantages of microfluidic systems include an increased flexibility of experimental control, allowing medium composition to be easily altered during an experiment. It also becomes conceivable to perform interorgan studies with minimal residence times, simply by investigating

the response of cells or tissue from different organs in connected chambers under flow conditions. Solution volumes are kept small in microfluidic devices, enabling increased sensitivity of metabolite detection with the possibility to perform in-line analysis. For all these reasons, various researchers have integrated primary hepatocytes in microfluidic devices.[9-11] The integration of cell lines from multiple organs into a single microfluidic device has already been shown by several researchers. Viravaidya *et al*.[12], for instance, describe a microsystem with connected microchambers containing animal lung and liver cells to probe interorgan naphthalene toxicity. However, as mentioned before, hepatocytes cultured alone do not resemble the complex architecture of the intact organ and cannot fully reproduce all liver functions. Several attempts have been made to co-culture hepatocytes with other cells, like Kupffer cells, in microfluidic systems to mimic the cell-cell interactions present in the liver.[13-15] We have chosen an alternative approach based on the integration of PCLS into microfluidic devices, to combine the advantages of both microfluidics and slices. To the best of our knowledge, there has been only one previous example published of the integration of liver tissue explants into a microfluidic device.[16] However, the tissue in this device exhibited very limited viability.

The aim of this paper is to describe the design and realization of a polymer microfluidic biochip for the incubation and metabolic study of live tissue slices. This is the first example of the integration of PCLS into a microfluidic device. The requirements for this low-cost microfluidic device are biocompatibility and the maintenance of constant oxygen concentration, pH, medium composition, and temperature conditions over time. In addition, flow conditions within the device should facilitate the fast diffusion of nutrients toward the inner cells of the slice. Hence, a well-defined microenvironment around the liver slice should be created, with a continuous supply of nutrients and oxygen and removal of waste products, in contrast to the conventional well plate system, where the medium usually is only refreshed every 24 hours. In well plates, medium parameters like pH, temperature, and oxygen are optimized by a continuous flow of carbogen gas (95% O_2 and 5% CO_2) into the wells plates. In a biochip, this requires a technically different approach, since a continuous supply of medium is applied at low flow rate. Moreover, though medium flow should be high enough to efficiently refresh the medium, it should not be too high because this could damage the tissue. To minimize the possibility of damage, the flow should be evenly distributed toward the liver slice. The dimensions should be kept small to minimize dilution in the chip due to the flow and thus achieve high sensitivity for analysis.

To address all the above-mentioned device criteria, we developed a flow-through polydimethylsiloxane (PDMS) microdevice for the perifusion of liver slices with polycarbonate membranes integrated to realize a well-characterized medium flow. The term “perifusion” is used here instead of “perfusion” since the medium flows

around the tissue slice rather than perfusing through it. The microdevice also incorporates PDMS membranes to act as "breathing" membranes. One of the advantages of using PDMS is its permeability to gasses.[17] However, this permeability is limited by the thickness of the PDMS. Therefore, a device was designed with a thin PDMS membrane to obtain sufficient oxygen and carbon dioxide supply while at the same time providing adequate pressure resistance to the flow. Thin, precision-cut rat liver slices were incubated in the new device, and their viability and metabolic capacity were compared with those in the conventional well plate system. The well plate system provides an excellent benchmark, as it has been shown that slices cultured in wells reflect the metabolic capacity of the liver very well over periods of at least 8 hours.[18] To test metabolic capacity, we chose to study the metabolism of 7-ethoxycoumarin. This compound is metabolized by cytochrome P450-mediated phase I metabolism to 7-hydroxycoumarin, which is then further metabolized by phase II enzymes to a sulfate and glucuronide conjugate (Figure 1).

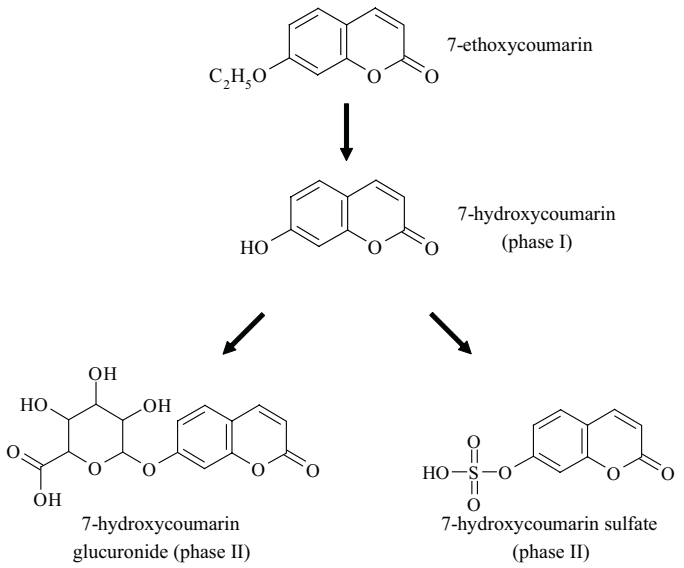

Figure 1. Metabolic pathway of 7-ethoxycoumarin (7-EC).

Materials and Methods

Animals

Male Wistar rats (HsdCpb:WU) weighing *ca.* 350 g were obtained from Harlan (Horst, The Netherlands). The rats were maintained under a 12-h light/dark cycle in cages in a temperature- and humidity-controlled room with free access to food (Harlan chow no. 2018, Horst, The Netherlands) and tap water. The use of animals for these experiments was approved by the Animal Ethics Committee of the University of Groningen.

Preparation of precision-cut liver slices

After anaesthetizing the rat with isoflurane/O_2, the liver was excised and placed in ice-cold Viaspan organ preservation solution (University of Wisconsin (UW) solution, Du Pont Critical Care, Waukegab, IL, USA). A 5-mm-diameter hollow drill bit was utilized to obtain cylindrical samples of liver tissue (cores). These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in oxygenated ice-cold (0-4°C) Krebs–Henseleit buffer. Liver slices were stored in ice-cold UW solution until incubation. The slices were approximately 4 mm in diameter, 100 µm thick (~6 cell layers), and had a wet weight of about 3.0 mg.

Microdevice fabrication

Figure 2 shows a schematic view of the microdevice, consisting of 10 layers of PDMS. All PDMS layers were made by thoroughly mixing Sylgard 184 prepolymer with the curing agent (Dow Corning, Mavom BV, The Netherlands) at a 10:1 mass ratio. All bonding steps between PDMS layers were performed using a UV ozone cleaning system (UVOCS, Montgomeryville, PA, USA). One slab of PDMS was placed in the UV ozone apparatus for one hour to make the surface glassy. After cooling down this PDMS slab, it was again placed in the UV ozone system together with a new, untreated PDMS slab for 15 minutes to activate (oxidize) the surfaces of both. The slabs were then removed from the system, immediately brought into contact with each other and left to react with each other. An irreversible bond between the two PDMS pieces was formed after approximately three hours.

The device consists of ten layers of PDMS, with two polycarbonate membranes inserted in the microchamber, one just after the inlet, the other before the outlet. Starting from the bottom of the microdevice, the first layer is 2-3 mm thick and ensures leakage-free connection of the tubing to the inlet of the chip. The inlets were made by pouring PDMS into a Petri dish with Ø1.5 mm stainless steel rods placed at the inlet positions. To form the aeration window of the microchamber, a 4-mm-diameter hole was punched with a biopsy puncher (D-Care BV, Houten, The Netherlands). On top of this first layer, a thin, 250 µm PDMS membrane was placed to act as the "breathing" layer forming the bottom of the chamber. The PDMS membrane was fabricated by spin-coating 6 grams of a PDMS prepolymer mixture on a 4″ bare silicon wafer at 600 rpm for 20 seconds. After these two layers were bonded, the 250-µm-thick membrane covering the inlet for the medium flow was perforated with a 1.5-mm-diameter biopsy punch. The third layer is the microfluidic channel connecting the inlet to the microchamber. This channel is 3 mm long, 500 µm wide and 100 µm deep, and was produced using a standard soft lithography procedure which we have adapted from Duffy *et al*.[19, 20] The end of the channel widens into a chamber with a diameter of 4 mm having a droplet shape (see Figure 2b). In earlier versions of the device, the 500-µm-wide channel was directly connected to a circular

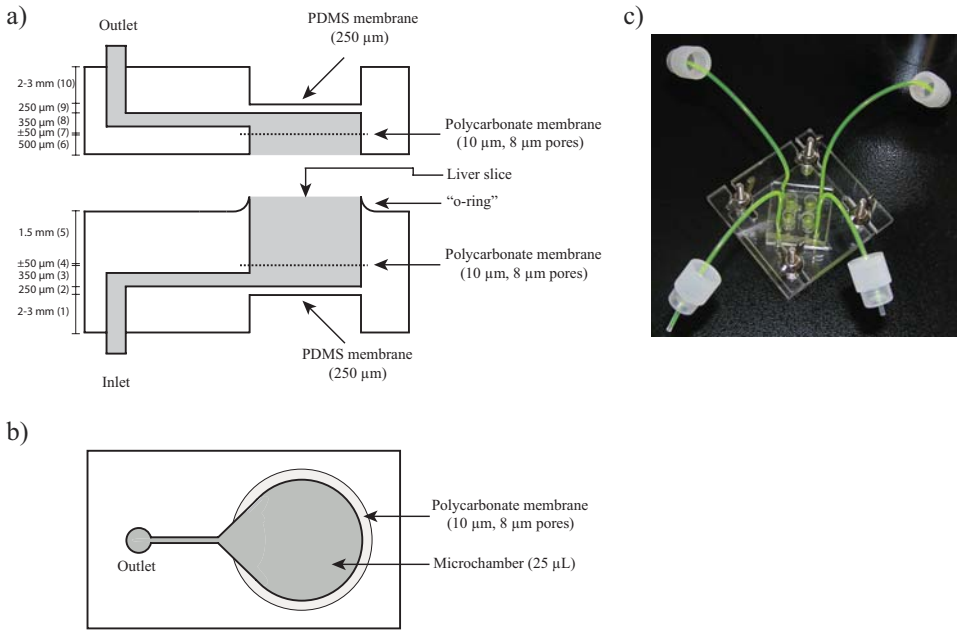

Figure 2. Schematic diagrams ((a) cross-sectional view and (b) top view) and (c) photograph of the PDMS biochip with integrated membranes. The microchamber dimensions are Ø4 mm x 2 mm. The dimensions of one chip, containing four microchambers, are 20 mm x 20 mm x 12 mm (L x W x H).

4-mm-diameter chamber. This geometry is problematic, however, as medium flow fails to follow the walls of the chamber as it enters this element, separating instead from the walls at the chamber entrance and flowing initially through the center. When filling an empty chamber with liquid for the first time, this flow separation results in air being trapped between solution flow and wall. This leads to the formation of air pockets or bubbles in the regions directly to the left and right of the entrance; once formed, these are extremely difficult to remove. The droplet-shaped structure ensures an even, more gradual flow of medium into the chamber underneath the inlet filter, without the occurrence of flow separation. The formation of air bubbles just beyond the chamber entrance can be overcome in this way. Sugiura *et al*. observed the same air bubble problem in their perfusion device, and were also able to solve it by changing the in- and outlet chamber geometries to cone-shaped channels to ensure liquid flows were guided along chamber walls.[21]

The fourth, very thin PDMS layer fixes the polycarbonate filter within the device, while the fifth layer contains the microchamber in which the liver slice is perifused. The filter ensures an equal distribution of medium to the entire liver slice, to suspend it horizontally in the flow in the chamber. The 1.5-mm-thick microchamber (Layer 5) was made by pouring a PDMS prepolymer solution into a Petri dish with 4-mm-

diameter stainless-steel rods placed at the location of the microchambers. During the curing process, PDMS crept up around the stainless-steel rods to produce a raised edge upon removal of the pins. This PDMS ridge around the chamber proved to be an advantageous feature in this layer, as it functions as an O-ring to ensure a leakage-free reversible assembly with the other half of the chamber device formed in Layers 6 through 10 (see Figure 2a). A 10-µm-thick polycarbonate membrane (Millipore, Amsterdam, The Netherlands) was bonded to the microchamber as described by Chueh *et al*.[22] Briefly, a 50-µm-thick PDMS layer was spin-coated on a bare silicon wafer, and the bottom of the PDMS microchamber layer was placed on this uncured PDMS layer for 30 seconds to generate a "mortar" layer (Layer 4). The microchamber layer was then removed from the wafer and a polycarbonate membrane with the desired size (Ø6 mm, 10 µm thick) was placed on top of it. The uncured PDMS then served as glue, penetrating into the pores of the membrane and creating a stable and leakage-free bond. The microchamber with polycarbonate filter was bonded to the other three PDMS layers described above using UV ozone activation.

To create a reversibly bonded microchamber, the stack comprising Layers 1 to 5 was closed with a similar 5-layer PDMS stack. The only difference is that the 1.5-mm-thick microchamber layer (Layer 5) is replaced by a 0.5-mm-thick PDMS layer (Layer 6). The openings for the chambers in this layer were punched with a Ø4 mm puncher rather than molded using pins. The resulting holes therefore were not surrounded by a built-in O-ring-like structure. Assembly of the two stacks resulted in a microchamber having a volume of 25 µL. It should be noted that each chip contained 4 microchambers, each perfused separately with medium.

During incubation experiments, the assembled chip was held in a polycarbonate clamp to realize a closed, leakage-free seal (see Figure 2c). The polycarbonate clamp was fabricated in-house with a Sherline CNC milling system (Vista, CA, USA). Windows were drilled in the polycarbonate layers to align with the microchambers, to give direct access of the "breathing" layers to the surrounding environment. These windows ensured efficient diffusion of oxygen and carbon dioxide through the PDMS membrane into the microchamber. Four screws with wing-nuts at the corners of the clamp were tightened carefully to fix the microchamber device in the clamp without deforming it.

Incubation system

Well plates

Incubation of liver slices in 12-well plates was performed as described before.[23] In brief, the slices were individually incubated in wells of a 12-well plate (Greiner bio-one GmbH, Frickenhausen, Austria) in 1.3 mL Williams Medium E with Glutamax-I (Gibco, Paisley, UK), supplemented with 25 mM D-glucose (Sigma-Aldrich, St. Louis, MO, USA) and 50 µg/mL gentamicin (Gibco, Paisley, UK). The well plates

were placed in plastic boxes, which were in turn placed in a 37°C incubator with continuous supply of carbogen (95% O_2 and 5% CO_2). The plastic boxes were put on a shaker with a shaking speed of 90 cycles per minute.

Biochip

Liver slices were placed in separate chambers in the biochip which had been filled with medium that had been pre-equilibrated with carbogen gas at 37°C. The chip was placed in an incubator set at 37°C. Plastic disposable syringes (Omnifix, B. Braun, Oss, The Netherlands) placed in a syringe pump delivered pre-carbogenated Williams Medium E (with Glutamax-I), supplemented with 25 mM D-glucose and 50 µg/mL gentamicin, to individual chambers in the biochip *via* PEEK tubing (200 mm x 0.75 mm ID, Bester BV, Amstelveen, The Netherlands). PTFE tubing (70 mm x 0.5 mm ID, Polyfluor Plastics, Oosterhout, The Netherlands) was connected to the outlet of the chip where medium fractions were collected. The chip was placed in a plastic box with continuous supply of carbogen to compensate for the loss of O_2 and CO_2 in the syringes and the biochip during the experiment. A schematic view of the system is given in Figure 3. The flow rate used in the experiments was always set at 10 µL/min, so that the medium in each microchamber was refreshed once every 2.5 minutes.

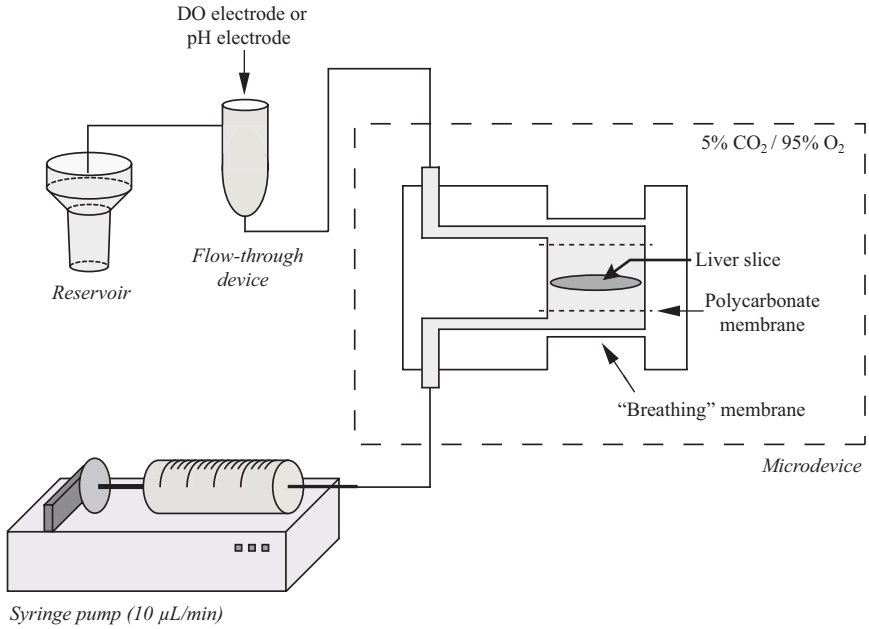

Figure 3. Schematic view of the set-up. The flow rate during incubation experiments was 10 µL/min.

Gas diffusion

To check whether the diffusion of O_2 and CO_2 through PDMS was sufficient to

realize a stable environment for the liver slice, the pH and dissolved oxygen (DO) were measured at the outlet of the biochip with no slice present during perfusion of the chip for 24 hours. For this, a polycarbonate (relatively impermeable for gasses) flow-through device was fabricated in-house with a medium inlet at the bottom and an outlet at the side (see Figure 3). An opening at the top allowed the insertion of a semi-micro-pH electrode (Thermo Orion, Beverly, MA, USA) or micro-DO electrode (Lazar Research Laboratories, Los Angeles, CA, USA). The flow-through device had a fluid compartment of 150 µL or 300 µL for the pH or DO measurements, respectively. A two-point calibration was performed for the pH electrode (pH 4 and 7), and a three-point calibration for the DO electrode (0%, 21%, 95% DO).

Viability testing

To assess the viability of rat liver slices in the microchamber and compare the results with the well plate system, leakage of the enzyme lactate dehydrogenase (LDH) was measured. LDH was analyzed using the Roche/Hitachi Modular system (Roche, Mannheim, Germany) according to a routine laboratory procedure. Immediately after slicing, the slices were washed in prewarmed medium and then placed in the well plates and in the microchamber. During 24 h of incubation, fractions were collected at the outlet of the chip at 1, 2, 4, 6, 8, 12, 20, and 24 hours. In the well plates, 150 µL of medium was pipetted out of the well and replaced by 150 µL fresh medium at the same times as for the biochip. All experiments were performed with three slices per rat for three rats (9 slices in total). Liver slices were collected after incubation, homogenized in 1 mL medium and centrifuged. A supernatant volume of 200 µL was used to assess the LDH content of the slice.

Metabolite analysis

For metabolite studies, slices were incubated with 7-ethoxycoumarin (7-EC) (Fluka Chemie, Buchs, Switzerland) at a final concentration of 500 µM or 75 µM for 3 hours. After incubation with 500 µM, the medium samples (well plates, 1.3 mL, and chip, 1.8 mL) were collected and stored at -20°C until analysis. During perifusion with 75 µM 7-EC in the chip, 300 or 600 µL medium fractions were continuously collected every 30 or 60 minutes, respectively, over a period of 3 hours, and fractions were stored at -20°C till further analysis. Experiments with 500 µM 7-EC were performed in triplicate for five rats, and with 75 µM 7-EC in triplicate for three rats.

Analysis of the collected medium fractions was performed using a modified version of an HPLC method described previously.[24] The system consisted of two Waters 510 pumps, a Waters automated gradient controller 680, a WISP 710 autosampler (Millipore, Milford, MA, USA), a Croco-cil column oven set at 60°C (Varian, Paris, France), and a Spectroflow 757 UV detector set at 320 nm (Kratos, Kanagawa, Japan). Separations were performed on a BDS Hypersil C8 (250 m x 4.6

mm, 5 µm) column (Thermo, Bellefonte, PA, USA) preceded by a C18 GuardPak precolumn (Waters). The mobile phase consisted of 7.5% acetonitrile/1% acetic acid/water containing 5 mM tetrabutylammonium hydrogensulfate, with an 18-minute linear gradient to 45% acetonitrile/1% acetic acid/water containing 5 mM tetrabutylammonium hydrogensulfate. The run time was longer than the 10-minute run time mentioned by Walsh *et al*. This was needed to separate an impurity present in 7-ethoxycoumarin from the 7-hydroxycoumarin sulfate peak. The flow rate was set at 1.5 mL/min.

Sodium azide (1 mg/mL, final concentration) was added to the thawed medium samples to inhibit bacterial growth during HPLC analysis. Samples were first centrifuged using an Eppendorf centrifuge for 3 minutes at 16,000 *g* and 4°C, and then directly analyzed on the HPLC system. Stock solutions of 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC) (Sigma-Aldrich), 7-hydroxycoumarin sulfate (7-HC-S) (Sigma-Aldrich), and 7-hydroxycoumarin glucuronide (7-HC-G) (Sigma-Aldrich) were used as standards. Pilot experiments showed that the percentage of metabolites retained in the liver slices were negligible (data not shown).

Results and Discussion

Flow performance of the biochip

The polycarbonate filters at the bottom and top of the microchamber were necessary to ensure an equal distribution of medium flow across the entire chamber cross-section upwards towards the liver slice. The hydrophilic filter below the slice acts as an up-side-down showerhead, with the 8-µm pores serving as an array of nozzles with essentially equivalent flow resistances. Simulations with Comsol Multiphysics (Comsol, UK) confirmed the hypothesis that a uniform medium velocity distribution is obtained across the chamber by using a filter (see Supplementary Information online). Without a filter, a high flow rate was obtained at the microchamber entrance (left side of Figure 2, Layer 3), while at the other side of the microchamber entrance the medium was only refreshed by diffusion (right side of Figure 2, Layer 3). The velocity distribution of individual flows of medium entering the chamber through the pores of the filter is thus very uniform across the chamber, and we observed that the slice is suspended horizontally in the flow. Because the slice experiences the same pressure over its entire surface, it did not curl up in the microchamber, which was the case when the filter was not present. Efficient delivery by diffusion of nutrients and oxygen to inner cells in the slice is not guaranteed when slices are folded or curled up, since diffusion distances to inner cells are increased when slices assume irregular three-dimensional shapes. The array of pores in the exit filter established a uniform flow resistance across the top of the chamber to maintain a uniform velocity gradient around the slice. This filter also prevented loss of the slice through the outlet, thereby simplifying operation of the device, particularly when medium flows were initiated

at the beginning of an experiment. The integration of membranes into microfluidic devices often results in leakage due to poor fixation of membranes between layers composed of other materials.[22] Therefore, the microdevice was checked for proper sealing of the polycarbonate membranes by flushing it with a fluorescein solution. No leakage was observed at the edges of the membranes when viewed under a fluorescence microscope and fluorescein was only found in the liquid stream, indicating proper sealing.

In addition to the PDMS-polycarbonate bonding, the quality of the reversible bonding between the two clamped PDMS stacks was also checked for the possibility of leakage. Using a microdevice without the extra molded PDMS "O-ring" (see Figure 2), it proved impossible to clamp the two planar contact surfaces together in such a way that a good seal was achieved, and leakage occurred between the two layers. However, with the "O-ring", the pressure applied in the clamp is more evenly distributed around the entire microchamber opening where it is required, and proper bonding can be obtained. Under these circumstances, no leakage was observed between the reversibly bonded PDMS stacks, even when much higher flow rates (250 μL/min) were applied.

By making use of thin liver slices (approximately 100 μm, or ~6 cell layers), the diffusion distance of nutrients and oxygen toward the inner cells of the slice is minimized. Application of a medium flow to perifuse the slice ensures that medium is constantly refreshed in the proximity of the tissue, as it would be in the body. This will also enhance nutrient and oxygen delivery to inner cells not directly exposed to medium flow. Consequently, when the medium around the slice contains enough nutrients, oxygen, and has the right pH and temperature, all cells in the slice will function optimally.[25, 26] To date, the only other reported microfluidic device used to incubate tissue, to the best of our knowledge, has been that of Hattersley *et al*.[16] The main differences between our system and this published example are twofold. Firstly, these researchers used relatively thick tissue biopsies (4 mm^3) which had been frozen in liquid nitrogen before analysis without any cryoprotectant, whereas in our case, very thin, fresh slices were used in the experiments, which is absolutely necessary for proper viability and metabolic function.[6] Secondly, though the tissue was bathed by medium, it was not placed directly in a perfusing stream as in our case, and supply of oxygen and nutrients was not guaranteed. This, together with the thickness of the biopsy, may have contributed to the low tissue viability observed by Hattersley *et al*. An albumin synthesis of 0.006 ng/min/mg liver tissue was reported, as compared to 10-70 ng/min/mg liver tissue published to be normal for rat liver by others.[7, 27, 28]

Gas diffusion

The advantage of PDMS gas permeability can also be a disadvantage. The pre-carbogenated medium used in biochip experiments contained higher concentrations

of both O_2 and CO_2 than are found in ambient air. Therefore, if these concentrations are not controlled in the biochip's surroundings, gasses will tend to diffuse into the PDMS from the high-concentration medium side toward areas of lower concentration outside the device. When perfusing a PDMS device with pre-carbogenated medium in ambient conditions (<5% CO_2), the CO_2 present in the medium diffused into the PDMS, resulting in an increase in pH. Even in thick PDMS devices (3 mm) without aeration membranes, CO_2 loss was significant, with a pH increase from 7.4 to 7.6 being observed.

The stability of pH and dissolved oxygen (DO) values was measured over a period of 24 hours using pre-carbogenated solutions and placing our system in an incubator containing a carbogen atmosphere. As can be seen in Figure 4a, the pH was very stable over the 24 hour period, with a value of 7.41±0.01, which indicates equilibrium with 5% CO_2 at 37°C. The DO concentration was also very high (92.5-94.5 %) and stable over the period measured (Figure 4b). These results indicate an optimal diffusion of gasses through the PDMS membranes and a stable environment in the microchamber, which is very important for cell viability and functioning.[26] The pH and DO values were also measured in the well plate system to be 7.4 and 95%, respectively.

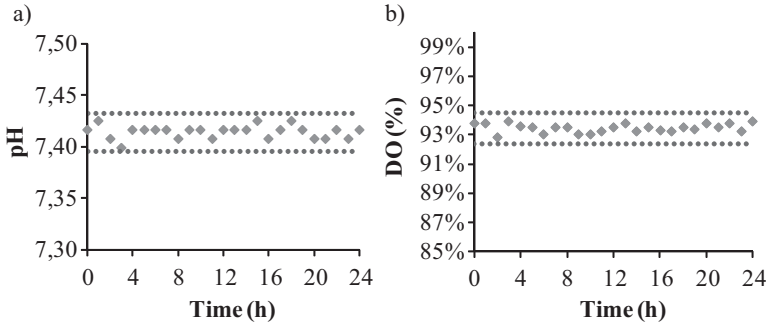

Figure 4. (a) pH measured with a semi-micro-pH electrode and (b) dissolved oxygen (DO) measured with a micro-oxygen sensor, during 24 h of perfusion with medium at the outlet of the microchamber.

To ensure that pH was also constant during liver slice perifusion experiments, the medium was supplemented with phenol red, a pH indicator. No color change, and hence no pH change, was observed during experiments, indicating that the CO_2 level was sufficient, and moreover, constant over time. The concentration of oxygen in the medium should also be sufficient during a perifusion experiment, since the medium is initially saturated with oxygen, and the constant flow of medium through the microchamber ensures a continuous supply. When liver slices consume oxygen, oxygen will also be replenished by diffusion through the PDMS into the medium (recall that experiments are performed with the PDMS device placed in an incubator

under a controlled carbogen atmosphere). It is also known that the oxygen diffusion coefficient and oxygen solubility are higher in PDMS than in medium,[29] leading to the PDMS acting as an oxygen reservoir for the microflow system and ensuring sufficient and constant DO during experiments.

The oxygen concentration used in both the well plate and biochip experiments is high compared to the *in vivo* situation. However, several authors[30-32] have shown that high oxygen concentrations are necessary for long-term viability, despite possible associated oxidative stress. However, we could find no signs of tissue damage due to oxidative stress between 50% and 95% of oxygen (unpublished observations). Moreover, the requirement for high oxygen concentrations appears to be similar in different conventional incubation systems.[30-32] Therefore, it was decided to also use this high concentration of oxygen in the microdevice for this initial study. If a lower percentage of oxygen were needed in the biochip incubation system, it could easily be achieved by changing the oxygen concentration in both the incubator environment and medium.

Liver slice viability

To assess liver slice viability in the two incubation systems, leakage of the enzyme, LDH, was determined over a 24-hour period in both systems, using liver slices from the same three rats (see Figure 5). A relatively high percentage of LDH leakage was found in the first hour (first fraction). This is probably due to slicing and slice handling procedures, since the effect was the same in well plates as in microchambers. Cells at the surface of the slices are damaged during the slicing procedure, and most likely

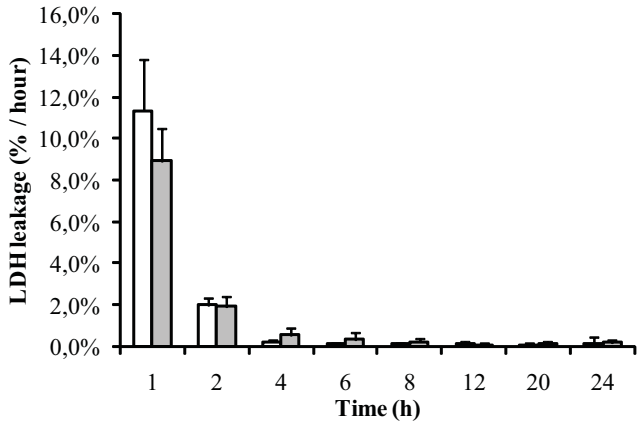

Figure 5. Lactate dehydrogenase (LDH) leakage as a percentage of the total LDH present in the slice measured during perifusion in biochips and incubation in well plates. White bars represent the leakage in well plates and gray the leakage in the biochip. Results are mean ± standard error of the mean (SEM) of 3 rats, 3 slices per incubation system per experiment.

enzymes are still leaking out of these damaged cells in the first hour of incubation. The relatively high percentage of LDH leakage in the first hour was also observed by Obatomi *et al*., in a study that showed that damaged cells were removed during a pre-incubation period of 1 hour.[33] As shown in Figure 5, the LDH leakage drops substantially after the first fraction to less than 0.5% of the slice content per hour. After 24 h of incubation, more than 95% of the LDH that was present after the first hour was retained in the slices. This indicates that the cells are not damaged by the flow, and that slices can be used for incubations up to at least 24 h. It is well known that PDMS is a relatively hydrophobic polymer.[26] To verify that the protein LDH was not adsorbing onto the PDMS, the total amounts of LDH remaining in the slice after incubation was assessed and summed with amounts measured in the medium fractions. The total amount was shown to be equal to the total slice content at the start of the incubation, and was also equal to that found after incubation in the well plates. It can be concluded that LDH was not adsorbed onto PDMS.

The leakage pattern obtained in the microchamber is very comparable to the LDH leakage obtained in the conventional well plate system, indicating high viability in both systems.

Metabolite analysis

Figure 6 shows the metabolism of 500 μM 7-EC in well plates compared to the microdevice. This concentration is well above the concentration required to achieve a maximum metabolic rate for phase I metabolism.[34] We can therefore assume that the rate of metabolism is constant over the course of a well plate experiment, despite a relatively small decrease in substrate concentration during incubation. This allows comparison of the metabolic rate of 7-EC in well plates with that in the microdevice during a 3-hour incubation. Liver slices incubated in the microchamber were able to produce both phase I as well as phase II metabolites. The total phase I formation of 7-HC was 214 ± 5 pmol/min/mg protein and was comparable in both systems. Moreover, the observed metabolic rates were the same as previously reported for the incubation of liver slices in well plates with 500 μM 7-EC.[34] When comparing the conventional incubation system with the biochip, a similar sulfation and glucuronidation rate was found in the microchamber compared to the well plates.

As noted above, PDMS is a relatively hydrophobic polymer and can adsorb 7-EC. However, the concentration in the outflow of medium was still well above the concentration required for the metabolizing enzymes to attain their maximum rate. Therefore, even though some 7-EC adsorption onto the PDMS did occur, it did not negatively affect the metabolic rate.

An advantage of the perifusion system is that the environment is very stable due to the continuous influx of fresh medium. As a result, the metabolite formation could be maintained constant, even at nonsaturating 7-EC concentrations where enzymes no

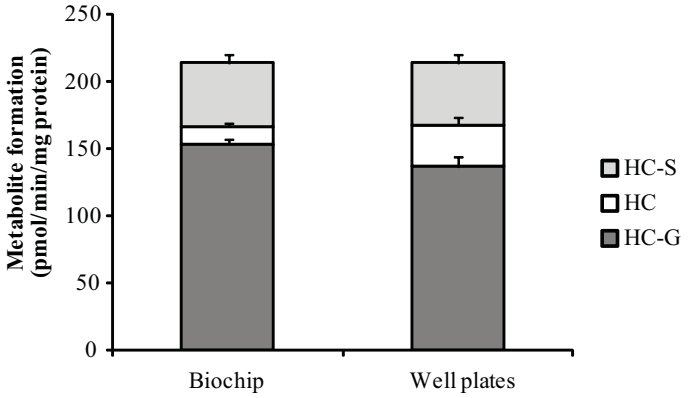

Figure 6. Metabolic activity of 7-ethoxycoumarin (7-EC, 500 µM). 7-EC is converted into the phase I metabolite 7-hydroxycoumarin (7-HC), and further metabolized into the two phase II metabolites; 7-hydroxycoumarin glucuronide (7-HC-G) and 7-hydroxycoumarin sulfate (7-HC-S). Results are mean ± standard error of the mean (SEM) of 5 rats, with 3 slices per treatment.

longer operate at their maximum turnover rate (see Figure 7). In this experiment, the metabolism of 75 µM 7-EC was monitored in the perifusion system. Fractions were collected over a period of 3 hours, and the constant concentration of metabolites in the fractions indicates a constant rate of formation in the time period measured. It can be concluded from Figure 7 that the total formation of metabolites for 75 µM 7-EC was 1.1 x 10^4 pmol/mg protein/180 min, or 18 pmol/min/slice (a liver slice contains around 0.3 mg of protein). The concentration of metabolites formed during the whole incubation period was constant at 1.8 pmol/µL. This means that after 30 minutes, 540 pmol/300 µL was formed in the biochip, while in the well plates the 540 pmol formed would be diluted in 1300 µL. Thus, the total metabolite concentration in the fraction collected from the biochip over 30 minutes, 1.8 pmol/µL, was 4.3 times higher than expected in the well plate. Analysis of total metabolite concentration is thus more sensitive in the biochip samples than in well plate samples obtained after 30 min. In fact, metabolite concentrations will remain higher in biochip samples until 130 minutes have elapsed in the experiment. At that point, the same volume will have passed through the biochip as is present in the well plate, and metabolite concentrations will be equal in the two systems (both 1.8 pmol/µL). Thereafter metabolites start to accumulate above this concentration in a well plate experiment. In the microdevice, analysis might be possible after incubation times which are even shorter than 30 minutes, though this was not tested in this study. However, preconcentration of metabolites might be required after collection when incubating substrates with low metabolic rates. This preconcentration can easily be achieved with conventional systems[35] or on-chip[36]. The most essential difference

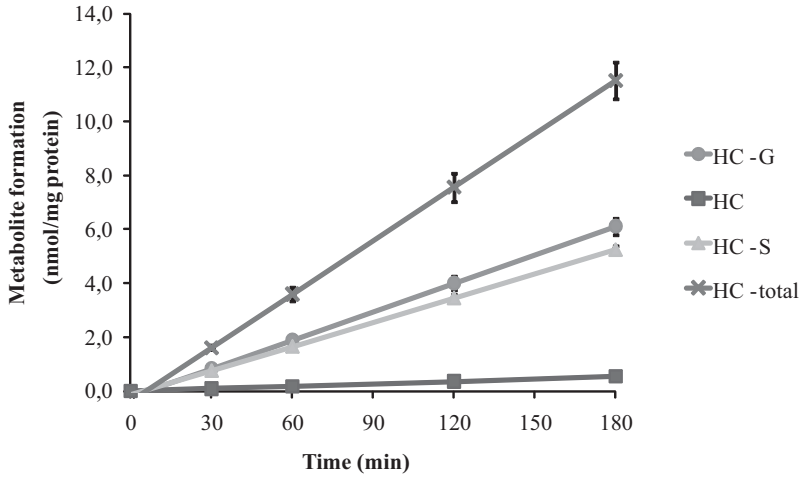

Figure 7. Cumulative metabolite formation for 75 µM 7-ethoxycoumarin in the microdevice, measured over time. 7-EC is converted into the phase I metabolite 7-hydroxycoumarin (7-HC, squares), and further metabolized into the two phase II metabolites, 7-hydroxycoumarin glucuronide (7-HC-G, dots) and 7-hydroxycoumarin sulfate (7-HC-S, triangles). Total metabolite formation is indicated as HC-total (cross). Results are mean ± SEM of 3 rats, 3 slice per experiment.

between the biochip and the well plates is the continuous flow of medium in the biochip. This means a constant medium composition for tissue slice incubation, which has been shown to be necessary to maintain protein expression and histo-architecture according to Schumacher *et al*.[8] The conclusion that medium composition remains constant was supported by the observation that metabolite formation was constant at a substrate concentration which was below the saturating concentration (see Figure 7). In contrast, substrate concentrations in wells decrease over time as enzyme consumption of substrate causes its depletion in the medium, resulting in decreasing metabolic rates.[23] Nutrients are also depleted from the medium in wells over time, resulting in a nutrient gradient. Refreshing the medium, normally after 24 h, can thus result in a shock for the tissue slices as nutrient concentrations are abruptly increased. Sudden changes in medium composition can be avoided in the biochip through the use of continuous flow, ensuring that tissue slices do not undergo periodic shock through abrupt modifications of their environment. Moreover, medium composition can be easily and gradually changed throughout the course of an experiment, if desired.

Conclusion

We have developed a new *in vitro* system for PCLS incubation. Incubations were performed in a 25-µL microchamber (as opposed to a 1.3-mL well) under conditions of constant flow (10 µL/min). Medium was fully refreshed in the microchamber every

2.5 minutes, enough to realize a constant and sufficient delivery of oxygen and nutrients to the liver slice. In contrast to the well plate format, the microfluidic device is hermetically sealed, necessitating a different approach to control the transport of CO_2 and O_2 into the microchamber. The incorporation of thin, gas-permeable PDMS membranes as aeration windows in the floor and ceiling of the microchamber facilitated this transport, provided the device was placed in an atmosphere containing 5% CO_2 and 95% O_2. In-line monitoring of medium exiting the chamber proved that the microdevice offers a well-controlled environment for liver slices, as the pH and DO were stable over the 24 h period measured. The polycarbonate filters are also an important and novel feature of the biochip, as they ensure uniform perifusion of the liver slice with medium.

The viability of PCLS remained high during 24 h, as evidenced by the low percentage of LDH leakage. Moreover, the observed LDH leakage was comparable to that in the conventional well plate incubation system, the benchmark for our experiments. Further experiments have to be performed to determine the maximum attainable lifetime of liver slices in the microdevice. The metabolic rate of the slices in the biochip was fully comparable to those in the well plate, and constant over 3 h. As a consequence, metabolite concentrations in the medium flowing from the microchamber were also constant over time, in sharp contrast to wells, in which metabolite concentrations increase over time. Analysis of metabolites produced by slices in wells is thus often not possible early on in an incubation experiment, as concentrations are too low to be detected. In the flow system, analysis is possible as soon as sufficient sample has been collected to allow for detection by HPLC, which is determined by the rate of metabolism and detection limits. In this study, we calculated that 10 minutes of incubation should have been enough to assess the metabolism rate, making this device more efficient than conventional systems. Because of continuous medium flow, the system could also be coupled to an HPLC system for performing on-line analysis to further increase efficiency, which will be further investigated in the near future. The continuous flow of medium will remove the waste products formed by the tissue slices which might affect liver metabolism, thereby making this system more predictive of the *in vivo* situation than well plates. Nutrient concentrations were also constant in the biochip and not being depleted as in the case of well plates. As a result, the slice does not have a nutrient “shock” in the biochip as occurs in well plates when the medium is replaced only every 24 h.

In conclusion, this newly developed microdevice is very suitable for addressing a variety of both pharmacological and toxicological questions. In particular, it becomes possible to take into account the effects of flow on the viability and ADME-Tox properties of precision-cut tissue slices. Moreover, it enables studies on possible interactions between two or more types of tissue by sequentially perfusing several chambers with slices from different tissues. Since the perifusion system better mimics

the *in vivo* situation, the system has the potential to significantly contribute to drug metabolism and toxicology studies of novel chemical entities.

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4

On-Line HPLC Analysis System for Metabolism and Inhibition Studies in Precision-Cut Liver Slices

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Abstract

A novel approach for on-line monitoring of drug metabolism in continuously perifused, precision-cut liver slices (PCLS) in a microfluidic system has been developed using HPLC-UV. In this approach, PCLS are incubated in a microfluidic device made of polydimethylsiloxane (PDMS) by continuous, single-pass perifusion with fresh medium. Two syringe pumps are incorporated into the system to infuse substrates or inhibitors at varying concentrations into the perfusion medium just before the chip entrance. The medium containing the metabolites produced by the PCLS is directed towards an injection loop. Once filled, the content of this injection loop is automatically injected onto an HPLC for analysis.

The on-line analysis of metabolites was tested by using the substrate, 7-hydroxycoumarin (7-HC). Rapid switching between substrate and solvent control was possible, and a direct metabolic response of the liver slice to perifusion with substrate was detected. Very stable phase II metabolism over a period of 24 h was observed. The inhibitory effect of phloxine B on the formation of 7-hydroxycoumarin glucuronide (phase II product of 7-HC) was also investigated. Phloxine B was injected into the incubation medium in increasing concentrations varying from 0 to 200 μM. The results showed a concentration-dependent inhibition of 7-HC glucuronide formation and allowed the calculation of an IC50 value of ~85 μM using one single liver slice. On-line detection was also shown to be advantageous for the detection of unstable metabolites. This was demonstrated by determination of the metabolites of the drug, diclofenac. The reactive metabolite, acyl glucuronide, was detected at relatively high concentrations which remained very constant over a period of 4 hours. In contrast, only low and decreasing amounts of diclofenac acyl glucuronide could be measured in the conventional well-plate incubation system.

The advantages of this novel on-line analysis system for PCLS include the capability to obtain direct information about tissue function, assess the concentration dependence of drug-drug interactions in one single slice, and detect unstable metabolites. The system also enables fast analysis without the need to store samples, thus eliminating the associated freeze-thaw problems, and allows the simultaneous analysis of multiple metabolites.

Introduction

Studies on drug metabolism in the liver have most often employed microsomes prepared from liver cells, as they are easy to handle and one of the best characterized *in vitro* systems. However, for proper prediction of metabolism, it is a disadvantage that microsomes do not contain all the metabolizing enzymes present in the cells, and that the cofactors are not present at their physiological levels. The use of primary hepatocytes or precision-cut liver slices (PCLS) is therefore preferred for quantitative analysis. One of the advantages of PCLS over hepatocytes is that all the different cell types are present, including their metabolizing enzymes, in their natural tissue-matrix configuration. This makes liver slices a good organomimetic model complete with intact cell-cell interactions, reflecting a more physiological situation. Commonly, liver slices are incubated in well plates or in a dynamic organ culture system.[1] However, recently we and others have reported examples of incubation systems with continuous liver slice perifusion.[2-4] The incubation environment is kept stable over time due to the flow, with a continuous influx of nutrients and removal of waste products. Another advantage of using perifusion is that it allows easy coupling to other flow-based analytical methods such as HPLC to perform on-line analysis. Fractions collected at the outlet of the incubation system can in principle be injected directly onto an HPLC system to analyze the metabolites formed. Various on-line analysis systems have been developed for microsomal fractions using liquid chromatography[5-7] and capillary electrophoresis[8, 9]. Sung *et al.* even showed in-line monitoring of cytochrome P450 activity of a hepatoma cell line (HepG2/C3A) *via* fluorescence detection on chip.[10] Until now, however, no examples exist for the on-line analysis of metabolites using precision-cut tissue slices (PCTS), though this capability clearly would facilitate metabolism studies. We recently developed a novel microfluidic biochip for the perifusion and incubation of precision-cut tissue slices.[4] The results showed that it is possible to keep liver slices viable for at least 24 h and intestinal slices for at least 7 hours in the biochip, and that 7-ethoxycoumarin metabolism is comparable with the conventional well-plate system and thus with the *in vivo* situation as well.[11, 12] Analysis, however, was still done off-line.

The aim of this study is to develop an on-line analysis system for the microfluidic biochip to perform metabolism and inhibition studies on-line in PCLS. With this system, it is possible to 1) inject a substrate into the medium just before the inlet of the biochip and 2) measure the metabolism in liver slices directly by automatic injection of fractions collected at the chip outlet onto an HPLC system. Switching between substrates, or addition of an inhibitor or inducer, is accomplished by two syringe pumps incorporated into the system. These pumps also allow the injection of a substrate or inhibitor multiple times at increasing concentrations to measure concentration-dependent effects on metabolism. Other advantages of this system are the speed of analysis and elimination of the necessity to store samples, enabling the

analysis of unstable metabolites. The quantification of these types of unstable compounds in conventional well plate incubations is impeded, as detectable concentrations are not achieved due to the relatively large incubation volume and decomposition during the incubation. In addition, PCTS function and stability of metabolic enzymes can be monitored directly over time. To show the applicability of the system, the phase II metabolism of 7-hydroxcoumarin (7-HC) into 7-hydroxycoumarin sulfate (7-HC-S) and 7-hydroxycoumarin glucuronide (7-HC-G) was determined over time.

The versatility of this system was further demonstrated by performing an inhibition study on-line in which phloxine B was infused together with 7-hydroxycoumarin. Phloxine B is a phenyl-xanthene dye which is known to inhibit the enzyme UDP-glucuronosyltransferase (UGT), especially the iso-enzyme UGT1A6.[13, 14] This UGT enzyme is the main catalyst for the glucuronidation of 7-HC. According to Mizutani *et al.*, phloxine B is a non-competitive inhibitor.[14] In this study, the tissue slices were perifused with different concentrations of phloxine B to assess the IC50 value (concentration in which 50% of the enzyme is inhibited) and confirm the mechanism of inhibition.

Finally, the ability to analyze unstable metabolites was demonstrated by incubating PCLS with diclofenac, a non-steroidal anti-inflammatory drug for the treatment of inflammation and pain. In the rat, diclofenac is mainly converted into 4′- and 5-hydroxydiclofenac and diclofenac acyl glucuronide.[15, 16] The last metabolite is considered responsible for serious idiosyncratic hepatotoxicity[17], but is, as many other acyl glucuronides, chemically unstable. This impedes the quantification of this type of compound in conventional well plate incubations, as discussed above. As a consequence, these metabolites can easily go undetected in the early stage of drug development, causing serious problems during clinical trials. Since the on-line system analyzes metabolites present in medium fractions directly after their formation, it is suitable to detect this unstable metabolite simultaneously with other metabolites.

Experimental section

Chemical reagents

Acetonitrile (ACN, HPLC grade) and methanol (MeOH, HPLC grade) were purchased from BioSolve BV (Valkenswaard, The Netherlands). Glacial acetic acid was obtained from VWR international BV (Amsterdam, The Netherlands). HPLC-grade water was prepared by passing demineralized water through an Arium 611 Ultrapure water system (Sartorius, Göttingen, Germany). Gentamicin and Williams medium E (WME) with Glutamax-I were obtained from Gibco (Paisley, UK). D-glucose monohydrate, ammonium formate, diclofenac (DCF) sodium salt, phloxine B, 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin sulfate (7-HC-S) and 7-hydroxycoumarin glucuronide (7-HC-G) were purchased from Sigma-Aldrich (St.

Louis, MO, USA). Diclofenac acyl glucuronide (DCF-AG), 4′-hydroxydiclofenac (4′-OH-DCF) and 5-hydroxydiclofenac (5-OH-DCF) were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Stock solutions of 7-HC in methanol (50 mM), DCF in DMSO (20 mM) and phloxine B in WME (20 mM) were prepared in-house. 7-HC and DCF solutions were stored at -20°C, and phloxine B at 4°C until further use.

Precision-cut rat liver slices

Liver tissue from male Wistar rats (HsdCpb:WU) weighing *ca.* 300 g were used for the experiments in this study. Animal use was approved by the Animal Ethics Committee of the University of Groningen. Precision-cut liver slices were prepared as described before.[4] Slices had a wet weight of 5 mg and were approximately 4 mm in diameter and 250 µm thick (~14 cell layers). They were stored for a maximum of 2 hours in ice-cold University of Wisconsin (UW) solution (Du Pont Critical Care, Waukegab, Il, USA) until placement in the biochip.

Instrumentation

A schematic view of the developed on-line analysis system is given in Figure 1. The system consists of five different parts: 1) a peristaltic pump (medium delivery), 2) an incubation box including the microfluidic biochip which has three chambers to perifuse 3 slices simultaneously, 3) three injection valves, 4) two syringe pumps (substrate / inhibitor delivery) connected to two two-position micro electric actuator injection valves, and 5) an HPLC system. An Ismatec IPN peristaltic pump (Ismatec SA, Zurich, Switzerland) equipped with Pharmed tubing (ID 0.25 mm, VWR international BV, Amsterdam, The Netherlands) was used for medium flow (10 µL/min). The medium itself was placed in a water bath kept at 37°C. Carbogen (95% O_2 / 5% CO_2) was continuously bubbled through the reservoir containing medium to maintain a constant pH of 7.4 and a high level of oxygen. Williams medium E with Glutamax-I (WME), supplemented with 50 µg/mL gentamicin and 25 mM D-glucose, was used as medium for all the experiments described. The medium was delivered to a plastic container (incubation box, 200 mm x 100 mm x 100 mm (L x W x H)) by means of Pharmed tubing. Inside this incubation box, a PEEK T-piece (Upchurch Scientific, Murrieta, CA, USA) was placed right before the inlet of the biochip to rapidly mix the medium with substrate added from the syringe pump(s). PTFE tubing (0.50 mm ID, Polyfluor plastics, Oosterhout, The Netherlands) connected the T-piece with the biochip. The incubation box was equipped with a Peltier element (Okaphone, Groningen, The Netherlands), a temperature controller (Horst temperature regulator HT 30, Sigma-Aldrich, Zwijndrecht, The Netherlands), and two computer ventilators to maintain a temperature of 37°C. The box was continuously supplied with humidified carbogen to ensure a well-controlled environment for tissue slice

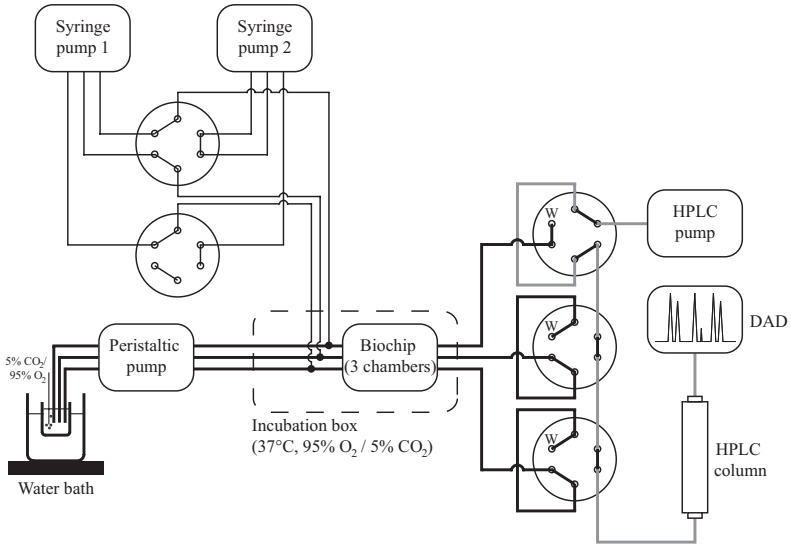

Figure 1. Schematic overview of the on-line analysis system for metabolism and inhibition studies. The system consists of a peristaltic pump for medium flow (10 µL/min), two syringe pumps for substrate or inhibitor introduction (100 nL/min), two substrate valves to switch between syringe pumps, temperature-controlled humidified incubation box containing the biochip, and three injection valves to inject the samples onto an analytical HPLC column. A more detailed description of the system may be found in the text. Note that the topmost injection valve has been switched to bring the injection loop filled with perifusate in-line with the HPLC pump for introduction of this sample onto the HPLC column. W= waste. DAD= diode array detector.

incubations. Individual slices were introduced into each of the three chambers of the polydimethylsiloxane (PDMS) chip, which was then placed in the incubation box. The fabrication of the PDMS chip has been described extensively elsewhere.[4] The chip incorporates microfluidic channels to deliver medium to each of the three microchambers containing slices. Two polycarbonate membranes (10 µm thick) form the ceiling and floor of each microchamber to realize an even distribution of medium flow around the tissue slice and to ensure that the slice is horizontally suspended in the flow. The chip also contains two 250-µm-thick PDMS membranes above and below each chamber acting as "breathing" membranes to keep the pH and oxygen levels stable in the incubation environment. CO_2 and O_2 exchange into the medium as it passes by these membranes on its way into and out of the microchamber. A schematic cross-sectional view of a microchamber is given in Figure 2; each chip contained three microchambers, as mentioned above. The outlet of each chamber was connected with PTFE tubing to a separate injection valve (Automated Cartridge Exchange unit, Spark Holland, Emmen, The Netherlands). The injection valves were

equipped with 75-µL PEEK injection loops (0.50 mm ID). At a flow rate of 10 µL/min, it takes 7.5 minutes to fill each loop with medium containing substrate and metabolites.

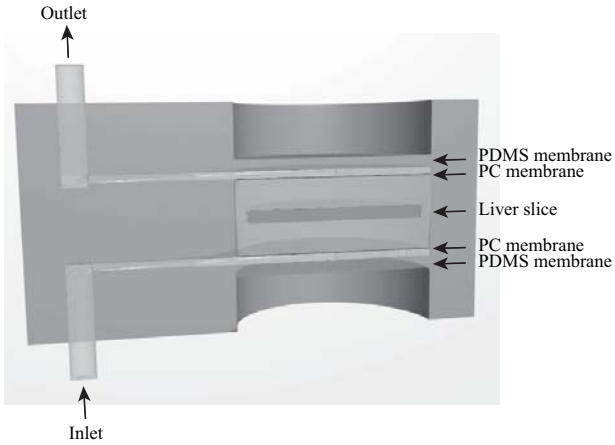

Figure 2. Cross-sectional view of the microfluidic device incorporating a liver slice, PDMS "breathing" membranes (250 µm thick) and polycarbonate (PC) membranes (10 µm thick, pore size of 8 µm). The dimensions of the microchamber are Ø4 mm x 2 mm (25 µL).

To add substrate to the medium *via* the T-piece as mentioned above, two New Era syringe pumps (New Era Pump Systems Inc., Farmingdale, NY, USA) equipped with 500-µL Hamilton syringes (Hamilton, Bonaduz, Switzerland) were used. The syringe pumps were computer-controlled to deliver 7-hydroxycoumarin (7-HC), phloxine B, or solvent as control (methanol or WME) at a flow rate of 100 nL/min. Silica capillaries (ID 75 µm, Aurora Borealis, Assen, The Netherlands) were used to connect the syringes to the two-position micro electric actuator injection valves (VICI Valco, Schenkon, Switzerland). These valves were used to switch between the two syringe pumps to realize a rapid change from one reagent solution to the other. After stopping a syringe pump, there is always pressure on the syringes, resulting in continued delivery of a solution for a short time. With the substrate valves, substrate delivery could be instantaneously discontinued when the pump was switched off. Silica capillaries were used to connect the substrate valves with the T-pieces located just before the inlet of the chip.

Samples from each chamber were injected sequentially onto an HPLC system by switching the corresponding injection valve to bring the 75-µL PEEK injection loop in-line with the HPLC system.

HPLC analysis of 7-hydroxycoumarin metabolites

The analysis was performed on an automated HPLC-UV system consisting of an

Agilent 1100 series LC system with low-pressure mixing (500 µL/min), column thermostat (30°C) and diode array detection (DAD) at 320 nm (Palo Alto, CA, USA). Separations were performed on a Hypersil 3.2 x 100 mm (5µm) BDS C18 column (Alltech, Breda, The Netherlands), preceded by a 2.1 x 12.5 mm XDB-C8 guard column (Agilent, Palo Alto, CA, USA). After switching the injection valve, the sample was loaded onto the trap column with 85% solvent A (3% (v/v) acetonitrile, 1% (v/v) glacial acetic acid and 5 mM tetrabutylammonium hydrogensulfate in water (pH 2.5)), and 15% solvent B (50% (v/v) acetonitrile, 1% (v/v) glacial acetic acid and 5 mM tetrabutylammonium hydrogensulfate in water (pH 2.5)). With a flow rate of 500 µL/min it takes 9 seconds to flush the injection loop. After injection, gradient elution of the analytical column was performed starting at 15% solvent B (85% solvent A) and increasing to 100% solvent B (0% solvent A) over 5 min. Before the next sample was injected, the column was flushed with 15% solvent B and 85% solvent A for 4 minutes. The injection valve was then switched back to the load position to refill the sample loop, and the next injection valve was switched to inject the next sample. The analysis of one sample takes 10 minutes. The procedure was performed sequentially for the three sample loops, which meant that the contents of each loop was injected once every 30 minutes.

Metabolism studies with 7-hydroxycoumarin

Slices were perifused with medium containing ~500 µM 7-HC. This was achieved by filling the three substrate syringes with stock solution containing 50 mM 7-HC dissolved in methanol. Because the infusion flow rate of this solution was 100-fold lower than the medium flow, the final concentration of 7-HC in the perifusion medium before entering the chip was ~500 µM. The second syringe contained methanol as solvent control.

During a metabolism experiment, the slices were pre-incubated for 2 hours in medium without substrate. The substrate valves were then switched to deliver 7-HC, and slices were further incubated with this compound for 2 hours. Thereafter, the slices were incubated with medium containing a small amount of solvent control (1%) for 4 hours and then again incubated with 500 µM 7-HC for 2 hours. This procedure for 7-HC delivery was performed four times over a period of 24 h. At the end of the experiment, the slices were taken out of the biochip and subjected to total protein content determination. Each experiment involved measurements in triplicate using 3 slices from one rat, and was performed with slices from three rats. Before and after each experiment, a calibration curve was made by injecting 7-HC, 7-HC-G and 7-HC-S standards onto the HPLC column.

For the inhibition studies, the system was slightly modified, since the intention was to increase the concentration of the inhibitor phloxine B over time. The substrate valves to switch between solvents were replaced with PEEK T-pieces, making it

possible to infuse substrates from both syringe pumps at the same time. One syringe pump was equipped with three syringes containing 20 mM phloxine B, the other syringe pump with three syringes containing solvent control (Williams medium E). The syringe pumps were programmed to change the flow rate every two hours. The flow rate of the pump delivering phloxine B was increased stepwise, while the flow rate of the pump delivering the solvent control was decreased accordingly. In this way, the concentration of phloxine B was changed every two hours, while the overall flow rate (100 nL/min) was kept constant. The concentration of phloxine B was varied between 1 and 200 µM (final concentration) (Figure 3). The slices were continuously perifused with WME containing 100 µM 7-HC. A concentration of 100 µM was chosen instead of 500 µM as in the previous experiments, to prevent cofactor depletion during the experiment. Slices were first incubated with 7-HC only, that is, without inhibitor. When stable metabolite formation was obtained, as determined by the on-line analysis system, the syringe pump delivering the inhibitor, phloxine B, was started.

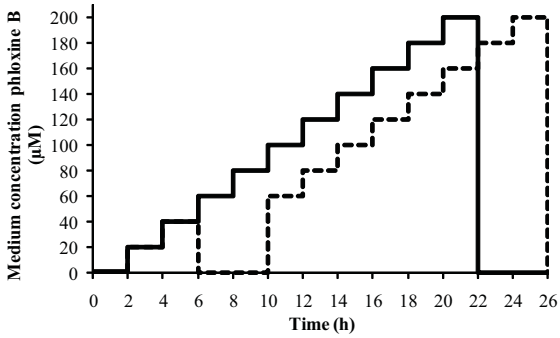

Figure 3. Calculated concentration of phloxine B in medium over time when changing the flow rates of syringe pumps 1 and 2. Though the total flow rate remained constant at 100 nL/min, individual pump flow rates were changed every two hours to create a step-wise phloxine concentration gradient with a range from 0 to 200 µM. The dashed line represents the concentration profile of phloxine B in the experiment incorporating the 4-h pause in phloxine delivery (from the 6-h to 10-h time point), performed to determine if this inhibitor is competitive.

Experiments were performed without inhibitor to measure the stability of 7-HC-G production over time, and with increasing phloxine B concentrations over time, as shown in Figure 3. To determine whether the inhibitor was competitive, experiments incorporating a pause in phloxine B delivery were also performed. This was accomplished after a two-hour incubation with 40 µM phloxine B by switching the syringe pumps back to ~100% solvent control for four hours. Slices were perifused with medium containing 100 µM 7-HC during this time. After this 4-hour period, the syringe pump program was resumed to further increase the concentration of phloxine

B. Again, each experiment involved measurements in triplicate using 3 slices from one rat, and was performed with slices from three rats.

To verify the IC50 value obtained in the on-line system, the inhibition experiment was also performed in a conventional 12-well plate incubation system. For this purpose, PCLS were pre-incubated for 2 hours in wells filled with 1.3 mL WME supplemented with 25 mM D-glucose and 50 µg/mL gentamicin. Subsequently, slices were transferred to pre-warmed plates into wells containing fresh WME, 100 µM 7-HC and various concentrations of phloxine B (0, 20, 40, 60, 80, 100, 150, 200 µM), and incubated for 4 hours. Samples were stored at -20°C until analysis by HPLC. In this case, 3 PCLS were required for each concentration of phloxine B, so that each experiment required 24 slices from one rat. Three experiments, one for each of three rats, were performed.

Metabolism studies with diclofenac

The equipment used to separate and detect diclofenac (DCF) and its metabolites was similar to the separation and detection of 7-HC, with the exception of the column. Isocratic separation was performed on a 150 x 4.6 mm Hypersil gold column with 5 µm C18 particles using a mobile phase consisting of 25% (v/v) acetonitrile and 0.05 M ammonium formate (pH 6.2) dissolved in water. The flow rate was set at 1 mL/min and the UV-detector at 282 nm. The run time was 30 minutes. Pilot experiments showed that DCF was not adsorbed onto the peristaltic tubing used for the medium delivery. Therefore DCF was added directly to the medium at a final concentration of 200 µM, and slices were continuously exposed to this substrate for four hours. The three injection valves were switched sequentially, once every 30 minutes, to inject samples of medium containing DCF and its metabolites onto the HPLC column. Again, one experiment was performed per rat, with each experiment involving the simultaneous testing of three liver slices in the tri-chamber microfluidic biochip device. Three rats were used.

As control, 12 liver slices per experiment were also incubated for four hours in well plates containing 200 µM DCF. Every hour, three slices were taken out of their respective wells, and the medium was directly collected and stored in the freezer at -20°C. Samples were taken out of the freezer the day after incubation and analyzed with the same HPLC as was used for on-line analysis. Three experiments, one for each of the rats used in the microfluidic system and each employing 12 slices, were carried out.

Results and Discussion

On-line analysis system

Chromatographic run times for 7-HC and DCF metabolism studies

The limiting factor that determines the frequency of the measurement per slice in this

system is the run time of the HPLC. However, this run time necessary to analyze two of the three chambers should not be shorter than the time to fill the loop of the third chamber. For the 7-HC metabolite samples it took 7.5 minutes to fill an injection loop with sample, and there were three injection valves per experiment to sequentially inject samples from the three chambers. This meant that with run times shorter than 3.75 minutes per sample, the injection loop will not be filled completely before the next analysis. The chromatographic method was based on a standard method used in our labs, which takes 20 to 25 minutes.[4, 18] By using a shorter column with a smaller internal diameter and optimizing the gradient parameters, however, the total run time was decreased to 10 minutes, including column equilibration. The optimized HPLC gradient resulted in fast and efficient separation of the metabolites, substrate and medium supplements, with low carry-over. A chromatogram obtained from the on-line HPLC system is presented in Figure 4, with the peaks for the metabolites, substrate and phenol red indicated.

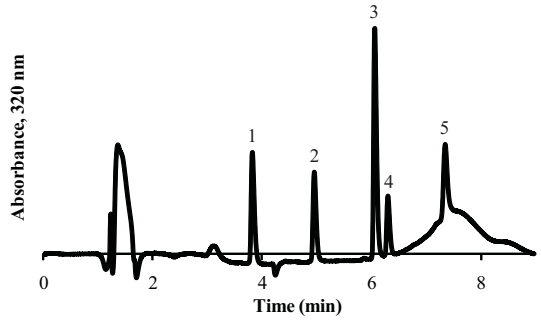

Figure 4. Chromatogram of the on-line HPLC system with efficient separation of (1) 7-hydroxycoumarin glucuronide, (2) 7-hydroxycoumarin, (3) phenol red, (4) 7-hydroxycoumarin sulfate, and (5) 7-ethoxycoumarin. Separation was performed on a Hypersil C18 column (500 µL/min) using a gradient with solvent A containing 3% (v/v) acetonitrile, 1% (v/v) glacial acetic acid and 5 mM tetrabutylammonium hydrogensulfate in water (pH 2.5), and solvent B containing 50% (v/v) acetonitrile, 1% (v/v) glacial acetic acid and 5 mM tetrabutylammonium hydrogensulfate in water.

For DCF metabolism studies, an existing HPLC method was used developed in our laboratory with a run time of 30 minutes. This was sufficient to study the unstable metabolite DCF-AG. However, the HPLC method can be optimized when shorter run times are required using a gradient elution for example.

On-line system diagnostics

Phenol red was present in the medium in all experiments to monitor the pH. The presence of the phenol red peak in the chromatogram could be used to advantage to assess the stability of both the HPLC run and the flow rate of the peristaltic pump.

When blockage or leakage in the system occurred, it was immediately detected as a decrease in the peak area of phenol red, since the injection loop was not completely filled with medium in these situations. In all the experiments described in this report, the relative standard deviation (RSD) of the area of the phenol red peak recorded over a 24-h period did not exceed 1%, with a RSD of <0.05% in retention time. This indicates a robust HPLC analysis over the 24-h period of the measurement.

To check the stability of the syringe pumps, the addition of 7-HC substrate was also monitored, since a change in the ratio of the peak areas for substrate and phenol red enabled the detection of potential problems. An increase in the peak area (substrate): peak area (phenol red) ratio indicated a problem with medium delivery, whereas a decrease indicated a problem with the substrate addition. A decrease in the areas of both peaks generally meant that leakage had occurred after the incubation chamber. If both peak areas remained constant but the metabolite peak areas were low, the tissue slices were no longer viable.

7-Hydroxycoumarin metabolism

In well plates, slices are normally pre-incubated in medium without added xenobiotics to replace the tissue-slice storing solution (UW) with medium and remove the damaged cells on slice surfaces which inevitably arise from the slicing procedure.[19] Enzymes released by damaged cells, like proteases, might negatively affect liver function.[20] Therefore, this pre-incubation was also performed in our on-line analysis system. UW solution, cell debris and leaked enzymes were flushed out of the micro-chamber with the continuous flow over the course of 2 hours before metabolism experiments were started. The use of flow also has an advantage over well plates at this stage of these experiments, since slices remain in the same chambers after pre-incubation; a simple switch of a valve or pump upstream is enough to change the solution to which the slices are exposed. In contrast, slices must be removed from wells after pre-incubation and placed in new wells containing fresh medium when well plates are used, an extra handling step which is eliminated in the microflow case. The pre-incubation period also serves as a control for peaks in the chromatogram due to endogenous metabolites or medium components. No extra peaks were apparent during these two hours compared to the blank runs recorded at 320 nm in the absence of tissue in the microchamber.

After two hours, the syringe pump with 50 mM 7-hydroxycoumarin was turned on to start the addition of 7-HC to the medium at 500 μM final concentration. The profile of 7-HC concentration in the outflow during the experiment is given in Figure 5a. The calculated time needed to transport the substrate through the tubing and chambers and to fill the injection loops was 21 minutes (dead time). In practice, after the initial two-hour equilibration period, the 7-HC concentration achieved its maximum value within 30 minutes and was stable for the two-hour incubation period. This is in line

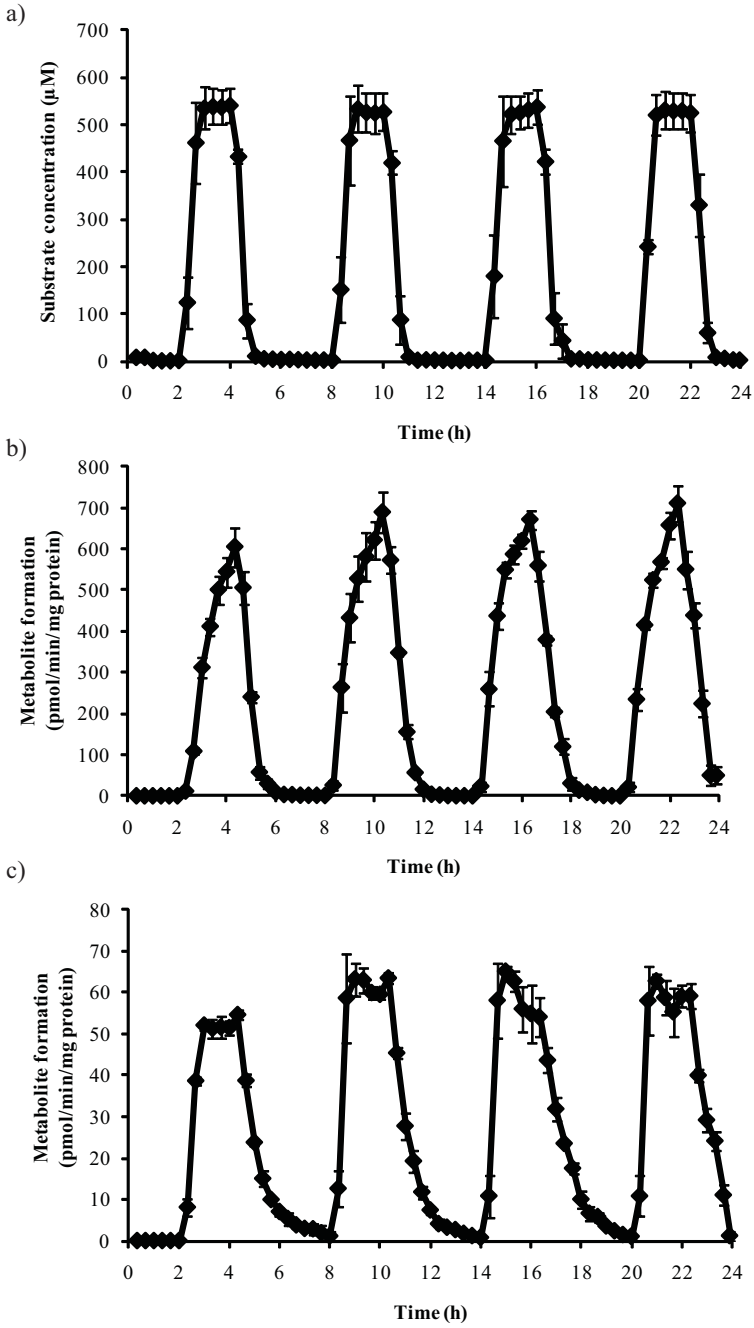

Figure 5: On-line metabolism study of 500 µM 7-hydroxycoumarin in rat liver slices. (a) 7-hydroxycoumarin (average ± standard deviation) is converted into (b) 7-hydroxycoumarin glucuronide and (c) 7-hydroxycoumarin sulfate. The results plotted are the mean ± standard error of the mean (SEM) of three rats, three slices per rat.

with the calculated dead time. After two hours, the syringe pump with 7-HC solution was turned off and the syringe pump with solvent controls was turned on, resulting in a direct decrease in the measured 7-HC concentration. This on-line substrate injection procedure was repeated three times with very repeatable results, as can be seen in Figure 5a.

When incubating the liver slices with 7-HC, the two phase II products were formed, as expected.[12] During the addition of substrate, the formation of 7-hydroxycoumarin glucuronide (7-HC-G, Figure 5b) and 7-hydroxycoumarin sulfate (7-HC-S, Figure 5c) could be clearly detected. At this high concentration of 7-HC, the glucuronide is the major metabolite formed, as described previously.[21] Moreover, the metabolic rate for glucuronidation was around 600 pmol/min/mg protein, which was similar with rates found previously.[12, 21] Importantly, the formation of metabolite was found to be repeatable over the 24 h period measured. 7-HC was administered for four 2-h periods and the same pattern for the formation of 7-HC-G was obtained, indicating that the slices remain functionally intact. Surprisingly, the peak area of 7-HC-G continuously increased over the 2-h period though the 7-HC concentration was constant. The same trend was obtained in all three experiments and for all four 7-HC administrations performed per experiment, as can be seen in Figure 5b. An increase in 7-HC-G production over time has also been observed by others in rat[22] and human liver slices[23], but the mechanism of this increase is not clear. The formation of 7-HC-S, on the other hand, was very stable over time. This metabolite was also formed immediately after the addition of 7-HC was started, and a plateau in concentration was observed. The amount of 7-HC-S formed was approximately 10 times lower compared to 7-HC-G, which can be explained by the well-known higher maximum turnover rate of UGT enzymes compared to sulfotransferase.[24]

The finding that metabolite formation was constant indicates that this on-line system is suitable for induction and/or inhibition studies.

7-Hydroxycoumarin glucuronide inhibition

The formation of 7-HC-G was relatively constant during 24 h of continuous perifusion of PCLS with 100 µM 7-HC, although again a slight increase over time was observed (Figure 6 (squares)). We can conclude that slices are viable and exhibit a constant rate of metabolism over 24 h under these conditions, which is a prerequisite for the inhibition studies performed in the present on-line set-up.

When starting the syringe pump program for phloxine B substrate addition, as shown in Figure 3, the metabolite formation was constant for four hours (Figure 6, dots). This corresponds to a phloxine B concentration of 20 µM, indicating that at this concentration 7-HC-G formation was not inhibited. When the concentration was increased to 40 µM phloxine B, the metabolite formation began to decrease gradually and continued to do so as the phloxine B concentration was increased stepwise every

2 h. After 20 hours, when the phloxine B concentration had reached 200 µM, hardly any 7-HC-G metabolite was detected. An IC50 value (phloxine B concentration at which 50% of the enzyme is inhibited) of 81 µM was calculated from this data. In a second experiment, the influx of phloxine B was interrupted after the 40 µM infusion period (indicated with arrows in Figure 6) and slices were perifused with only medium with 7-HC for 4 hours. The 7-HC-G formation remained constant during this period, and the observed metabolite formation did not increase. If inhibition were competitive, we would expect 7-HC-G formation to increase again as substrate gradually displaced inhibitor. However, the phloxine B inhibitor apparently remained bound to the enzyme even when no phloxine B was being added, leading to the conclusion that phloxine B is not a competitive inhibitor. This data is in agreement with the observations made by Mizutani *et al.* that phloxine B is a non-competitive inhibitor for the human UGT1A6 isoenzyme.[14] However, from the data obtained with the on-line analysis system, no conclusion can be drawn as to whether phloxine B inhibition is reversible or irreversible. When the infusion of phloxine B was resumed in the experiment, the rate of formation of 7-HC-G continued to decrease, to the same extent as for experiments performed without interruption of inhibitor delivery (see Figure 6, triangles versus dots). The IC50 value measured with the interruption was

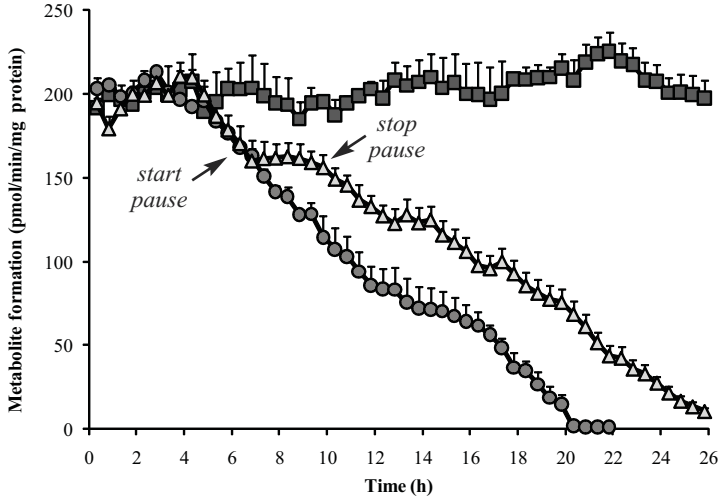

Figure 6. On-line inhibition study with 100 µM 7-hydroxycoumarin (7-HC) and varying concentrations of phloxine B in rat liver slices. Squares represent the formation of 7-HC-G without inhibitor; dots the 7-HC-G formation with increasing phloxine B concentration ranging from 0 to 200 µM; and triangles the 7-HC-G formation with increasing phloxine B concentration, including an interruption in phloxine delivery for 4 hours at 40 µM phloxine B (mean ± standard error of the mean, for three experiments, 3 slices per experiment). The start and end of the interruption in phloxine B delivery are indicated with arrows.

89 μM, which was not significantly different from the value obtained with continuous infusion. The IC50 value measured for human UGT1A6 was 40 μM[13, 14, 25], which is lower than the 80-90 μM found in this study for rat liver UGT1A6 enzymes. Possibly, this reflects a species difference in the affinity of the phloxine B for the respective isoforms of UGT. To verify the IC50 value obtained in the on-line system, comparable experiments were performed in well plates, with a co-incubation of 7-HC (100 μM) and phloxine B concentrations ranging from 0 to 200 μM. An IC50 of 83 μM was found (data not shown), confirming our biochip data. Therefore, it can be concluded that the IC50 of phloxine B for rat UGT1A6 is around 85 μM.

Diclofenac metabolism

A study investigating the metabolism of diclofenac, a non-steroidal anti-inflammatory drug taken to reduce inflammation and pain, was undertaken, as this drug is known to produce a chemically unstable but presumably hepatotoxic metabolite, diclofenac acyl glucuronide (DCF-AG). Other important metabolites formed when liver slices are incubated with diclofenac are 4′- and 5-hydroxydiclofenac (4′-OH-DCF and 5-OH-DCF). The metabolite formation over a period of four hours for the biochip on-line analysis system is shown in Figure 7a, and for well-plate incubations in Figure 7b. Both phase I products, 4′-OH-DCF and 5-OH-DCF, were formed at a constant rate for at least four hours in the on-line system and the well plates, with comparable formation rates in both systems. DCF-AG was also formed at a very constant rate in the biochip, as can be seen in Figure 7a (triangles). In well plates, however, the amount of DCF-AG decreased over time. To determine the cause of the disappearance of DCF-AG in well plates, this compound was incubated at 37°C in WME for 5 hours in the absence of liver slices. Samples were taken and analyzed by HPLC. Results showed that the DCF-AG concentration decreased over time with a half life of 30 min, with the simultaneous appearance of new peaks in the chromatogram (data not shown). A shoulder was also observed on the diclofenac peak. Similar peaks also appeared in the chromatograms recorded for medium samples collected after incubation of slices with diclofenac in well plates. It is likely, then, that these peaks are due to breakdown products of DCF-AG. These extra peaks did not appear when medium samples for incubation of slices with diclofenac were analyzed using the on-line system. It can therefore be concluded that the DCF-AG formed and excreted by the liver slices decomposes in the medium in the well-plate system and is not formed by the liver slices. Decomposition products may include diclofenac and glucuronic acid, as mentioned by Grillo *et al.*[26]

Conclusion

A novel on-line HPLC system for the analysis of the metabolism of precision-cut liver slices was developed and tested with two substrates and an UDP-glucuronyl-

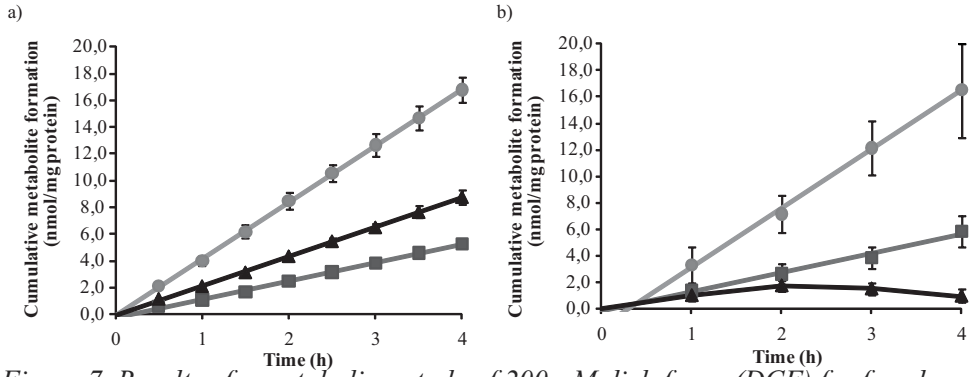

Figure 7. Results of a metabolism study of 200 µM diclofenac (DCF) for four hours in (a) the biochip and (b) well plates. DCF is mainly converted into 4'-hydroxy-diclofenac (4'-OH-DCF) (dots), 5-hydroxydiclofenac (5-OH-DCF) (squares) and diclofenac acyl glucuronide (DCF-AG) (triangles). The results plotted are the mean ± standard error of the mean (SEM) of three rats, three slices per experiment.

transferase inhibitor. Slices were incubated in small microchambers in a flow-through microfluidic device. The use of microfluidic technologies meant that metabolite concentrations were constant in the medium outflow of the device. Moreover, metabolite concentrations were not diluted in the early stages of an experiment as they are in well plates, ensuring concentrations which were high enough for detection with a conventional HPLC-UV system over all time points of the experiment. While the system used in this study employed a UV detector, detection sensitivity could also be improved in the future through use of fluorescence detection or mass spectrometry (MS). MS would additionally allow metabolomics studies on-line. Since medium samples do contain salts, the possibility of ion-suppression would need to be taken into account when using MS, but there are simple approaches for solving this problem (e.g. nanoLC[27]).

With the on-line system, it was possible to monitor the conversion of 7-HC to its metabolites by liver slices over time, with phase II metabolism of 7-HC proving to be very stable over a period of at least 24 h. Given this level of performance, the system should allow the investigation of circadian rhythms[28], and in fact, a small circadian variation could be observed for 7-HC-G. The ability to measure an inhibition-type drug-drug interaction on-line was demonstrated using the inhibition of rat UGT1A6 by phloxine B as example. The decrease in metabolite formation was not competitive in nature, and an IC50 value of ~85 µM was determined for this particular inhibitor. The IC50 value was verified with the conventional well-plate system, which also yielded an IC50 value of 85 µM. Interestingly, 24 slices per experiment were needed to obtain the same result in the well plate system study as in the on-line system, where only three slices were used per experiment. This system is

thus also very interesting for experimentation with tissue which is not readily available, such as human material.

Due to the incorporation of two syringe pumps, it was possible to switch easily between two substrates with a relatively fast response (<30 min dead time). The ability to mix two solutions at different flow rate ratios to create a concentration gradient over time was also demonstrated with the rat UGT1A6 - phloxine B enzyme inhibition example. This demonstrates the flexibility of this system and its potential suitability for many other applications, such as the measurement of enzyme conversion rates at different substrate concentrations with one tissue slice.

The ability to measure unstable metabolites was demonstrated with the incubation of PCLS with diclofenac. The three major metabolites, 4′-OH-DCF, 5-OH-DCF and DCF-AG were detected. The on-line system clearly revealed the very stable formation of the DCF-AG metabolite. This is in contrast to well plates, in which DCF-AG was observed at much lower initial concentrations which then also decreased over time. As the peak area corresponding to DCF-AG decreased, other unidentified peaks appeared in the chromatograms. With the on-line system, it was possible to show that these unknown peaks were not metabolites produced by the liver, but most likely break-down products of the DCF-AG formed in the medium.

Advantages of this on-line analysis system include speed of analysis, direct information about tissue slice activity, elimination of sample storage problems, and the capability to detect unstable metabolites. Use of an automated system for switching syringe pumps and injection valves means that much of the original manual labour associated with this type of analysis is circumvented. The use of HPLC enables the analysis of complex mixtures for a number of components simultaneously over a wide concentration range. Several measurements can be performed with one slice, which is beneficial for applications requiring scarce tissue. This on-line system therefore also could potentially contribute to the reduction of the number of experimental animals needed for drug development.

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5

Hydrogel Embedding of Precision-Cut Liver Slices in a Microfluidic Device Improves Drug Metabolic Activity

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Submitted

Abstract

A microfluidic-based biochip made of polydimethylsiloxane was recently reported for the first time by us for the incubation of precision-cut liver slices (PCLS). In this system, PCLS are continuously exposed to flow, to keep the incubation environment stable over time. Slice behavior in the biochip was compared with that of slices incubated in well plates, and verified for 24 hours. The goal of the present study was to extend this incubation time. The viability and metabolic activity of precision-cut rat liver slices cultured in our novel microflow system was examined for 72 hours. Slices were incubated for 1, 24, 48, and 72 hours, and tested for viability (enzyme leakage (lactate dehydrogenase)) and metabolic activity (7-hydroxycoumarin (phase II) and 7-ethoxycoumarin (phase I and II)). Results show that liver slices retained a higher viability in the biochip when embedded in a hydrogel (Matrigel) over 72 hours. This embedding prevented the slices from attaching to the upper polycarbonate surface in the microchamber, which occurred during prolonged (>24 h) incubation in the absence of hydrogel. Phase II metabolism was completely retained in hydrogel-embedded slices when medium supplemented with dexamethasone, insulin, and calf serum was used. However, phase I metabolism was significantly decreased with respect to the initial values in gel-embedded slices with medium supplements. Slices were still able to produce phase I metabolites after 72 hours, but at only about ~10% of the initial value. The same decrease in metabolic rate was observed in slices incubated in well plates, indicating that this decrease is due to the slices and medium rather than the incubation system.

In conclusion, the biochip model was significantly improved by embedding slices in Matrigel and using proper medium supplements. This is important for *in vitro* testing of drug metabolism, drug-drug interactions, and (chronic) toxicity.

Introduction

Various *in vitro* models exist which are able to mimic *in vivo* liver metabolism.[1, 2] Of these, primary hepatocytes and precision-cut liver slices (PCLS) exhibit the best correlation with the *in vivo* situation, with each model having its own particular advantages and disadvantages.[1] The main advantage of precision-cut liver slices (PCLS) is that the tissue architecture remains intact even when cut into thin slices.[3] All cell types are present in their original matrix, making PCLS a good model to study multicellular processes. For example, the role of stellate cells in the development of liver fibrosis,[4, 5] or the role of Kupffer cells in mediating hepatic toxicity can be investigated.[6] PCLS are usually incubated in well plates or in a dynamic organ culture (DOC) system.[7] Although slices are generally incubated for a short period of time (<24 hour), incubations of 72 hours are possible in well plates or in a DOC system.[5, 8, 9] Slices retained their viability over a 72 h period in both systems, although a rapid decrease in phase I metabolism was observed.[7]

Recently, we developed a microfluidics-based incubation system incorporating rat PCLS with continuous perfusion.[10] Tissue slices were incubated in small micro-chambers made of polydimethylsiloxane (PDMS), in which the incubation environment could be well controlled. The continuous flow of medium ensures that the incubation environment is kept stable over time, with a continuous influx of nutrients and removal of waste products. In this way, no depletion of nutrients or accumulation of waste products occurs, which is the case in well plates and DOC systems. Moreover, microfluidic technologies enable very precise control of medium flow and composition. The advantages of this microfluidic device are diverse, including 1) the ability to study the effect of flow on liver metabolism, 2) easier on-line analysis to enable the measurement of unstable metabolites,[11] and 3) the chambers containing different organ slices can be coupled and perfused sequentially to study interorgan interactions.[12] Previously, we showed that rat liver slices remain viable and metabolically functional for at least 24 hours.[10, 11] However, no studies have been performed to date with incubations longer than 24 hours, though this would be beneficial for studying the effect of prolonged exposure to drugs on liver metabolism, toxicity and fibrosis.

The aim of this study was to monitor and improve the maintenance of viability and metabolic activity of rat PCLS incubated in our microfluidic device over an extended period of 72 hours. The viability was assessed by measuring the leakage of the enzyme, lactate dehydrogenase (LDH). LDH is mainly present in the hepatocytes, and leaks out of cells which have been injured.[13] Phase I metabolism was determined by incubating PCLS with 7-ethoxycoumarin (7-EC). This substrate is converted mainly by CYP1A1 and CYP2B of the cytochrome P450 (CYP) enzyme complex into 7-hydroxycoumarin (7-HC).[14] After formation of 7-HC, this compound is further converted into the phase II products 7-hydroxycoumarin glucuronide (7-HC-G) by

UDP-glucuronosyltransferase, and 7-hydroxycoumarin sulfate (7-HC-S) by sulfotransferase.[10] This phase II metabolic activity was also assessed directly by incubating slices with 7-HC. The stability of phase I and II metabolite formation was assessed daily, and compared to well plates as control. As mentioned above, a decrease in phase I metabolism was observed in conventional systems over time, with phase II metabolism proving to be more stable than phase I.[7]

Pilot experiments demonstrated that the viability of PCLS decreased with time when incubated in our microfluidic device, and that they lose their metabolic activity as well. It was observed that PCLS were attached to the upper polycarbonate membrane forming the ceiling of the microchamber after incubations longer than 24 hours. As we hypothesized that this might be the cause of the decreased viability, it was decided to embed the liver slices in a hydrogel to prevent attachment to the membrane to improve the viability and stability of metabolite formation. A hydrogel is colloidal gel (mixture with properties between those of a solution and fine suspension) in which water is the dispersion medium. Thus slices embedded in hydrogel are still sufficiently wetted by medium. The hydrogel used in this study was Matrigel, which is normally used as culture overlay or to create three-dimensional hepatocyte cultures.[15] It is a basement membrane consisting mainly of laminin, collagen IV, heparin sulphate proteoglycan, and entactin,[16] and is therefore very similar to the native extracellular matrix in the rat liver.[15] The use of Matrigel for hepatocyte cultures and hepatoma cells (HepG2/C3A) has been shown to substantially improve morphology and functionality.[17-20] Various supplements were also added to the medium to further improve the metabolic stability of the PCLS during culturing, as mentioned previously.[7]

Materials and Methods

Chemicals

William's medium E (WME) supplemented with Glutamax I was purchased from Gibco (Paisley, UK). D-glucose, 7-ethoxycoumarin, 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide, 7-hydroxycoumarin sulfate, stabilized antibiotic antimycotic solution (100x), and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Matrigel™ was obtained from BD Biosciences (Bedford, MA, USA). Dexamethasone was purchased from Genfarma BV (Maarssen, The Netherlands). Hyclone heat-inactivated bovine calf serum was supplied by Thermo Scientific (Logan, UT, USA).

Preparation of precision-cut liver slices

Male Wistar rats (300-350 gram) obtained from Harlan (Horst, The Netherlands) were used for all experiments. Liver slices were prepared as described previously.[10, 21] Briefly, the liver was excised after anaesthetizing the rat with isoflurane/oxygen.

Subsequently, cylindrical cores of liver tissues with a diameter of 4 mm were made by utilizing a hollow drill bit. Cores were placed in a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) to produce reproducible PCLS with a thickness of ~250 µm and a wet weight of approximately 5 mg.

Incubation of precision-cut liver slices

Slices were pre-incubated for 1 h in individual wells of a 12-well plate, each of which contained 1.3 mL WME with Glutamax I. Medium was supplemented with 25 mM D-glucose, 100 units/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B (solution has both antibiotic and antimycotic properties). This pre-incubation removes cell debris and enzymes originating from damaged cells at the cutting surfaces. The damaged cells result during preparation of the tissue slices, and are only present in the outer layers of the slice. The energy status of the cells also recovers during pre-incubation, as the intracellular adenosine triphosphate (ATP) is restored to the value of fresh tissue after being reduced during the slicing procedure at 4°C.[22, 23]

After pre-incubation, slices were incubated in two different incubation systems: 1) well plates and 2) the microfluidic biochip. When incubated in well plates, the slices were placed individually in 1.3 mL medium, as described in detail elsewhere.[21] The well plates were placed in humidified plastic containers and shaken back and forward 90 times per minute in an incubator set at 37°C. The containers were continuously supplied with humidified carbogen gas (95% O_2 / 5% CO_2). Unless otherwise stated, the medium was supplemented with D-glucose, penicillin, streptomycin and amphotericin B at the same concentrations as used for the pre-incubation. In well plates, the medium was refreshed every 24 hours by transferring the slices to a new well plate containing fresh medium.

Slices incubated in the biochip were placed in small microchambers made of polydimethylsiloxane (PDMS). An extensive description of the fabrication process of the biochip was published recently.[10] In the PDMS device, each slice was incubated in a 25 µL microchamber (2 mm x Ø4 mm) in which polycarbonate membranes (10 µm thick, Ø8 µm pore size, Millipore, Bedford, MA, USA) formed the top and bottom surfaces of the chamber. Integration of these membranes into the device ensured a well-controlled, even flow of medium from the bottom of the chamber around the tissue slices and out the top. PDMS membranes were integrated above and below these polycarbonate membranes to act as “breathing” membranes. Dissolved CO_2 and O_2 concentrations in the medium could be regulated by exchange of these gases through these membranes when the chip was placed in an environment with a controlled atmosphere (humidified carbogen supply consisting of 95% O_2 and 5% CO_2). Medium was introduced by a syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA) using syringes filled with WME supplemented with glucose,

penicillin, streptomycin and amphotericin B, which had been pre-equilibrated with carbogen gas at 37°C. The syringes were connected to the biochip with PEEK tubing (DaVinci Europe, Rotterdam, The Netherlands). PTFE tubing (Polyfluor Plastics, Oosterhout, The Netherlands) was connected to the outlet of the biochip to collect fractions for further studies. The flow rate was set at 10 µL/min for all experiments.

Slices were incubated either directly in medium or embedded in Matrigel. The procedure to embed the slices in Matrigel is shown in Figure 1. The Matrigel was diluted 1:3 with WME to obtain a porous structure which could be perfused with medium, since concentrated Matrigel results in a dense polymer through which medium does not easily pass. Ten microliter of diluted, ice-cold Matrigel (4°C) was introduced into a microchamber. Subsequently, the slice was placed on top of this first layer of Matrigel and covered with another 10 µL of ice-cold Matrigel. The whole device was then closed by assembling the gel-filled bottom half with the top half of the chamber. The insertion of liver slices into the PDMS device occurred in an incubator set at 37°C. Hence, the polymer immediately polymerized.[24] The flow (10 µL/min) was started approximately 10 minutes after insertion of the liver slices.

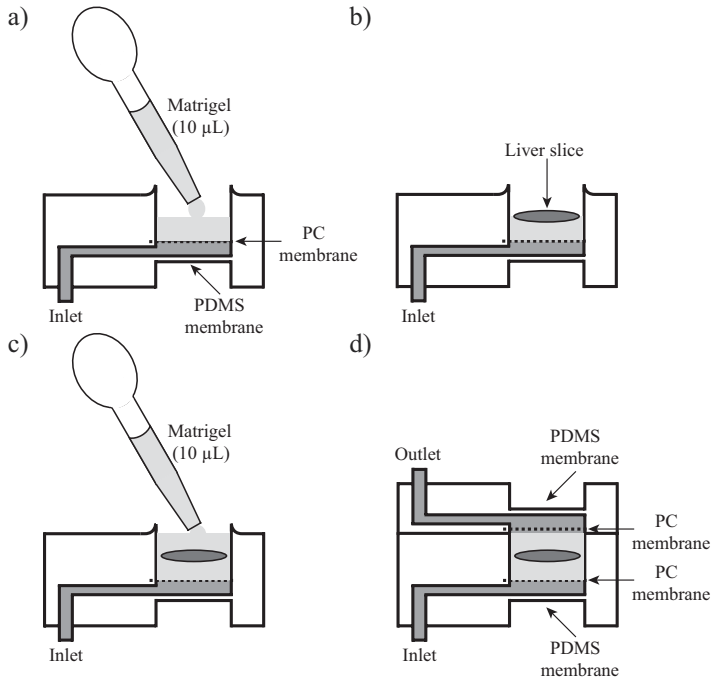

Figure 1. Schematic diagram of the procedure for embedding liver slices in Matrigel in the microfluidic device. (a) 10 µL Matrigel (diluted with medium in a 1:3 ratio) is added to an open chamber. Subsequently, (b) the slice is placed on top of the Matrigel, and is covered with (c) another 10 µL Matrigel. Finally, (d) the device is assembled and ready for use.

Viability testing

The viability of liver slices incubated for 72 hours in both well plates and the biochip was assessed by measuring the leakage of the enzyme, lactate dehydrogenase (LDH), over time. Analysis was performed on a Roche/Hitachi Modular System (Roche, Mannheim, Germany), according to a routine laboratory procedure. PCLS were pre-incubated for 1 hour; subsequently, fractions of medium were continuously collected after 3, 24, 48, and 72 hours and snap-frozen and stored at -80°C until analysis. To assess the total LDH content in fresh liver slices, three liver slices were collected after pre-incubation. These were homogenized in 1 mL of medium, which was then centrifuged for 3 min at 16,000*g* and 4°C. The supernatant was then analyzed to determine the initial LDH content in fresh liver slices. The supernatant was stored at -80°C until analysis. Each experiment was performed in triplicate using slices from 3 different rats.

Metabolism studies

The metabolic activity of liver slices was assessed after one hour pre-incubation (0 h), and after 24, 48 and 72 hours of incubation by measuring the metabolite formation in medium containing 100 µM 7-ethoxycoumarin (7-EC) or 100 µM 7-hydroxy-coumarin (7-HC). Slices were exposed to these substrates for three hours starting at the indicated time points. Pilot experiments showed that liver slices could be exposed repeatedly to 7-hydroxycoumarin without affecting the metabolic rate. In well-plate experiments, liver slices were transferred directly after the pre-incubation to well plates containing fresh medium with 100 µM 7-HC and exposed to 7-HC for 3 h. Subsequently, the slices were transferred to a new well plate containing fresh medium and no substrate for further incubation for 21 h. This procedure was repeated and the slices were exposed to 7-HC four times during the 75-hour time period (72 h plus 3 h incubation with 7-HC). In the biochip experiment, slices were transferred to the biochip after 1 h pre-incubation in a well plate and perifused with medium containing 100 µM 7-HC. After 3 h of exposure to 7-HC, syringes filled with 100 µM 7-HC in medium were replaced with syringes filled with medium only and perifusion was continued. After 21 hours, a switch back to medium containing 7-HC was made, and slices were perifused for a second period of 3 hours. This was repeated until the slices had been exposed four times in total to 7-HC.

When 7-EC was the selected substrate, each slice was only exposed once over a period of 3 hours, after a pre-incubation in medium without 7-EC of 1, 24, 48 or 72 h respectively. This is because earlier experiments showed that the metabolite formation decreased rapidly upon repeated exposure to 100 µM 7-EC, indicating that this concentration is most likely toxic for the cells.

Medium fractions (1000 µL) collected after all 7-EC and 7-HC exposure experiments were stored at -20°C until analysis. Experiments were performed with

four rats, with three to four slices per treatment per rat.

The amounts of metabolite formed from 7-EC and 7-HC were determined using an HPLC with UV detection, as described earlier, using 7-EC, 7-HC, 7-hydroxycoumarin glucuronide (7-HC-G), and 7-hydroxycoumarin sulfate (7-HC-S) as standards.[10]

The metabolic rate was expressed per milligram protein. Slices pre-incubated for one hour were used to determine the protein amount. The Bio-Rad protein assay (Bio-Rad, Munich, Germany) was employed according to the manufacturer's protocol using bovine serum albumin as standard.

Statistical evaluation

The results were analyzed for significant differences using the Student *t*-test, with $p < 0.05$ considered as significantly different.

Results and Discussion

In our previous studies, slices were incubated in the biochip for a maximum of 24 hours.[10, 11] To be able to measure the effect of prolonged exposure of xenobiotics on liver metabolism and toxicity, this incubation period should be increased to at least a few days, and preferably to weeks. However, pilot experiments showed that increasing the exposure time resulted in an even more decreased metabolic activity compared to PCLS incubated in the well plates. We hypothesized that this might be due to attachment of the slices to the polycarbonate ceiling of the microchamber which was observed at exposure times >24 hours, as mentioned above. To avoid this damage, it was decided to embed slices in Matrigel, a matrix with hydrogel properties. Indeed, the embedding of slices in Matrigel prevented the slices from coming in contact with the polycarbonate surface.

Viability testing

The leakage of the enzyme, LDH, was determined in slices incubated in well plates, the biochip without gel embedding, and in the biochip with slices embedded in Matrigel for 72 h. Pilot experiments showed that Matrigel did not adsorb the enzyme LDH (data not shown). The cumulative leakage is given in Figure 2. The results indicated that liver slices retained a higher viability in the biochip with Matrigel over 72 hours. Around 90% of the cells remained intact after the incubation period. The cumulative leakage of LDH in liver slices which were not embedded in gel reached a maximum value of 15% at 48 h, with no further increase observed afterwards. This was surprising, given the fact that after the experiment slices were found attached to the microchamber ceiling, and it was impossible to remove them intact from the device. Though earlier experiments indicated that the metabolic activity decreased strongly, the LDH leakage was relatively limited for non-gel-embedded slices in the

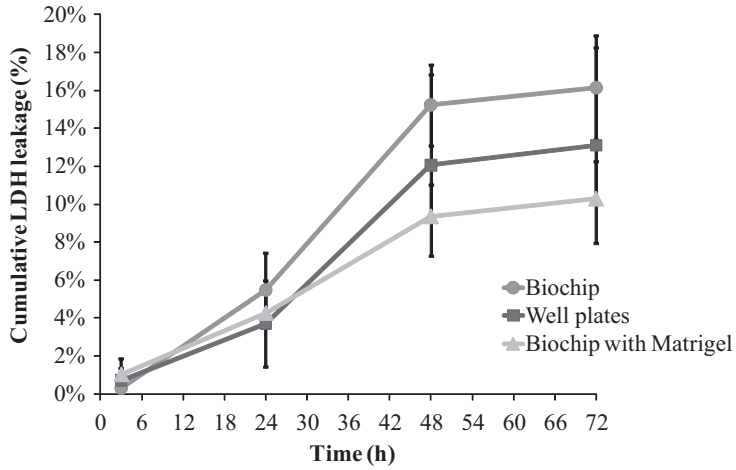

Figure 2. Cumulative leakage of the enzyme lactate dehydrogenase (LDH) as a percentage of the total LDH present in a typical slice, measured over a period of 72 hours. The results presented are the mean ± standard error of the mean (SEM) of three rats, with three slices per rat per experiment.

chip. This indicated that the cell membranes of the hepatocytes remained intact, despite attachment of the slices to the upper polycarbonate surface of the micro-chamber. In the case of gel-embedded slices, there was no problem removing slices after 72-hour incubation in the biochip, although they were more fragile compared to fresh tissue slices. Slices could also be removed from the wells without damaging them, although they were also more fragile than fresh tissue slices.

Phase II metabolism with and without Matrigel-embedding

The metabolism studies were performed with a 7-HC concentration below the concentration required to achieve a maximum metabolic rate,[12] in order to limit the consumption of co-factors during the course of the experiments. Liver slices were exposed daily to 100 μM 7-HC in the biochip with and without gel-embedding of slices (Figure 3). Both 7-HC-G and 7-HC-S were formed. As expected, no significant differences were obtained for fresh slices with or without gel-embedding on the first day. The metabolic rate was around 190 pmol/min/mg protein for 7-HC-G and 25 pmol/min/mg protein for 7-HC-S, which was similar to data obtained previously.[11] After 24 hours, similar metabolic rates were again observed. This indicated very stable phase II metabolism for 24 hours both with and without gel embedding, as observed previously in medium alone by us in the biochip,[11] and in Erlenmeyer flasks by others.[25] However, at 48 hours, the metabolite formation in slices without Matrigel significantly decreased to a value which was 7% of the initial value for 7-HC-G and 14% for 7-HC-S. In contrast, gel-embedded slices retained a much higher metabolic

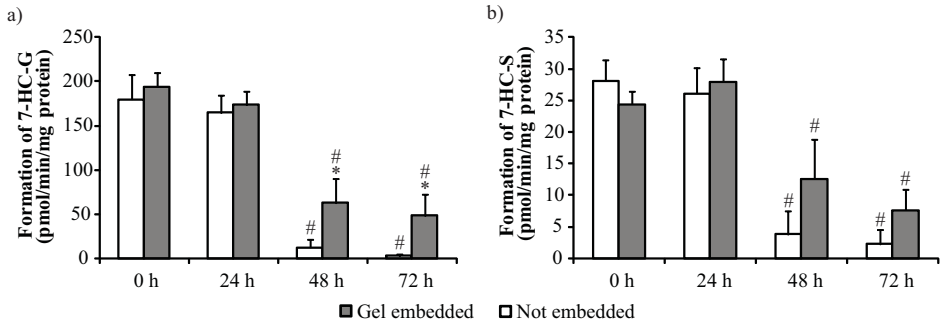

*Figure 3. Liver metabolism of 100 μM 7-HC over a period of 72 h, measured once a day in the biochip for slices embedded in Matrigel (gray bars) and without Matrigel (white bars). Slices were exposed for 3 h to 7-HC starting at each time point indicated along the horizontal axis. (a) Formation rates for 7-HC-G. (b) Formation rates for 7-HC-S. Results are the mean ± SEM of four rats with four slices per rat per experiment. Significant differences with respect to 0 h are indicated with # $p < 0.05$. Significant differences between slices embedded in Matrigel and without Matrigel are indicated with * $p < 0.05$.*

rate, though a decrease in phase II metabolism was observed in this case as well. The 7-HC-G formation rate decreased to 32% of the initial value, while that of 7-HC-S dropped to 52% of its initial value after 48 hours of incubation. This was also the case after 72 h of incubation, with a significantly higher amount of 7-HC-G being formed in gel-embedded slices compared to slices without gel. The formation rates of 7-HC-G decreased to 2% and 25% of the initial value for slices without gel and with gel, respectively. The 7-HC-S formation rates decreased to 8% and 31%, respectively, for the gel and no-gel situations.

In summary, even though metabolic rate decreased over time, this decrease was less in liver slices embedded in Matrigel compared to slices without gel. Slices were not attached to the microchamber ceiling after 72 h of incubation, and were able to produce metabolites. Embedding PCLS in Matrigel in the microfluidic device thus improves viability and metabolic functionality.

Phase II metabolism with medium supplements

Liver slices incubated in well plates exhibited a decrease in phase II metabolic rates during incubation which resembled the behavior of Matrigel-embedded slices in the biochip (data not shown). This indicated that the decrease was independent of the incubation system, provided slices did not adhere to the ceiling of the microchamber. Various medium supplements have been shown to improve the maintenance of the metabolic rate for longer periods of time.[26, 27] It was therefore decided to supplement the medium with 5% v/v heat-inactivated calf serum, as has commonly been done by

others when liver slices were incubated longer than 48 hours.[7] 1 µM insulin was also added to improve glucose uptake[28] and 0.1 µM dexamethasone as glucocorticosteroid to reduce inflammatory reactions[29, 30]. Phase II metabolism was again assessed by incubating liver slices with 7-hydroxycoumarin, comparing slices incubated in well plates and gel-embedded in the biochip.

As can be seen in Figure 4, very stable phase II metabolism was observed over the 72 hours measured. There was no significant difference between fresh slices and slices incubated for 72 h both in the biochip and in the well plates. These results were in line with those of the LDH leakage (Figure 2), which indicated that tissue slices remained intact for at least 72 hours. Hashemi *et al.* also measured phase II metabolism in well plates for 72 hours.[9] In contrast to the results presented here, they reported a decrease in UDP-glucuronosyltransferase (UGT) activity over time, using medium supplemented with insulin, serum, and a glucocorticosteroid (hydrocortisone 21-hemisuccinate). A decrease in sulfotransferase activity was also reported, albeit a decrease which was slower over time compared to UGT. The difference in observed metabolic rates between this study and that of Hashemi *et al.* might be explained by the low concentration of oxygen used in the latter case. They incubated the slices in an environment consisting of 5% CO_2 in air, while it is known that slices need an oxygen concentration >70% to culture them longer than 24 hours.[7, 8, 31]

Khong *et al.*, on the other hand, demonstrated an increase in metabolic rate for phase II metabolism with their intra-tissue perfusion system.[32] The slices incubated in a static environment showed a decrease in metabolic rate, while perfusion through the tissue resulted in an increase. We did not find a difference in phase II metabolic rates between flow (biochip) and no flow (well plates) conditions. On the contrary, a

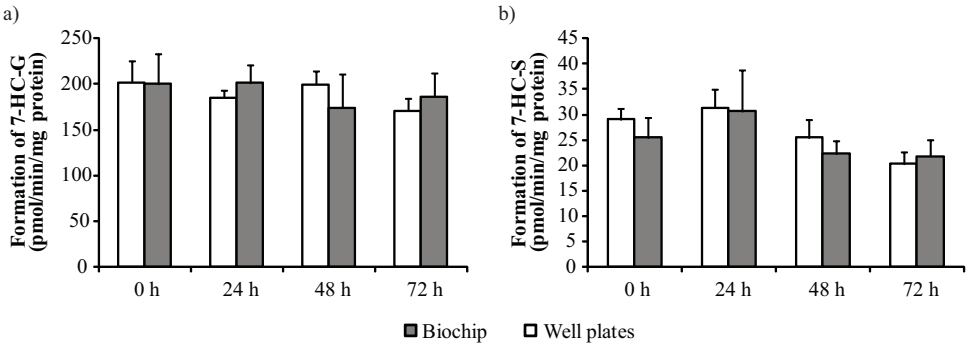

Figure 4. Liver metabolism of 100 µM 7-HC over a period of 72 h measured every day for slices in well plates (white bars) and Matrigel-embedded slices in the biochip (gray bars). (a) Formation rates for 7-HC-G. (b) Formation rates for 7-HC-S. Results are mean ± SEM of four rats with three slices per rat per experiment. No significant differences were observed between results obtained in well plates and results in the biochip, or between fresh slices and those incubated for 24, 48, and 72 h.

stable formation of metabolites over 72 hours was found in both systems. It should be noted that the well plates were not cultured under static conditions in this study, since the plates were moved back and forward 90 times per minute. In neither situation, however, was upregulation of UGT activity found, as reported by Khong *et al.*[32]

Phase I metabolism with medium supplements

The phase I metabolism in gel-embedded slices in the biochip and in slices incubated in well plates was assessed by determining the metabolism of 7-ethoxycoumarin. All three metabolites 7-HC, 7-HC-G, and 7-HC-S, were formed. The total phase I metabolism is the sum of 7-HC, 7-HC-G and 7-HC-S produced by the slices. However, the concentration of 7-HC was low and hardly detectable after 24 hours of incubation, and negligible compared to 7-HC-S and 7-HC-G, suggesting that phase I metabolism is the rate-limiting step in 7-EC metabolism. This is the reason only the formation rates of 7-HC-G and 7-HC-S are given in Figure 5. Unfortunately, the metabolism decreased upon culturing. A significant decrease in phase I metabolism was observed after 24 hours of incubation in both well plates and biochip. Only 60-70% of cell activity was retained at this point, which further decreased to 25-30% after 48 hour of incubation in both systems. After 72 hours a significant difference between well plates and biochip was observed, with slices incubated in the well plates retaining 15% of their cell activity while the biochip exhibited only 5% of the initial

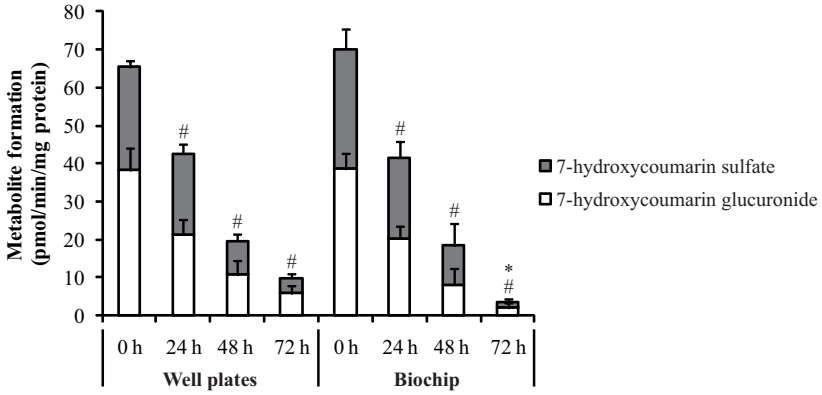

*Figure 5. Liver metabolism of 100 μM 7-EC over a period of 72 h, measured once a day in well plates and in the biochip. White bars represent the formation of 7-HC-G, and gray bars the formation of 7-HC-S. Slices were exposed only once to 7-EC for a 3-h period, starting at the time point indicated along the horizontal axis, due to the toxicity of 7-EC for the slices. Results are mean ± SEM of four rats with three slices per rat per experiment. Significant differences of total phase I metabolism with respect to 0 h are indicated with # $p < 0.05$. Significant differences of total phase I metabolism between well plates and biochip are indicated with * $p < 0.05$.*

value. Overall, the decrease in formation of 7-HC-S during culturing occurred at a slower rate than that of 7-HC-G. This is probably due to the lower concentrations of 7-HC formed over time, as the ratio of 7-HC-G to 7-HC-S formed decreases as 7-HC concentrations decrease.[33]

The decrease of phase I metabolism in liver slices over time has been previously reported.[7] In isolated hepatocytes, CYP-mediated metabolism also declines during culturing.[34, 35] Hashemi *et al.* demonstrated a 90% reduction in the phase I metabolism of ethoxyresorufin in rat liver slices within 24 hours, with hardly any metabolites formed after 72 hours.[36] In our study, a decrease of 30-40% in metabolic activity was observed after 24 hours, and slices were still able to produce metabolites after 72 hours (5-15%). The differences in results are probably due to differing experimental conditions in the two studies. The rats used by Hashemi *et al.* were treated with inducing agents, and the oxygen concentration during incubation was relatively low, as mentioned above, which might explain the discrepancy in the two sets of results. Another important issue is that the loss in CYP activity does not affect every isoenzyme equally.[36] Our results are more in line with data shown by Vandenbranden *et al.*, who demonstrated a decrease in coumarin 7-hydroxylase activity to about 10-40% of the initial value after 24 hours.[37] However, VandenBranden *et al.* used human liver slices instead of rat liver slices. Apparently, our incubation medium lacks components that maintain the expression or activity of CYP1A1 and 2B in the liver slices at their physiological level.

Conclusion

In this study it was demonstrated that the biochip model for liver-slice incubation was significantly improved by embedding the liver slices in Matrigel, allowing liver slices to remain viable up to 72 hours. The Matrigel embedding prevents the attachment of PCLS to the upper polycarbonate membrane of the biochip's micro-chamber during incubations longer than 24 h. Results show that liver slices retained a higher viability in the biochip with Matrigel over 72 hours, and that ~90% of cells were intact after 72 hours. With the proper medium supplements, the metabolic phase II activity could also be retained in PCLS for over 72 hours. No significant decrease in the metabolic rates for 7-HC-G and 7-HC-S production was observed over this period of time. However, the phase I metabolic rate did fall within 24 h to a value which was 30 to 40% less than the initial rate, which though considerable, was less than that found by Hashemi *et al.*[36] The same results for phase I and phase II metabolism were obtained in well plates, which is the benchmark for these experiments. It should also be noted that the flow conditions under which incubations were carried out did not influence the observed metabolic behavior of slices, contrary to what was reported by Khong *et al.*[32] We thus hypothesize that the lowered metabolic rates measured were not linked to the incubation system, but rather due to

the combination of PCLS and incubation media. More research has to be done to better maintain metabolic phase I activity using supplemented medium. Key to this would be the selection of medium supplements which influence the transcription factors that might be responsible for the decline in CYP isoforms, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and farnesoid X receptor (FXR). However, the current system can be used to assess phase II metabolism. The induction of phase I and phase II metabolism can also be studied in biochips for 72 h.

This is the first example of embedding liver slices in Matrigel. This is also the first example of incubating rat liver slices in a microfluidic device for a period longer than 24 hours. We do believe that this perifusion system could be a good addition to the conventional well-plate system. As mentioned above, this perfusion system can also address questions related to the effect of flow on metabolism. The flow rate can be varied between 4 and 50 µL/min, while maintaining a high metabolic activity during the first 3 hours.[12] Unstable metabolites which are difficult to detect in well plates can be analyzed by coupling the microfluidic culture systems directly to an HPLC.[11] Finally, interorgan interactions can be studied by coupling microchambers containing different organ slices and perfusing them sequentially, as demonstrated by the interplay of liver and intestine in the regulation of bile acid synthesis published by our labs recently.[12] We therefore believe that this improved system has the potential to significantly contribute to drug metabolism and toxicology studies of novel chemical entities.

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6

Perifusion of Human Precision-Cut Liver Slices in a Microfluidic Device for Metabolism and Toxicology Studies

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Elisabeth Verpoorte
Geny M.M. Groothuis

Abstract

Early information on metabolism and toxicity properties of new drug candidates is crucial for selection of the proper candidate for further testing. *In vitro* tests are used to gain information about new chemical entities (NCE) with minimal use of animals. Due to species differences in metabolism and toxicity, *in vitro* systems are needed which permit the incorporation of human material. Knowledge of how *in vitro* animal data extrapolates to *in vivo* data allows, under some circumstances, the extrapolation of *in vitro* data gained using human material back to the human *in vivo* situation. We and others showed that rat and human precision-cut liver slices (PCLS) can be used to perform these studies. These slices are commonly incubated in well plates or dynamic organ culture systems. Recently, we reported a novel microfluidic-based (biochip) perfusion system for rat liver slices, also with metabolism and toxicity studies in mind. This report describes the verification of the microfluidic system for studies involving human liver slices. Slice viability has been tested by morphological evaluation and measurement of the leakage of liver-specific enzymes. All experiments were verified by comparison with well plates, which is an excellent benchmark for these experiments. The metabolism of four different substrates, lidocaine, 7-hydroxycoumarin (7-HC), 7-ethoxycoumarin (7-EC) and testosterone, was also tested in both systems. Finally, the ability to form phase I and II metabolites after 24 hour of incubation was assessed by measuring the metabolism of 7-EC and 7-HC. For all the experiments performed, the results in both systems were not significantly different. Human liver slices were as viable and metabolically active in the biochip as in the well plates. The biochip system can thus be used in addition to the well plate system for human liver tissue incubation, thereby enabling to questions related to the effect of flow on metabolism to be addressed. We believe this system will yield more information about new drug candidates in an early stage of development compared to well plates alone.

Introduction

Only one in nine newly developed drugs entering clinical trial will be approved by European and/or the US regulatory authorities.[1] Many of the drugs that fail are withdrawn due to unforeseen toxicity or lack of efficacy.[2] This means that preclinical tests, which include both *in vitro* and *in vivo* methods, are not able to adequately predict how new drugs will behave in the human body. There is thus a continuing demand to develop *in vitro* systems which are able to better mimic the *in vivo* environment. Preclinical tests normally use animals (*in vivo* tests) and human and animal tissue or cells (*in vitro* tests).[3] By extrapolating results obtained *in vitro* to those obtained *in vivo* for the tested animals, a prediction of the human *in vivo* behavior of a drug can be made based on the *in vitro* data of human material. However, as interspecies differences are often evident with respect to the behavior of animal and human cells or tissue under culture conditions, each *in vitro* system developed with animal material should be verified for its applicability with human material. If an *in vitro* system based on human material with excellent correlation to the *in vivo* situation were to exist, animal experiments could be reduced and replaced. To develop an *in vitro* system with the ability to correlate accurately to the *in vivo* situation, attention should be paid to the selection of a biological system which represents the organ as closely as possible.

While there are various models to determine ADME-Tox profiles, only a few exhibit a good correlation to the *in vivo* situation, among them primary hepatocytes and precision-cut liver slices (PCLS).[4] The main advantage of PCLS compared to primary hepatocytes is that PCLS contain all liver cell types in their native environment.[5] This model is thus also very useful for toxicity studies which are mediated *via* multicellular processes. Normally PCLS are incubated in well plates or dynamic roller culture systems.[6] In both systems, the slices are cultured in an excess of medium which is refreshed only once every 24 h. This leads to an accumulation of waste products and depletion of medium components. By using microfluidic technologies to maintain a constant flow to perifuse slices in microchambers, the environment around the slices remains unchanged. The presence of a constant flow over the tissue has also been suggested to be more consistent with the *in vivo* situation.[7-9] We therefore recently developed a new *in vitro* system based on microfluidic technologies for the incorporation of precision-cut tissue slices.[10] The polydimethylsiloxane (PDMS) device contains a microchamber of 25 µL into which liver slices and intestinal slices can be incorporated. The chamber is then continuously perfused with fresh medium to ensure the stability of the slice environment during incubations. The system has been verified for rat liver and intestinal slices with respect to the optimized conventional well plate system, in which slices retain their *in vivo* metabolic rate at least for 8 h (intestine) and 24 h (liver). In the case of intestinal slices, only short incubations of 3 h have been performed.[11] However, liver slices retain their viability

for at least 72 h in the microfluidic device (see Chapter 5).

In the present study, the incorporation of human material in the microfluidic biochip is demonstrated. Though metabolism studies have been successfully performed with rat liver slices,[10] it is not a given that human liver slices will also be metabolically active in the microfluidic device, due to the interspecies differences that exist. This study thus set out to verify that human tissue could also be tested in the microfluidic system, using well-plate experiments as controls, as these serve as an excellent benchmark. To assess the viability of the slices, slice morphology was assessed after incubation, and the leakage of liver-specific enzymes was measured. Since the main purpose of this system is to perform metabolism studies, the ability of human material to convert substrates into metabolites was also measured. 7-Ethoxycoumarin, 7-hydroxycoumarin, testosterone, and lidocaine were chosen as substrates, since they are involved in various phase I (hydroxylation, oxidation, N-deethylation, and O-deethylation) and phase II (glucuronidation and sulfation) metabolic routes.[12, 13] 7-Hydroxycoumarin (7-HC) is a phase I metabolite of 7-ethoxycoumarin (7-EC), which is further converted into 7-hydroxycoumarin glucuronide (7-HC-G) and 7-hydroxycoumarin sulfate (7-HC-S), both phase II products. Lidocaine is converted by the liver mainly into monoethylglycinexylidide (MEGX). Finally, testosterone (TT) is converted into several hydroxytestosterone metabolites and into androstenedione (TT-A), which is the major metabolite of TT. In this study, only the metabolites 6β-hydroxytestosterone (6β-OH), 2β-hydroxy-testosterone (2β-OH), and TT-A were measured.

Materials and Methods

Human liver tissue

Pieces of human liver tissue were obtained from redundant donor tissue as surgical waste remaining after split-liver transplantation. The pieces of human liver were perfused with and stored in ice-cold UW solution (liver preservation solution).[14] The research protocols were approved by the Medical Ethical Committee of the University Medical Center in Groningen, The Netherlands.

Preparation of precision-cut liver slices

The preparation of precision-cut tissue slices followed exactly the same procedure as described previously for rat liver tissue.[10, 15] In short, a hollow drill bit was utilized to obtain Ø4 mm cylindrical samples of liver tissue (cores). These cores were placed in a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold, oxygenated Krebs-Henseleit buffer. After slicing, the slices were stored in ice-cold UW solution until incubation. Slices were approximately 4 mm in diameter and 250 μm thick, and had a wet weight of about 5.0 mg.

Incubation

Tissue slices were pre-incubated individually in wells of a 12-well plate (Greiner Bio-One GmbH, Frickenhausen, Austria) containing William's medium E with Glutamax-I (Gibco, Paisley, UK) without xenobiotics. For all described experiments, William's Medium E was supplemented with 25 mM D-glucose (Sigma-Aldrich, St. Louis, MO, USA) and 50 µg/mL gentamicin (Gibco). Slices were pre-incubated for 1 hour to remove cell debris and enzymes present in damaged cells at the edges and surfaces of the slice, due to the slicing procedure.[16] Thereafter, slices were removed from the well plate and introduced either into another well plate or into the microfluidic biochip for incubation.

For the well plates, the slices were individually placed in wells of a 12-well plate containing fresh 1.3 mL medium. The well plates were placed in humidified plastic containers with continuous exchange of carbogen (95% oxygen / 5% carbon dioxide) in a 37°C incubator. The plastic containers were in turn placed on a shaker with a speed of 90 cycles per minute. In the biochip, slices were placed individually into microchambers (25 µL) which were continuously perfused with medium using syringe pumps (10 µL/min, New Era Pump Systems Inc., Farmingdale, NY, USA). The fabrication process and verification of the biochip for metabolism studies with rat liver slices have been elaborated elsewhere.[10] Two porous polycarbonate membranes formed the floor and ceiling of the PDMS microchamber to create a well-

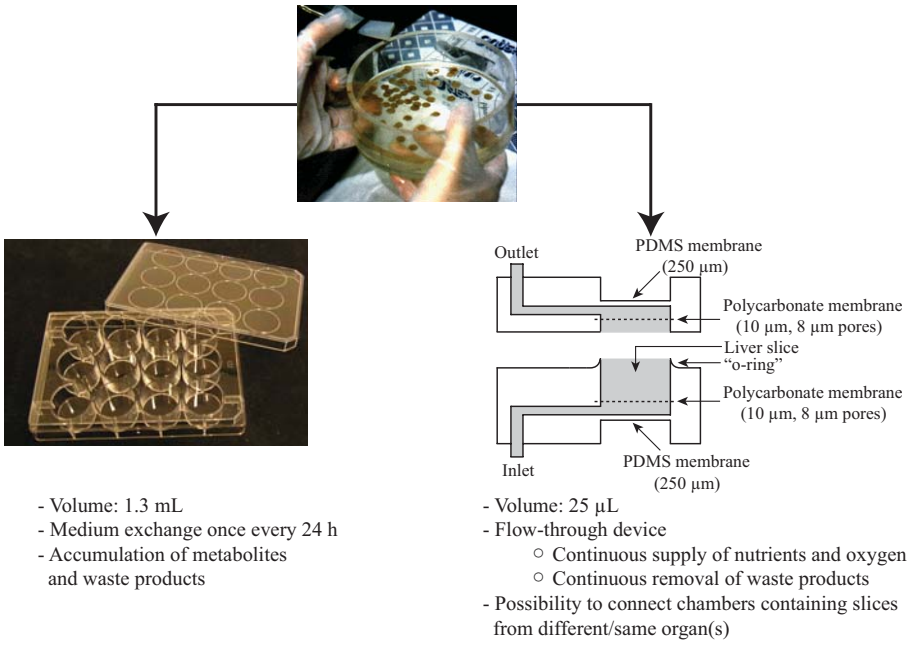

Figure 1. A comparison of two incubation systems, well plates and the microfluidic biochip, for tissue slice incubations to perform metabolism and toxicity studies.

characterized medium flow around the tissue slice. Two PDMS membranes served as aeration windows for the exchange of oxygen and carbon dioxide into the chamber when the biochip was placed in a humidified plastic container filled with carbogen gas, to maintain an optimal incubation environment. Both the plastic container and syringe pumps were placed in an incubator set at 37°C. A comparison of the two systems with their characteristics is given in Figure 1.

Viability testing

The viability of human liver slices was assessed in well plates and biochip by morphological evaluation, and by measuring the leakage of liver-specific enzymes after 4 h and 24 h. For morphological evaluation, slices were fixed in 4% formaldehyde in phosphate-buffered saline solution for 24 h at 4°C. Subsequently, they were placed in 70% ethanol until paraffin embedding, sectioning and haematoxylin and eosin staining (HE staining) as described previously.[17] The leakage of the enzymes lactate dehydrogenase (LDH), alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT) were measured using the Roche/Hitachi Modular System (Roche, Mannheim, Germany). Analysis was performed according to a routine laboratory procedure. Leaked enzymes were measured in medium fractions collected after 4 and 24 hours, and compared to the total enzyme content present in intact fresh tissue slice (after 1 h pre-incubation).

Metabolism of 7-EC, 7-HC, TT and Li

The metabolic activity of the human tissue slices was assessed by measuring the metabolites formed from four different substrates, 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), testosterone (TT) and lidocaine (Li). Liver slices were incubated with 500 μM 7-HC and 500 μM 7-EC for 3 hours, and with 5 mM lidocaine for two hours. In the biochip case, the medium outflow containing the metabolites from 7-HC, 7-EC, and Li was continuously collected and stored at -20°C until analysis. Medium samples were obtained from the well plates after incubation was completed. Earlier experiments showed that the percentage of metabolites retained in the liver slices were negligible (data not shown). The HPLC-UV analysis of 7-EC and 7-HC was performed as described previously[10], and the analysis of Li with its metabolite was the same as described by Olinga *et al.*[18] The incubation of PCLS with testosterone was performed at a concentration of 250 μM, for an incubation period of 2 hours. In this study, only the metabolites 6β-hydroxytestosterone (6β-OH), 2β-hydroxytestosterone (2β-OH) and TT-A were measured. Since the amounts of metabolites retained in the liver slices for testosterone were not negligible, the slice together with medium was stored after incubation at -20°C to assess metabolite formation. 11β-hydroxytestosterone was used as an internal standard for sample preparation. Tissue was homogenized, and the metabolites and testosterone were

extracted from the medium with liquid-liquid extraction using dichloromethane. After evaporating the dichloromethane, the residue was reconstituted in 50% methanol and 50% water; a 50-µL aliquot was injected onto an HPLC system as described previously.[13]

After 24 h incubation in both systems, PCLS which had not yet been exposed to xenobiotics were incubated with 500 µM 7-ethoxycoumarin or 500 µM 7-hydroxycoumarin to assess the stability of phase I and phase II metabolic activity. All experiments were performed using slices prepared from tissue originating from three human livers, using at least three slices per treatment.

Metabolite formation was expressed per mg protein (pmol/min/mg protein). Protein amounts in control tissue slices were determined in five representative slices using the Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) using bovine serum albumin as standard.

Statistical evaluation

Significant differences between slices were determined using the Student's *t*-test, with $p < 0.05$ considered as significantly different. The measured metabolic rates were statistically evaluated.

Results and Discussion

Viability

Human liver slices were less fragile than rat liver slices. The removal of human liver slices after incubation from the biochip was thus easier compared to rat slices. When opening the biochip, extra preheated medium was added into the microchamber from the top. This resulted in the slices floating at the surface of the medium in the chamber, and made it possible to remove the slice with a spatula without damaging it for further processing. Figure 2a) and b) show the morphology of liver slices incubated for four hours in well plates and biochip, respectively. No difference in liver morphology was obtained between the two systems. Figure 2c) and d) shows the morphology of the liver slices incubated for 24 h in well plates and biochip, respectively. Here also no differences in morphology were observed, indicating that human liver slices remained intact in both systems for at least 24 h.

The enzymes LDH, ALAT and ASAT are present in very different amounts in human liver slices. While all three enzymes may be found localized in parenchymal cells, only ALAT and LDH are present in the cytosol of hepatocytes. Of the three enzymes, only ASAT is present in the mitochondria.[19-21] However, the leakage of these enzymes as a percentage of the total amount measured over time was very comparable in well plates and biochip, as shown in Figure 3. After four hours, only ~5% of the enzyme had leaked out of the tissue slices for all three enzymes, and in both incubation systems. This means that 95% of cells remained viable after 4 hours. Even

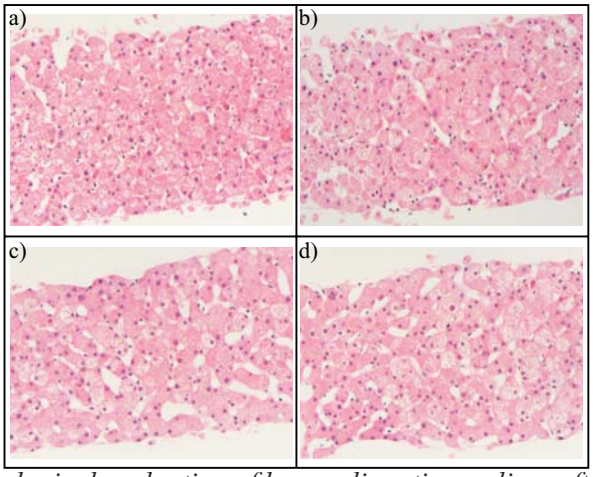

Figure 2. Morphological evaluation of human liver tissue slices after 4 h incubation in (a) well plates and (b) biochip, and after 24 h incubation in (c) well plates and (d) biochip (200x magnification).

after an incubation period of 24 h, the cumulative leakage of enzymes was still low. Less than 10% of the total ALAT had leaked out in both well plates and biochip, whereas about 15% leakage of ASAT in both well plates and biochip had occurred. About 10% LDH had leaked from the slices in the biochip, while this amount was higher at

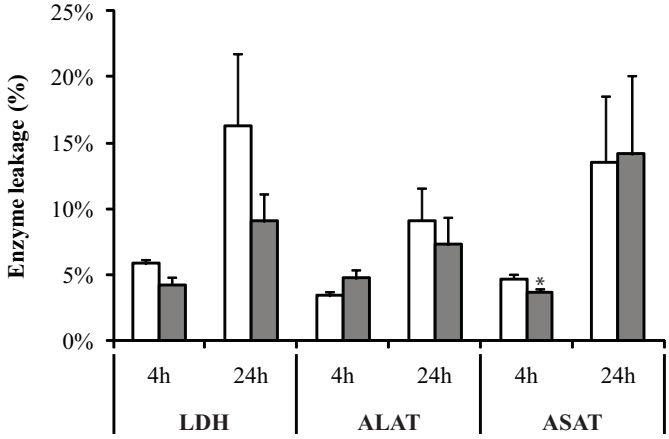

Figure 3. Cumulative enzyme leakage of lactate dehydrogenase (LDH), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) after 4 h and 24 h incubation, as a percentage of the total amount of each enzyme present in the tissue slice. White bars represent leakage in well plates, while gray bars indicate leakage in the biochip. Results are the mean of three organs ± standard error of the mean (SEM), with four slices per incubation system per experiment. Significant differences with respect to the well plates are indicated with an asterisk ($P< 0.05$).

15% in well plates. According to this data, about 85% of cells present in the human slices were still intact after 24 hours of incubation. This correlates well with the intact morphology observed for the slices. It could therefore be concluded that liver slices remain viable for at least 24 hours in both systems. It was demonstrated previously that human liver slices maintained their viability when incubated for 24 h in well plates.[22] These experiments have verified that liver slices also remain viable when incubated in the biochip under flow conditions.

Metabolism studies

As expected, large differences in metabolism were observed between livers from different patients, which is probably due to different diets and medications. Since rat liver slices incubated in well plates exhibit a good correlation to the rat situation *in vivo*,[23, 24] results obtained for human slices in the biochip were compared to results obtained for human slices in well plates. In order to do this, the metabolite formation in the biochip was normalized with respect to that obtained in well plates. Figure 4 presents the metabolism of four substrates in fresh tissue slices.

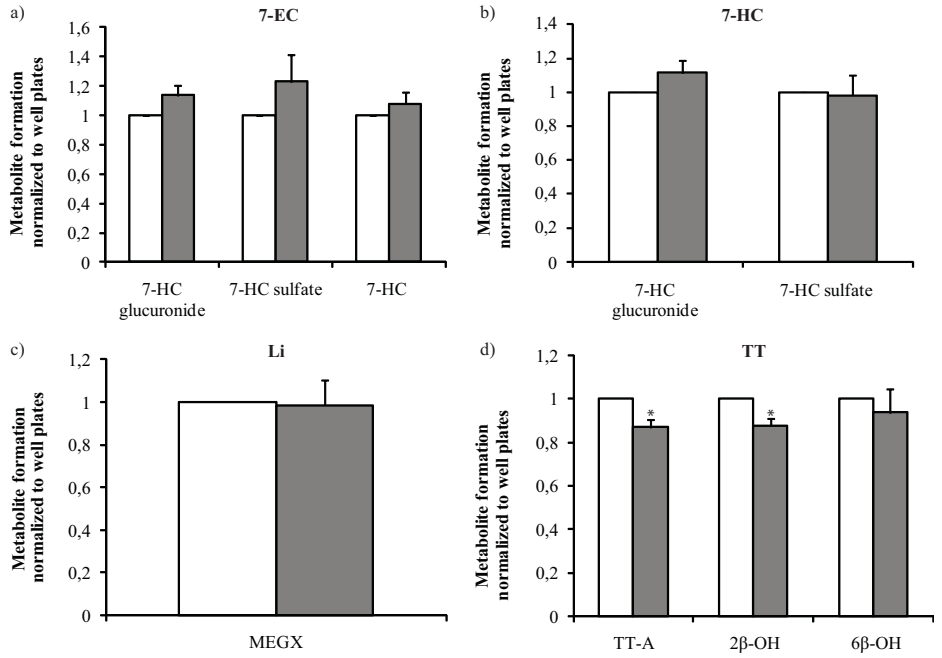

Figure 4. Metabolic activity of (a) 500 µM 7-ethoxycoumarin, (b) 500 µM 7-hydroxycoumarin, (c) 5 mM lidocaine, and (d) 250 µM testosterone in fresh human liver slices. White bars represent results obtained in well plates, while gray bars represent results obtained in the biochip. Results are mean of three organs ± SEM, with three slices per organ per incubation system per experiment. Significant differences of biochip results with respect to the well plates are indicated with an asterisk (P< 0.05)

Human liver slices were able to form both phase I and phase II metabolites from 7-EC (see Figure 4a), as shown previously.[22] These results indicate O-deethylation and the activity of sulfotransferase and UDP-glucuronosyltransferase in human liver slices. Though the metabolite formation in the biochip appeared to be slightly higher for all three metabolites compared to the well plates, the differences were not significant. The metabolism of 500 μM 7-HC into 7-HC-G also appeared to yield a slightly higher mean compared to well plates (see Figure 4b), although this difference proved not to be significant. Sulfation rates, on the other hand, were very similar. The formation of MEGX from lidocaine (see Figure 4c) demonstrated the ability of the human liver to N-deethylate a substrate, and was very comparable both in well plates and biochip for all three livers tested. Only in the case of testosterone metabolite formation was a significant difference between well plates and biochip observed (see Figure 4d). The means of the values for the measured formation rates for the three metabolites of interest were in all cases lower when compared to well plate results. Of these, however, only androstenedione and 2β-hydroxytestosterone exhibited significantly different behavior in the two systems. These two metabolites were also the most hydrophobic formed from testosterone, and are more hydrophobic than any of the other metabolites studied for 7-HC, 7-EC, and Li. One of the disadvantages of using PDMS is its tendency to absorb and adsorb hydrophobic compounds.[7, 25] To verify if adsorption/absorption occurred in the PDMS device, medium containing testosterone metabolites was flushed through a PDMS device which did not contain a liver slice. As expected, recoveries of circa 80% of the most hydrophobic compounds were obtained (see Chapter 8). This correlates well with the lower amounts of metabolites observed in the biochip when compared to well plates. It was therefore concluded that the metabolite formation for testosterone was also similar in well plates and biochip, but that 10 to 20% of the metabolites formed were adsorbed or absorbed onto or into the PDMS. The hydrophobicity of PDMS limits the applicability of the biochip and dictates that the possible adsorption or absorption of substrates and metabolites be taken into account when planning studies with this system.

The ability to form metabolites after 24 hours of incubation in well plates and the biochip was assessed by measuring the metabolism of 7-EC and 7-HC (see Figure 5a and b, respectively). The results were normalized to those obtained for fresh slices in well plates. After 24 h, the human slices were still able to form all three metabolites from 7-EC (see Figure 5a). A substantial amount of free 7-HC was also detected after 24 hours, while this was hardly detectable when incubating rat PCLS after 24 hours of incubation in both systems (see Chapter 5). However, the metabolite formation rates in human PCLS were only 35 to 40% of the formation rates measured for fresh slices. This decrease in metabolism is probably due to the decrease in CYP activity over time, something which is well known.[6, 26, 27] The loss in CYP activity does not

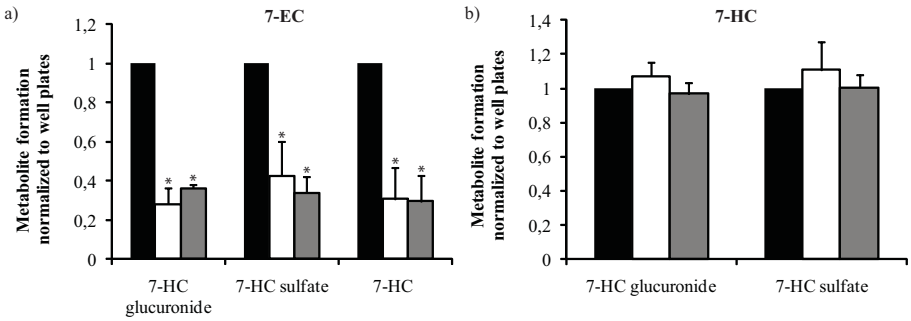

Figure 5. Phase I and phase II metabolic activity of slices incubated for 24 h in well plates (white bars) and biochip (gray bars). (a) 7-EC was used to asses phase I metabolism and (b) 7-HC to assess phase II metabolism. Results are the mean of three organs ± SEM, with three slices per organ per incubation system per experiment. Significant differences with respect to fresh slices incubated in well plates (black bars) are indicated with an asterisk ($P<0.05$). No significant differences were obtained between well plates and biochip after 24 h incubation.

affect every isoenzyme equally. However, Vandenbranden *et al.* found a decrease in coumarin 7-hydroxylase activity (CYP2A6) of 60 to 90% after 24 hours in three human livers.[26] This is in line with our results, which reveal a decrease of 60 to 65% in 24 h. The reduction in CYP enzyme activity is not limited to slices, as primary hepatocytes also exhibit this drop.[28] When metabolism in well plates and biochip are compared after 24 h of incubation, no significant difference can be discerned. Therefore, it can be concluded that the lower metabolic rates are due to the tissue and not the incubation system. This decrease can also be minimized by adding the right supplements, such as the inducer, dexamethasone, to the medium.[29] However, phase II metabolism after 24 hours is well maintained in human slices, at levels which are not significantly different from fresh slices in both well plates and biochip (see Figure 5b). This also indicates that human liver slices are viable for 24 hours, independent of the incubation system. Since the conversion of 7-HC into 7-HC-G and 7-HC-S was comparable after 24 hours of incubation to that observed in fresh slices in well plates, the decrease in 7-EC metabolism after 24 hours can only be due to the low CYP activity.

Conclusion

Before using a new incubation system to test new chemical entities, it is important to verify that the system will yield results which are reliable. Previously, we developed a microfluidic-based incubation system for the perifusion of PCLS.[10] This system has been verified with respect to the well-plate system for the incubation of rat liver slices. However, the ultimate goal is to incubate human tissue to predict the metabolic

pathway of xenobiotics without the need to sacrifice animals and with better extrapolation to the *in vivo* situation in humans. The microfluidic-based perifusion system was therefore also tested in conjunction with human tissue in this study. We have shown that it is possible to incubate viable human tissue in the biochip while maintaining its metabolic function. Slice viability was tested after 24 hours of incubation. The results showed intact structure and a low leakage of liver-specific enzymes, indicating viable slices. The metabolic function was also maintained in the biochip, as revealed by tests with four different model substrates (7-EC, 7-HC, Li, TT). Even after 24 h, the metabolic rate in the biochip was very comparable to the well-plate system, the benchmark for these kinds of experiments. Therefore, it can be concluded that the biochip system is appropriate for metabolism and toxicity studies using both rat and human liver slices.

Even though the results generated in the two systems were comparable, the biochip has several advantages compared to well plates. One of the advantages enabled by the continuous flow is that the chip outlet can be directly coupled to an HPLC injector for on-line analysis of metabolites (see Chapter 4).[30] It becomes possible to measure unstable metabolites formed by liver slices, such as acyl glucuronide, a metabolite of diclofenac, as shown previously for rat slices.[30] These types of metabolites often remain undetectable in well plates due to their rapid degradation. A second advantage is the very efficient use of scarce tissue that can be achieved. We have demonstrated previously, for example, that it is possible to perform a complete on-line inhibition study using only three rat liver slices.[30] Another advantage is the ability to measure the effect of flow on the metabolic pathways of xenobiotics, thereby mimicking the blood flow *in vitro*. Finally, it is possible to mimic first-pass metabolism with this system by exploiting the continuous flow feature. This can be accomplished by connecting the outlet of one chamber containing an intestinal slice to the inlet of a different chamber containing a liver slice from a different organ (see Chapter 7). In this way, it becomes also possible to investigate the effect of products formed by one organ on a different organ, the so-called interorgan effects. In well plates, this type of experiment involves placing two slices in one well; however, mutual interaction between slices can take place, and it is never clear which organ is responsible for the effect. In the biochip, the two organ slices are isolated from each other, making it far easier to characterize interorgan interactions. The preservation of human organs is a crucial issue for this type of experiment, since the chance of receiving surgical waste from two different organs on the same day is rather low.

This system, then, has the potential to contribute to drug metabolism and toxicology studies of novel chemical entities. Though it will not replace incubation in well plates, it will provide supplementary information about xenobiotics in an early stage of development.

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A Microfluidic Approach for *In Vitro* Assessment of Interorgan Interactions in Drug Metabolism Using Intestinal and Liver Slices

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Lab on a Chip 2010:10(20) 2778-2786

Abstract

Over the past two decades, it has become increasingly clear that the intestine, in addition to the liver, plays an important role in the metabolism of xenobiotics. Previously, we developed a microfluidic-based *in vitro* system for the perifusion of precision-cut liver slices for metabolism studies. In the present study, the applicability of this system for the perifusion of precision-cut intestinal slices, and for the sequential perifusion of intestinal and liver slices, all from rat, was tested to mimic the *in vivo* first pass situation. Intestinal and liver slices, exposed to the substrates 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC) and lidocaine (Li), exhibited similar metabolic rates in the biochip and in the well plates for periods of at least 3 h. The metabolic rate remained the same when two slices were placed in adjacent microchambers and perifused sequentially. In addition, the system has been adapted to sequentially perifuse intestinal and liver tissue slices in a two-compartment co-culture perfusion system with a continuous flow of medium. It becomes possible to direct metabolites or other excreted compounds formed by an intestinal slice in the first compartment to the second compartment containing a liver slice. The intestine does not influence liver metabolism for these substrates. The interplay between these two organs was demonstrated by exposing the slices to the primary bile acid, chenodeoxycholic acid (CDCA). CDCA induced the expression of fibroblast growth factor 15 (FGF15) in the intestinal slice, which resulted in a stronger down-regulation of the enzyme, cytochrome P450 7A1 (CYP7A1), in the liver slice in the second compartment than when the liver slice was exposed to CDCA in a single-microchamber biochip. We thus demonstrate in this paper that intestinal slices, in addition to liver slices, remain functional in the biochip under flow conditions, and that the two-microchamber biochip has great potential for the study of interorgan effects. This is the first example of the incorporation of both liver and intestinal slices in a microfluidic device. Use of this microfluidic system will improve our insight into interorgan interactions and elucidate as yet unknown mechanisms involved in toxicity, gene regulation and drug-drug interactions. Moreover, human-specific metabolism can be studied when human slices are available.

Introduction

It is generally known that the liver plays a major role in drug metabolism.[1] However, in the last twenty years it has become increasingly clear that the role of the intestine in drug metabolism cannot be neglected.[2] Increased insight into intestinal drug metabolism and active transport processes has revealed the importance of the intestine not only for drug absorption, but also for the metabolism of xenobiotics. As studies on drug metabolism in the intestine are difficult to perform *in vivo*, especially in man, various *in vitro* systems have been developed to study intestinal metabolism, as was recently reviewed by Van de Kerkhof *et al*.[2] These include subcellular fractions,[3] cell lines,[4] primary isolated enterocytes[5] or intact tissue preparations.[6] However, subcellular fractions and cell lines have their limitations for drug metabolism studies, since they do not contain all enzymes at the proper concentrations.[2] Therefore, primary cells or intact tissue are to be preferred for these studies. However, the isolation of primary cells results in the loss of normal cell-cell and cell-matrix contacts, which may induce changes in cellular function. Precision-cut intestinal slices, on the other hand, have proven to be a versatile *in vitro* system for metabolism studies and show a good correlation with the *in vivo* situation.[7] They closely resemble the complex architecture of the organ, with all cell types present in their natural tissue-matrix configuration. The common method to incubate intestinal slices is to place them in medium-filled well plates.[8] However, in this *in vitro* system, the medium is usually refreshed every 24 h, resulting in decreasing concentrations of nutrients and accumulation of metabolites and waste products. To prevent such variations in culture conditions, we recently developed a new system for the incubation of rat liver slices, consisting of a perfused microfluidic biochip where the liver slice is continuously perifused.[9] (Note: the term "perifusion" is used to emphasize that the medium flows around the tissue slice rather than through it, whereas the term "perfusion" applies to the medium flow through the microchamber.) The microenvironment of the slice could be very well controlled in this microfluidic system, and liver slices maintained their viability and metabolic function for at least 24 h. These results were as good as those obtained in optimized, conventional well plate assays, where drug metabolism rates are similar to those in the organ *in vivo*.[7] Such a microflow system offers a flexible platform for experimentation, as medium composition and flow can easily be varied throughout the course of an experiment, allowing the study of dose dependence and drug-drug interactions in one slice.

The first aim of this paper was to investigate whether intestinal tissue slices can also be cultured in the biochip, and to compare their metabolic activity with slices incubated in the conventional well plate system. To the best of our knowledge, this is the first report of the incorporation of intestinal tissue slices into a microfluidic device.

In vivo metabolites formed by the intestine and excreted to the basolateral side

are transported *via* the portal blood to the liver. It can be hypothesized that these metabolites are further converted in the liver and/or influence liver function. However, these so-called interorgan effects have proven difficult to verify in *in vitro* experiments. In well plates, two different tissue slices can be placed in one well; however, mutual interactions can take place in this case, and it would be impossible to conclude which organ was responsible if interorgan effects were to be observed. Thus, the second aim of this study was to construct a new *in vitro* system to sequentially perifuse precision-cut tissue slices (PCTS) from the intestine and the liver, to gain new insight into these potential interorgan effects.

There have been to date only a few examples of co-culture flow systems incorporating cells derived from different tissues that allow the investigation of interorgan effects. None of these has used freshly isolated cells, relying instead on cell lines. Flow systems containing Caco-2 cells (a colon-derived cell line, differentiated into small intestinal-like cells) and HepG2 cells (a liver carcinoma cell line) have been reported for absorption and biotransformation studies.[10-12] Recently, Mahler *et al*. developed a microscale cell culture analog (μCCA) with Caco-2 and HepG2 cells. Due to the low volume, this μCCA allowed for near *in vivo* fluid-to-tissue ratios and cellular shear-stress values.[13] These co-culture systems yielded interesting results for absorption and toxicity studies. However, the lack of normal expression levels of important metabolic enzymes in Caco-2 and HepG2 cells[14] makes this system less predictive for metabolism studies. Moreover, interorgan effects due to mediators excreted by cell types other than the enterocytes will not be detected. To ensure availability of these endogenous enzymes, the use of primary cells, or even better, tissue, is preferred in *in vitro* systems. Chen and Pang showed the sequential *ex vivo* perfusion of intact rat intestine and liver preparations for metabolism studies,[15] and studied the effect of flow on first-pass metabolism. However, two rats are needed for one experiment, one as intestine donor and one as liver donor, making the system labour-intensive and not particularly attractive from an animal usage point of view. Moreover, this technique is not applicable to the human case, since it is clearly ethically impossible to perform this experiment in the human context. To date, no microfluidic *in vitro* system has been developed which incorporates both liver and intestinal primary cells or tissue explants, such as biopsies or slices. Such a system would greatly increase our understanding of interorgan interactions as well as substantially reduce animal use.

We have therefore developed an *in vitro* microflow system for the purpose of investigating interorgan effects, made by coupling two microchambers, one containing an intestinal tissue slice, the other a liver slice, which can be sequentially perfused. In this way, metabolites formed by the intestine in the first chamber can be directed to the liver in the second chamber for further metabolism, thereby mimicking *in vivo*, first-pass metabolism. In addition, the possible influence of metabolites or

other mediators formed by the intestine on the liver can be studied, providing insight into interorgan effects.

To demonstrate the feasibility of the microfluidic system for metabolism studies, we tested whether rat liver and intestinal slices remain functional in such a sequential perfusion set-up. The metabolism of several model compounds was measured in a single liver slice, a single intestinal slice, two sequentially coupled liver slices, two sequentially coupled intestinal slices and a sequentially coupled intestinal and liver slice. The compounds 7-ethoxycoumarin, 7-hydroxycoumarin and lidocaine were used as model substrates to probe the activity of several phase I cytochrome P450 isoenzymes, and phase II UDP-glucuronyltransferase and sulfotransferase. Since the well plate provides an excellent benchmark with high viability for short-term metabolism studies, these experiments were also performed in well plates as control.[16] It was not expected that these compounds would show interorgan effects.

To demonstrate the application of the developed system to the investigation of interorgan effects, the interplay of liver and intestine in the regulation of bile acid homeostasis was simulated. In addition to the liver, the intestine also plays an important role in the regulation of enzymes and transporters involved in bile acid transport and metabolism.[17] One of the key events in the regulation of bile acid homeostasis is the regulation of the enzyme, cytochrome P450 7A1 (CYP7A1), expressed in the liver and the rate-limiting enzyme for the synthesis of bile acids from cholesterol. Several mechanisms have been shown to explain the bile acid-induced down-regulation of CYP7A1. In the first place, bile acids bind to the nuclear receptor, farnesoid X receptor (FXR), in the liver, thereby inducing the expression of small heterodimer partner (SHP), which in turn down-regulates the expression of CYP7A1 and hence the synthesis of bile acids. In addition, primary bile acids, like chenodeoxycholic acid (CDCA), induce the expression of fibroblast growth factor 15 (FGF15) in the rodent intestine (FGF19 in the human intestine). This results in the formation of the FGF15 protein, which is transported *in vivo via* the portal vein to the liver.[18, 19] In the liver, this protein causes an additional down-regulation of CYP7A1. In this study, the interorgan interaction was confirmed in the biochip incorporating sequential perifusion of precision-cut liver and intestinal slices by measuring the gene expression of FGF15 and CYP7A1 using real-time PCR.

This study demonstrates the first example of metabolism studies in intestinal slices under flow conditions. Moreover, this is also the first example of sequential perifusion of precision-cut tissue slices from intestine and liver to investigate interorgan interactions.

Material and Methods

Chemicals

Sodium chloride, low-temperature-gelling agarose, 7-ethoxycoumarin (7-EC), 7-

hydroxycoumarin (7-HC), 7-hydroxycoumarin sulfate (7-HC-S), 7-hydroxycoumarin glucuronide (7-HC-G), and D-glucose monohydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chenodeoxycholic acid (CDCA) was obtained from Calbiochem (San Diego, CA, USA). Gentamicin, Williams Medium E with Glutamax-I, and amphotericin B (Fungizone) solution were purchased from Gibco (Paisley, UK). Lidocaine (Li) was obtained from Centrachemie (Etten-leur, The Netherlands). Monoethylglycinexylidide (MEGX) was a kind gift from AstraZeneca (Södertälje, Sweden).

Animals

Male Wistar rats (HsdCpb:WU) weighing *ca.* 300 g were obtained from Harlan (Horst, The Netherlands). The rats were maintained under a 12-h light/dark cycle in a temperature- and humidity-controlled room with free access to food (Harlan chow no. 2018, Horst, The Netherlands) and tap water. The use of animals for these experiments was approved by the Animal Ethics Committee of the University of Groningen.

Preparation of precision-cut tissue slices

After anaesthetizing the rat with isoflurane/O_2, the intestine and liver were excised and placed in ice-cold oxygenated Krebs-Henseleit buffer (pH = 7.4) and ice-cold Viaspan organ preservation solution (University of Wisconsin (UW) solution, Du Pont Critical Care, Waukegab, IL, USA), respectively. Liver slices were made as described previously.[9] Slices were approximately 4 mm in diameter, 100 μm thick (~6 cell layers), and had a wet weight of about 3 to 4 mg. Intestinal slices (jejunum and ileum) were made as described by Van de Kerkhof *et al*.[8] The jejunum slices were used for metabolism studies, and ileum for the regulation of bile acids. Briefly, the intestine was cut into 3 cm segments and flushed with Krebs-Henseleit buffer. Segments were ligated at one end, and filled with 3% (w/v) agarose solution in 0.9% sodium chloride at 37°C. Subsequently, the segments were placed in ice-cold Krebs-Henseleit buffer to solidify the agarose. The gel-filled intestinal segment was then embedded in 37°C agarose solution, and sliced after gelling at 4°C. The total time needed for the preparation of the gel-filled intestinal segment is around 30 minutes, and slicing of 10-20 slices takes about 10-15 min. Intestinal slices were approximately 400 μm thick and had a wet weight of approximately 2 mg.

Microdevice

Figure 1 shows a schematic view of the microdevice made of 10 layers of poly-dimethylsiloxane (PDMS). Details of the biochip fabrication and medium distribution through the chamber have been extensively described elsewhere.[9] The biochip used in this study is the same, with one minor modification. Previously, the chips contained

4 microchambers. For this study, we designed biochips where each chip contained 6 microchambers, all of which could be perfused separately. The medium inlet at the bottom of the chip was connected *via* a microfluidic channel (500 µm x 100 µm (W x H)) with the tissue chamber. A polycarbonate filter (10 µm thick, 8 µm pores) was integrated at the entrance to the tissue chamber (Ø4 mm x 2 mm, 25 µL). This filter acts as an inverse showerhead, and ensures an equal distribution of medium flow towards the tissue slices, as described previously.[9] At the top of the microchamber a second polycarbonate filter was integrated, to ensure that the tissue slices are suspended in medium and that the medium flows all around the tissue slice (perifusion). The microchamber was connected to the outlet *via* a microfluidic channel. Connecting this outlet to the inlet of a neighbouring chamber allows sequential perfusion of these two chambers. An important feature of this device is the integration of two PDMS membranes to act as aeration windows to maintain a highly controlled microenvironment for tissue-slice incubations. PDMS is known to be gas permeable,[20] and by integrating 250-µm-thick PDMS membranes, oxygen and carbon dioxide from the environment can penetrate the PDMS and enter the medium. This enables the regulation of oxygen and carbon dioxide (important to maintain the pH at 7.4) concentrations in the medium.

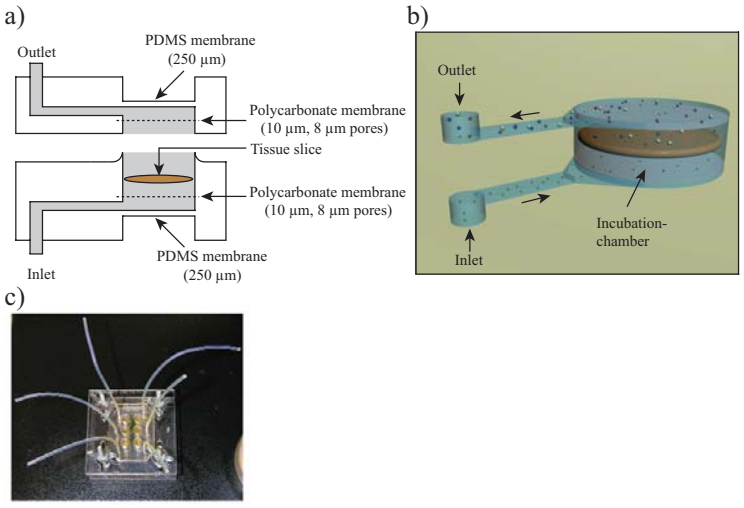

Figure 1. Schematic illustration and photograph of the PDMS biochip. (a) Cross-sectional view of one chamber with the integrated polycarbonate (10 µm thick) and PDMS membranes (250 µm thick). The dimensions of the microchamber is Ø4 mm x 2 mm high. (b) Animated illustration of how liquid flows through the biochip. A substrate is added at the inlet and converted into metabolites by the liver slice, which are then transported to the outlet by the flow. (c) Photograph of the PDMS biochip containing six microchambers in the polycarbonate holder. The dimensions of one chip are 30 mm x 20 mm x 12 mm (L x W x H).

Incubation system

Well plates

Tissue slices were incubated in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) in 1.3 mL Williams Medium E with Glutamax-I. For both liver and intestinal slices, the medium was supplemented with 25 mM D-glucose monohydrate and 50 μg/mL gentamicin. In addition, media for intestinal slices and co-incubation of liver and intestinal slices were supplemented with 2.5 μg/mL amphotericin B. Well plates were placed in humidified plastic boxes which were continuously supplied with humidified carbogen to obtain an atmosphere of 95% oxygen and 5% CO_2. The boxes were shaken at 90 cycles per minute. All experiments were performed in an incubator at 37°C. Co-incubation of liver and intestinal slices was performed with both slices placed in the same well in 1.3 mL medium.

Biochip

The same medium was used for the biochip experiments as for the well plates. Glass syringes (ILS, Stützerbach, Germany) were filled with medium which was saturated with carbogen at 37°C (pH of 7.4). Filled syringes were placed in a syringe pump. PEEK tubing was used to connect the syringes with the biochip (150 mm x 0.75 mm ID) (Da Vinci Europe, Rotterdam, The Netherlands). To sequentially perfuse two chambers, the outlet of the first chamber was connected to the inlet of the second chamber with PTFE tubing (150 mm x 0.5 mm ID) (Polyfluor Plastics, Oosterhout, The Netherlands). PTFE tubing was also connected to the outlet of the chip (75 mm x 0.50 mm ID) to collect medium fractions. The chip was placed in a humidified plastic container with a continuous exchange of humidified carbogen gas, and the box was placed in an incubator set at 37°C. After flushing the system with medium for at least half an hour, tissue slices were placed in the microchambers. A schematic overview of the system set-up for the sequential perfusion of two microchambers containing slices is given in Figure 2. The flow rate was set at 10 μL/min, as pilot experiments showed that high flow rates (>50 μL/min) resulted in damage to the tissue, whereas low flow rates (<4 μL/min) may result in insufficient diffusion and refreshment rate (results not shown). A flow rate of 10 μL/min results in complete refreshment of the medium in the microchamber every 2.5 minutes.

Morphology

Non-incubated (0 h) precision-cut tissue slices and slices incubated in both well plates and biochip for 3 hours (3 h) were subjected to morphological evaluation. Slices were fixed in 4% formaldehyde in phosphate-buffered saline solution for 24 h at 4°C. Thereafter, slices were placed and stored in 70% ethanol until paraffin embedding, sectioning and haematoxylin and eosin staining (HE staining). HE staining was performed as described previously by De Graaf *et al*.[21]

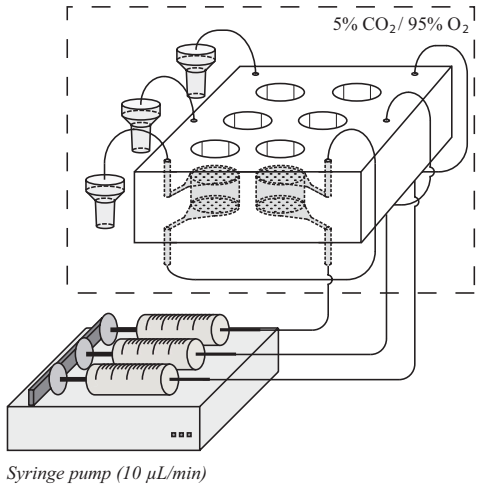

Figure 2. Schematic view of the set-up for a chip with three sets of two sequentially perfused chambers.

Metabolite analysis

Slices were pre-incubated for one hour in well plates. They were then either incubated in well plates with medium containing 500 µM 7-EC or 500 µM 7-HC for 3 hours, or 5 mM Li for 1 hour, or transferred to a biochip and perifused with medium containing these substrates.[22] After incubation, the medium samples were stored at -20°C until analysis. Analysis was performed on an HPLC system with UV detection. 7-EC, 7-HC, 7-HC-G and 7-HC-S were quantified as described previously,[9] as was Li and its metabolite, MEGX.[23] To compare the results obtained in well plates and biochip, and to compare two-slice with one-slice incubation, all results were reported in terms of amount of metabolite formed per minute per milligram protein in the tissue. Protein amounts in tissue slices were determined using the Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) using bovine serum albumin as standard.[8]

Bile acid-induced regulation of gene expression

Ileum and liver slices were incubated singly in a biochip or sequentially perifused with or without 50 µM CDCA. After 7 h of incubation, the slices were taken out of the chips and snap-frozen in liquid nitrogen. Subsequently, RNA was isolated from the slices using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the Promega Reverse Transcription System (Promega, Madison, USA), in which 1.25 µL sample were used in real-time PCR reactions using Sensi-Mix SYBR green (Quantace, London, UK). The primers used for real-time PCR are listed in Table 1. The comparative threshold cycle (C_T) was used to quantitate the expression of the genes of interest. For intestinal slices, the expression was calculated

relative to villin as reference gene (ΔC_T), and for liver slices relative to GAPDH as reference gene. The effect of treatment with CDCA was calculated relative to the control slices ($\Delta\Delta C_T$) and expressed as fold expression according to $2^{-(\Delta\Delta C_T)}$.

Table 1. Primer information of the rat genes used for real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	CGCTGGTGCTGAGTATGTCG	CTGTGGTCATGAGCCCTTCC
Villin	GCTCTTTGAGTGCTCCAACC	GGGGTGGGTCTTGAGGTATT
CYP7A1	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC
FGF15	ACGGGCTGATTCGCTACTC	TGTAGCCCAAACAGTCCATTTCCT

Statistical evaluation

Significant differences between control and treated slices were determined using the Student's *t*-test, with $p < 0.05$ considered as significantly different. For metabolism studies, it was of interest to determine what, if any, significant differences existed between experimentally determined metabolic rates; for gene expression, $\Delta\Delta C_T$ values were statistically evaluated.

Results and Discussion

Liver slices

Previously, we showed that liver slices remain viable for 24 h in the microchamber of our biochip. Here, it was important to ensure that a liver slice in the second microchamber, perfused with outflow medium from the first microchamber, retained its viability and received sufficient nutrients and substrates. Hence, the viability of a liver slice in the second chamber was compared with that in the first chamber.

Figures 3a), c) and e) show the morphology of liver slices directly after slicing and after 3 h incubation in well plates and in the biochip, respectively. No differences in liver morphology were observed in the biochip and well plates when compared to fresh tissue. The morphology was also not changed when two liver slices were coupled and sequentially perifused, indicating intact cells.

The metabolic characteristics of the slices in the first microchamber were subsequently compared with those in the second. The metabolic rates of liver slices after incubation for 1 h with 5 mM Li, and for 3 hours with 500 µM 7-EC or 500 µM 7-HC, are given in Figure 4 for single liver slices in the well plate and biochip, and for two liver slices sequentially perifused in the biochip. The substrate concentrations used were well above the concentrations needed to achieve the maximum metabolic rate,[16] resulting in a constant rate of metabolism despite a small decrease in substrate concentration during incubation in the well plates. This allows us to compare the metabolic rate in well plates with that in the biochip, where substrate concentrations remain constant.[9]

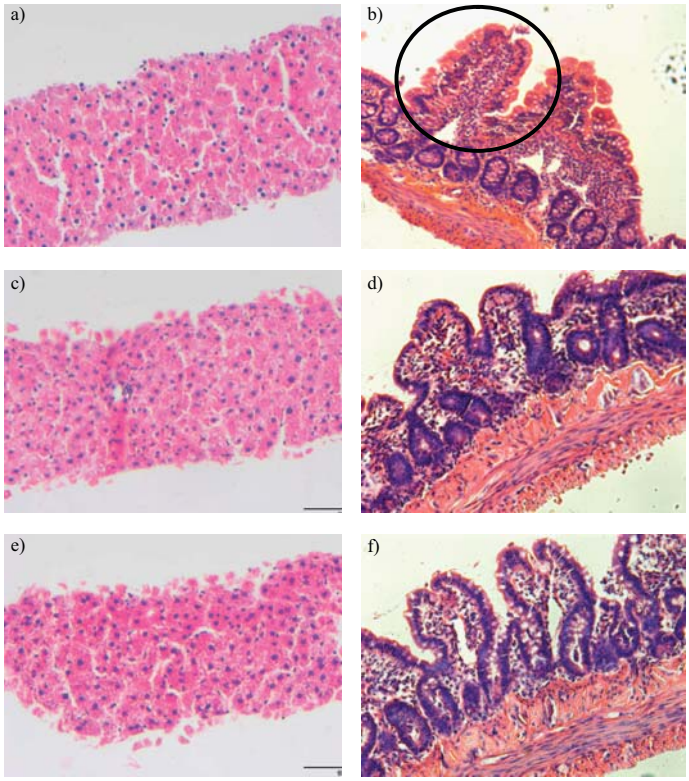

Figure 3. Morphological evaluation of liver and intestinal slices directly after slicing (a and b), after 3 h of incubation in well plates (c and d), and in the biochip (e and f). Magnification: 100x. The circle in b shows the villi of the intestine.

The formation of MEGX was 2.6 ± 0.2 nmol/min/mg protein, and was not significantly different in the well plates and biochip (Figure 4a)), indicating a similar viability in both systems. In addition, the formation rate of MEGX expressed per mg of protein was identical in one liver slice and two sequentially perifused liver slices. Therefore, we can conclude that the consumption of nutrients and substrates by the first slice did not negatively affect the metabolic rate of the second slice. Moreover, the substrate concentration in the second chamber was still high enough for the second slice to operate at the maximum metabolic turnover rate, despite the consumption of substrate in the first chamber. The same results were observed for 7-EC and 7-HC (Figure 4b) and 4c)). No significant differences were observed in specific metabolic activity between slices in well plates and in the biochip, and between one slice and two slices perifused sequentially. The slight, but not significant, difference in the level of 7-HC between the biochip and well plates, observed previously,[9] might indicate a somewhat more efficient metabolism of 7-HC into 7-HC-G in the biochip. The total metabolic conversion of 7-EC in rat liver slices was 218 ± 10 pmol/min/mg protein,

and of 7-HC was 594 ± 30 pmol/min/mg protein. These metabolic rates were comparable with metabolic rates found previously,[16] and thus correlate well to the *in vivo* situation.[16, 24]

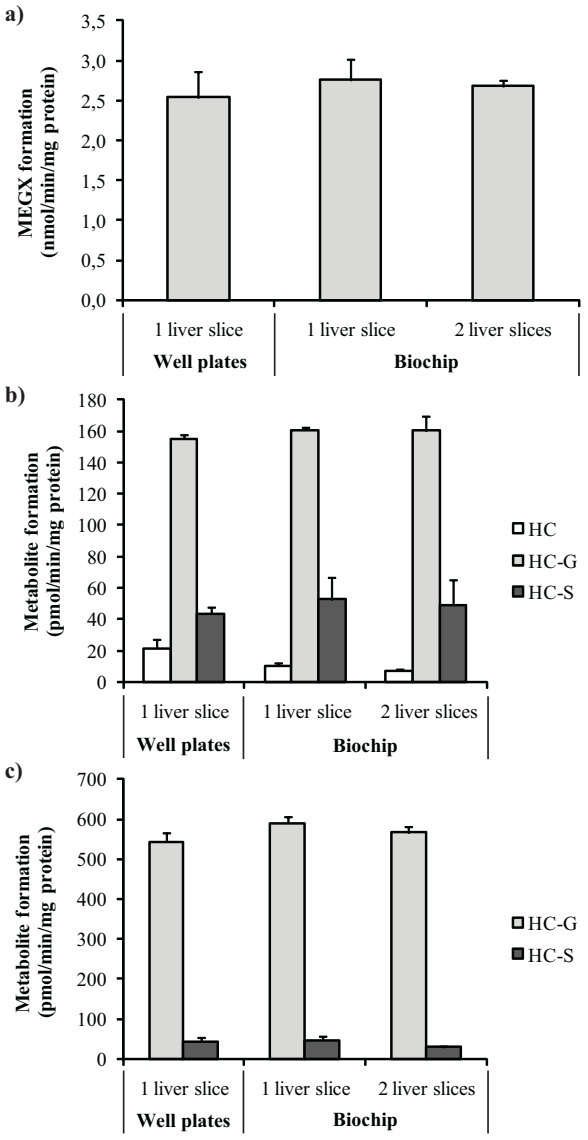

Figure 4. Metabolism of (a) 5 mM Li, (b) 500 µM 7-EC, and (c) 500 µM 7-HC in single liver slices and in two liver slices perifused sequentially in the biochip as indicated in Figure 2. Results are given as the mean ± standard error of the mean (SEM) of 3-4 rats, with 3 slices per experiment. No significant differences were observed in metabolic activity between liver slices perifused singly or sequentially, or between experiments in well plates and biochips.

Intestinal slices

Subsequently, we tested the incorporation of intestinal tissue slices in the biochip. As far as we know, this is the first attempt to perifuse intestinal slices and measure metabolism in a perifusion system. The viability and function of intestinal slices incubated in well plates up to 24 h have been shown by us and others.[25, 26]

In general, the morphology of intestinal slices (jejunum) as evaluated by an independent pathologist was retained intact compared to fresh intestinal slices, as can be seen in Figures 3b), d) and f). However, after incubation for 3 h in well plates, the villi, present at the apical side of the intestine, appeared somewhat flattened and broadened, as was also observed in earlier studies.[6] The villi of most of the intestinal slices perifused in the biochip were less flattened compared to those in the well plates (see Figure 3f)), although this was not the case for all slices evaluated. No changes in slice morphology were observed when incubating two intestinal slices instead of one, independent of whether incubation was performed under flow or no-flow conditions.

Figure 5 shows the metabolism of 5 mM Li, 500 µM 7-EC and 500 µM 7-HC by small intestinal slices (jejunum) incubated in well plates or perifused in the biochip, in a single microchamber and in two microchambers perfused sequentially. The formation of MEGX in the small intestine under all incubation conditions was 140 ± 38 pmol/min/mg protein. When intestinal slices were exposed to 7-EC, the metabolites 7-HC and 7-HC-G were formed, but 7-HC-S was undetectable (see Figure 5b)). When slices were exposed to 7-HC, both 7-HC-G and 7-HC-S were formed. The total metabolic rate for 7-EC in the intestine was 21 ± 2 pmol/min/mg protein, and for 7-HC 304 ± 40 pmol/min/mg protein, comparable to the rate found previously by Van de Kerkhof *et al*. in well plates.[26] As with liver slices, the specific metabolic rate of two intestinal slices perifused sequentially did not significantly differ from one individual slice, and no significant difference was observed between the well plates and the biochip. This indicates that intestinal slices in the biochip retain a similar viability as in well plates for at least 3 h.

The total phase I metabolism of 7-EC and Li in intestinal slices was 10- and 18-times lower, respectively, than in liver slices. This is in line with the ratio of the 10-20 fold difference in cytochrome-P450 protein expression in the liver and intestine as found by Matsubara *et al*.[27] and Lin *et al*.[28] The rate of formation of 7-HC-S from 7-HC in the intestinal slices was comparable to the liver slices, while the rate of glucuronidation was only two times lower. This indicates a high activity of sulfotransferase and glucuronyltransferase in the intestine as observed previously.[6, 8] The lack of detection of 7-HC-S after incubation with 7-EC can be explained by the low phase I biotransformation rate by cytochrome P450 isoenzymes, and thus low intracellular concentration of the phase I metabolite 7-HC.

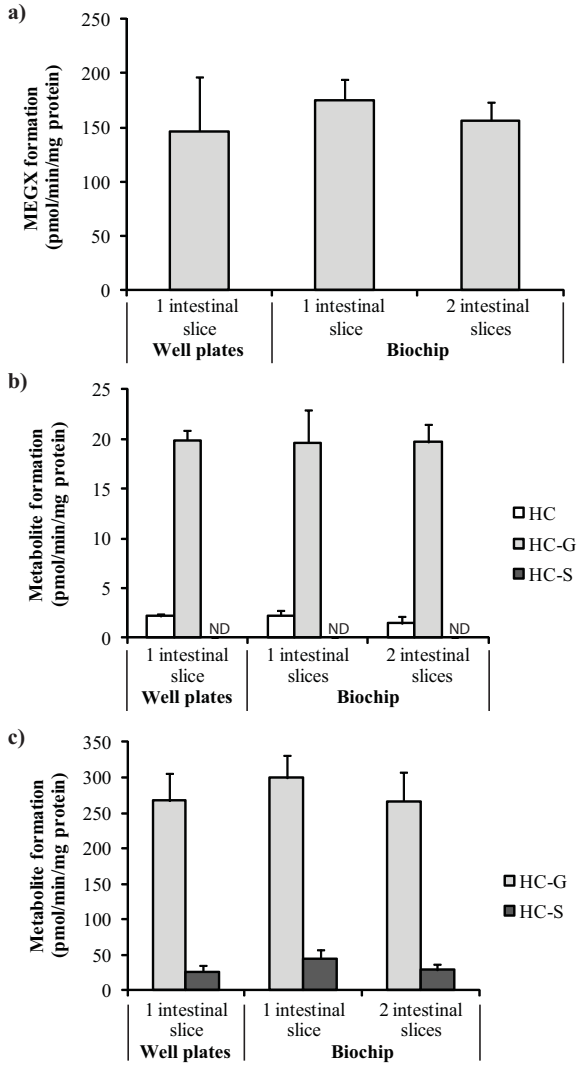

Figure 5. Metabolism of (a) 5 mM Li, (b) 500 µM 7-EC, and (c) 500 µM 7-HC in single intestinal slices and in two intestinal slices perifused sequentially. Results are the mean ± SEM of 3-4 rats, 3 slices per experiment. No significant differences were observed between intestinal slices perifused singly or sequentially, or between experiments in well plates and biochip. ND: not detectable.

Intestinal and liver slices

Sequential perifusion of intestinal slices and liver slices in the biochip can serve as a model to study first-pass metabolism. The weight of the tissue slices used for the experiments was 2 mg for intestine and 3-4 mg for liver. This ratio reflects the organ weight ratio in the rat *in vivo*.[16] Again, the morphology did not change in the two

incubation systems during the co-incubation of a liver and intestinal slice. Slice morphology in both the liver and intestinal samples was comparable with single-slice incubation or perifusion, as shown in Figure 3. This indicates that both liver and intestinal slices remain intact in the well plate system as well as in the biochip. Figure 6 shows the metabolism of the three substrates after co-incubation of liver and intestinal slices in well plates or sequential perifusion in biochips (indicated as "measured"). In addition, the data are given for single-incubation experiments with separate liver and intestinal slices, after which the total metabolic rate of a liver and an intestinal slice was calculated and corrected for inter-experimental differences in protein content of the slices (indicated in Figure 6 as "calculated").

The calculated and measured metabolite formation rates did not significantly differ for any of the measured substrates. The metabolizing enzymes in the liver slices were thus functioning at their optimal turnover rate. This means that the consumption of nutrients and the excretion of waste products by the intestinal slice did not affect liver-slice metabolism during the 3 h of incubation. We conclude from these data that coupling two chambers sequentially did not affect the metabolic competence of slices in the second chamber. From these results, we can also conclude that the intestine does not influence the liver's metabolism of the three substrates used in this study, at least not under the applied concentrations. Again a small, but now significant, difference was found for the amount of 7-HC measured in the well plates compared to the biochip. However, the total phase I metabolic rate, which is the sum of 7-HC-G, 7-HC-S and 7-HC, was not significantly different. As the 7-HC-G formed was slightly higher in the biochip compared to the well plates, the lower 7-HC concentration may be caused by a more efficient formation of 7-HC-G by the liver slice in the biochip.

From the data presented in Figures 4, 5 and 6, it can be concluded that the contribution of the small intestine to the metabolism of 7-EC and Li is small compared to the liver at the applied substrate concentrations. However, this may be different at lower substrate concentrations, where the ratio of the metabolic rate in the liver to that in the intestine is smaller, as shown by Martignoni *et al*.[25] The contribution of the small intestine to the phase II metabolism of 7-HC, on the other hand, was high at the concentration tested.

Interorgan interaction

The ability to monitor interorgan interactions was assessed by studying the bile acid-induced regulation of the enzyme, CYP7A1, responsible for bile acid synthesis. Single liver and intestinal slices were incubated with CDCA, to measure the direct effect of CDCA on the expression of CYP7A1 in the liver and FGF15 in the intestine. In addition, to determine the effect of products formed by the intestine on bile acid treatment of the liver, the outlet of the chamber containing an intestinal slice was

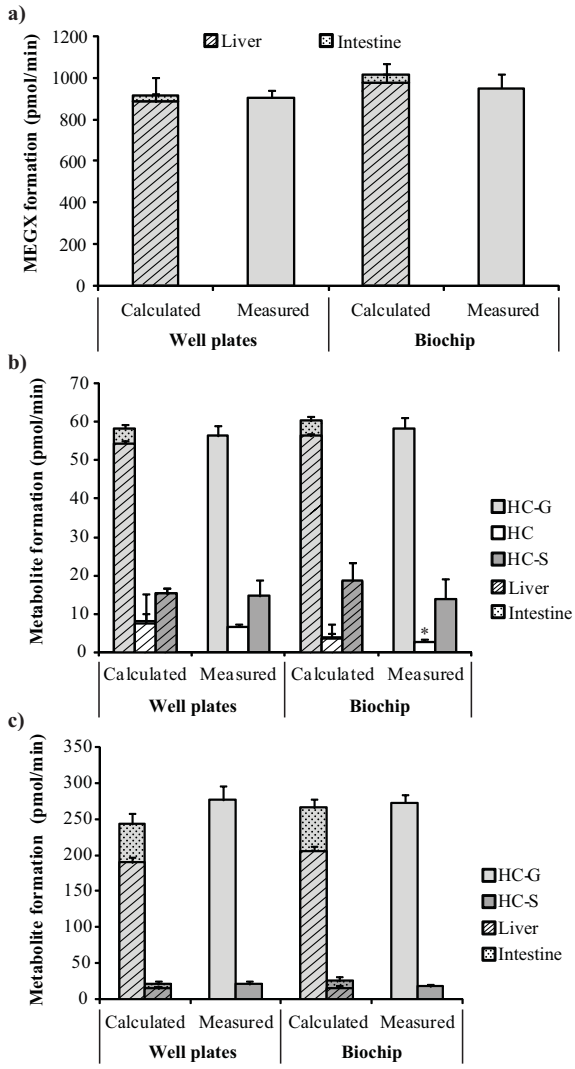

*Figure 6. Metabolism of (a) 5 mM Li, (b) 500 µM 7-EC, and (c) 500 µM 7-HC in liver slices and intestinal slices perifused sequentially or incubated together in the same well (measured). For the calculated values, the results for individual slice incubations were used. Results are the mean ± SEM of 3-4 rats, 3 slices per experiment. No significant differences were observed between calculated and measured metabolic rates. Significant difference between well plate and biochip is indicatd with * ($p < 0.05$).*

connected to the inlet of a chamber containing a liver slice. When intestinal slices were treated with CDCA, a significant up-regulation of FGF15 was observed (see Table 2). As expected, results obtained with single intestinal slices were not

significantly different from those in co-incubated slices, since the intestinal slice was always perifused in the first compartment during sequential perfusion. As shown in Table 2, the fold expression of FGF15 of the three rats was highly variable. This variability is probably due to the gradient of FGF15 expression along the intestinal tract[19] as it is difficult to prepare the slices from exactly the same location of the intestine. However, for all three rats there is a clear and significant up-regulation of FGF15 in ileum slices when perifusing them with CDCA, as shown by others.[29]

Table 2. Fold change of the expression of FGF15 in rat ileum slices after 7 h of exposure to CDCA compared to untreated control slices.

	Rat 1	Rat 2	Rat 3	Mean
Single	32	332	134	166
Co-incubated	30	290	134	151

When perifusing intestinal and liver slices sequentially without the addition of bile acids, the CYP7A1 expression in the liver was comparable to that in single-liver-slice incubation (see Figure 7). This indicates that intestinal slices do not release compounds which affect the expression of CYP7A1 in the liver under control conditions. The incubation of single liver slices with CDCA resulted in a small (25%) but not significant down-regulation of CYP7A1.[17, 18] However, after exposure of sequentially perifused intestinal and liver slices to CDCA, the CYP7A1 expression in the liver slices was decreased to 40% of its control value. Since exposure of liver slices alone to CDCA and sequential perifusion of intestinal and liver slices without CDCA did not result in such low CYP7A1 expression, this effect is most likely due

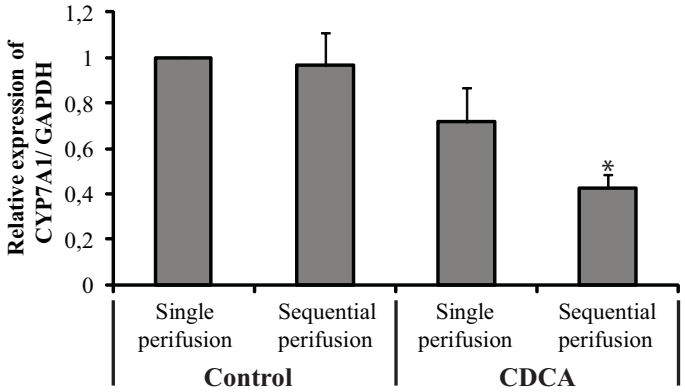

*Figure 7. Expression of CYP7A1 in liver slices treated with CDCA after single perifusion and after sequential perifusion with an intestinal slice. Results are the mean ± SEM of 3 rats, 3 slices per experiment. Significant difference with respect to control is indicated with * ($p < 0.05$).*

to a product formed by the intestine upon exposure to CDCA. As shown by Inagaki *et al*., bile acids induce the expression of FGF15 in the intestine[19] and this FGF15 can be transported in our microfluidic system to the liver by continuous perfusion. The exposure of the liver slices to both CDCA and FGF15 formed by the intestine resulted in the strongly reduced expression of CYP7A1. This will in turn result in a decrease of the synthesis of bile acids from cholesterol in the liver. This clearly demonstrates, for the first time, the utility of the dual-chamber microfluidic biochip for the investigation of the interplay between the intestine and liver, in this case for the control of bile acid synthesis.

Conclusion

We have developed a new *in vitro* system to study intestinal and liver metabolism in a microfluidic biochip under flow conditions. It was previously demonstrated that liver slices incorporated in this novel system retain their viability and functionality for at least up to 24 h.[9] Now we show that it is also feasible to maintain intestinal slices in this biochip with high viability and functionality for at least 3 h. Morphological evaluation of intestinal slices indicates a slightly better preserved intactness of tissue in the biochip compared to the well plates. This is the first example of the incorporation of intestinal slices into a microflow system. The results obtained in this new *in vitro* system were verified with the optimized conventional well plate system, in which slices retain their *in vivo* metabolic rate at least for 8 h for intestine and 24 h for liver. The results were similar in both systems for the model substrates tested, which shows that the newly developed system is well-suited for metabolism studies. It was not the goal of the present study to test the maximum lifespan of the intestinal slices in the biochip. Rather, the aim was to show its applicability to metabolism studies for single intestinal and liver slice incubations, and for intestinal slices and liver slices in a multi-organ incubation set-up. Experiments to assess the lifespan of intestinal slices in the biochip are currently ongoing.

The main advantage of applying microfluidics to these studies is the excellent control of medium flow achievable and the possibility to easily control and alter the medium composition during an experiment. Due to this excellent control of liquid flow, it is possible to direct metabolites formed by tissue slices to a second compartment for further incubation, or for on-line metabolite detection. The present data show that the microfluidic approach used also enables microchambers to be linked to one another for sequential perifusion of tissue slices from the same or different organs while maintaining a high metabolic activity. The applicability of the system to the study of interorgan interactions was demonstrated by revealing the interplay between the intestine and liver in the regulation of bile acid synthesis. Upon exposure to the bile acid, CDCA, the intestine expressed FGF15, which caused a larger down-regulation of the expression of CYP7A1 in the liver than observed when

liver alone was exposed to CDCA. It is thus possible to study the effect of metabolites and/or (waste) products from one tissue slice on another. The coupling of two tissue slices has been shown now for small intestinal and liver slices. However, lung, kidney and colon slices could also be integrated into the system in the future, as precise slicing techniques have been developed for these organs as well.[7] Therefore, this system makes it possible to investigate and gain insight into interorgan interactions *in vitro*. Another advantage of the continuous flow compared to the static well plate system is that the incubation environment is very stable over time. It is also possible to perform in- and on-line analysis in continuous flow systems, to study time-dependent drug-drug interactions and the concentration dependence of metabolism in one single slice, and detect chemically unstable metabolites (Chapter 4).

The advantage of the presented system compared to systems incorporating cell lines[13] is the use of freshly isolated tissue, which retains the physiological expression of metabolizing enzymes. However, it should be noted that the apical and basolateral sides of the intestinal tissue were not perfused separately in our system, which means that the apically excreted metabolites and other excreted compounds are also supplied to the liver.

By using small precision-cut tissue slices, this system will contribute to the minimization of animal testing. Multiple experiments can be performed with only one rat, which is more efficient compared to the system used by Chen and Pang, for example, where two rats were used per experiment.[15] Moreover, the ability to work with limited amounts of tissue enables the study of human-specific metabolism, when scarce human tissue is available.

As shown previously, incubation of tissue slices in well plates is a suitable *in vitro* system to measure the metabolism and toxicity of drugs with a good correlation to the *in vivo* situation.[16, 24] In this study, the results for the tested substrates in the metabolism studies performed in the biochip agreed consistently with those obtained in the validated well plate system. This is crucial for the validation of the biochip, assuring the reliability of future results obtained with this system for new substrates whose metabolism remains as yet uncharacterized *in vitro* or *in vivo*. Thus, we believe that this new biochip will serve as an excellent complementary system to the well plate system for three reasons: 1) questions related to the effect of flow on the viability and functionality of the tissue slices can be addressed, 2) the effect of intestinal response to drugs on liver metabolism and function can be investigated and 3) on-line analysis is facilitated, which also allows a better detection of chemically unstable, reactive metabolites. With this new tool, it should be possible to gain a better insight into interorgan interactions and elucidate unknown mechanisms involved in toxicity, gene regulation and drug-drug interactions. Accordingly, this microflow system will help minimize the gap between the *in vitro* and *in vivo* situation, and will moreover contribute to the reduction of the use of experimental animals.

Acknowledgement

The authors would like to thank pathologist Patricia Robbe for her valuable evaluation and scoring of the tissue sections.

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8

Biocompatibility and Adsorption Properties of Plastics to Fabricate Microfluidic Devices for Cell Experiments

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Submitted

Abstract

The elastomer polydimethylsiloxane (PDMS) is often used as polymer to fabricate microfluidic devices for cell culture experiments. The ease to fabricate and the low cost are ideal for rapid prototyping. However, PDMS has the disadvantage that the surface is hydrophobic and small compounds can penetrate into PDMS. Therefore PDMS can adsorb hydrophobic compounds and absorb small hydrophobic compounds, whereas surface treatment to reduce the hydrophobicity is not successful. Instead of PDMS, thermoplastic polymers (plastics) can be used to produce microfluidic devices in high quantities in the industry at low costs. They allow easy surface treatment, and most of them are transparent and biocompatible. This study focuses on the possibilities to fabricate biocompatible microfluidic devices with low absorptive characteristics which can be used to culture cells or tissue. The surface of the plastics polymethylmethacrylate (PMMA), polystyrene (PS), polycarbonate (PC), and cyclic olefin copolymer (COC, Zeonor®) was treated with oxygen plasma or ultraviolet light (UV) and ozone to oxidize the surface, thereby making it more hydrophilic. By contact angle measurements it was shown that 15-30 minutes of treatment with UV ozone or 30-60 seconds with oxygen plasma resulted in a surface with hydrophilic character. The hydrophilic surface was more stable after UV ozone (more than one week for all plastics tested) than after oxygen plasma treatment. The effect of surface treatment on adsorption of compounds was tested by flushing 7-ethoxycoumarin and testosterone with its metabolites through microfluidic devices, made of these 4 plastics with a hot embosser. After treatment with UV ozone, no adsorption of the hydrophobic substrates or their metabolites occurred on PC, PS and COC. However, a substantial amount of the hydrophobic compounds adhered to PMMA, as well as to PDMS. The effect of UV ozone treatment on the biocompatibility of the plastics was assessed by culturing human hepatoma HepG2 cells on the UV ozone treated surfaces. PDMS, PC, PS and COC were biocompatible after treatment, however, culturing on PMMA resulted in loss of viability. In conclusion, the plastics PC, PS and COC can be used to develop biocompatible microfluidic devices with a low adsorption profile after UV ozone treatment for cell culture experiments. However, when liquid chromatography is to be incorporated on the device, PS is less suitable due to the low solvent resistance compared to PC and COC. Production of UV ozone treated microfluidic devices from PC or COC will result in biocompatible low-cost devices with good surface characteristics which can easily be produced in large batches in the industry.

Introduction

Microfluidic technology has been utilized to develop advanced cell culture models which are useful *in vitro* tools to better understand human biology and disease.[1, 2] Various *in vitro* models have been developed incorporating endothelial, liver, lung, fat, bone tissue (cells) and other types of cells or tissue.[3] By applying microfluidic technology for cell culturing, the complex architecture of organs can be mimicked,[4] and medium flow can be applied and altered during an experiment mimicking the natural cell environment.[1, 2] Mostly, those new *in vitro* chips have been developed in polydimethylsiloxane (PDMS). PDMS has the advantages of biocompatibility, low cost, optical transparency, ease of fabrication, and gas (oxygen and carbon dioxide) permeability.[5] For these reasons PDMS is ideal as polymer to develop prototypes for the incorporation of cells or tissue on microfluidic chips. Recently, we developed a microfluidic-based PDMS device for the incubation of precision-cut liver slices.[6] The tissue slices were viable and metabolically active in the microdevice, however, adsorption of the hydrophobic substrates 7-ethoxycoumarin[6] and testosterone and its metabolites (Chapter 6) was observed, which prevented quantitative assessment of drug metabolizing capacity.

PDMS has a hydrophobic character, which is a disadvantage when studying drug metabolism and toxicity, since hydrophobic substrates and metabolites can interact with the material. Due to the high surface-to-volume ratio in microfluidic devices, the interaction between compounds present in the microchannels and the channel wall material will be more pronounced compared to lower surface-to-volume ratios (conventional systems). Therefore adsorption of hydrophobic compounds from the liquid to the PDMS, is relatively high.[7, 8] This effect is even used in the field of solid phase extraction in so called stir-bar sorptive extraction (SBSE).[9, 10] Stir-bar rods coated with PDMS are used to pre-concentrate and extract organic compounds from aqueous solutions, like sea water for example. PDMS has a porous structure which will also result in absorption of small hydrophobic compounds into PDMS.[8] Coatings with a hydrophilic compound can be applied to overcome these problems,[5] however, stability of coatings is problematic and coatings might influence cell responses.[11]

Various materials can be used to produce microfluidic chips like glass, silicon, plastics, and flexible polymers. However, glass and silicon are rather expensive compared to the other materials, and flexible polymers like PDMS have the disadvantage of adsorbing/absorbing hydrophobic compounds as mentioned above. Therefore plastics might be a good alternative to PDMS for the development of microfluidic chips. In conventional cell culture experiments, plastics like polycarbonate and polystyrene are also used as material to produce culture flasks and well plates. These plastics are hydrophobic in their native form, however, it is easy to reduce their hydrophobicity by oxidizing the surface.[12] In conventional systems, the flasks are so-called tissue culture treated, which means that the surfaces are oxidized.[13]

In general, in every laboratory that fabricates chips an UV ozone or oxygen plasma apparatus is usually available, which can be used to oxidize substrates resulting in hydrophilic surfaces. However, after both UV ozone or oxygen plasma treatment, the substrate will recover from its oxidative situation and recover its hydrophobic state again, so called hydrophobic recovery.[14] This hydrophobic recovery is known for PDMS,[15] however, less information about the hydrophobic recovery of plastics is available in the literature.

By making the surface hydrophilic, hydrophobic compounds will not adsorb onto the material, which makes them suitable for metabolism and toxicity studies with hydrophobic compounds. However, extremely hydrophilic surfaces are charged, which may adsorb charged compounds. Therefore a balance is needed in surface hydrophobicity. Another issue of concern is that surface treatment by UV ozone might be toxic to cells or tissue, due to products, like peroxides, formed on the surface during or after treatment.

The aim of this study is to test several plastics for their applicability as alternative material to PDMS for the incubation and cultivation of cells and tissue without significant adsorption of hydrophobic compounds present in the medium or formed by the cells (e.g. metabolites). A proper plastic should: 1) be suitable to produce multiple chips in a high-throughput manner, 2) not adsorb hydrophobic compounds, and 3) not be toxic to cells after surface treatment to reduce hydrophobicity.

In this study, polycarbonate (PC), polystyrene (PS), polymethylmethacrylate (PMMA), and cyclic olefin copolymer (COC, Zeonor®) are tested. All polymers are thermoplastics and therefore can be used in injection molding or hot embosser to produce chips in bulk in the industry.[16, 17] The plastic surfaces are altered using UV ozone and oxygen plasma treatment, and the hydrophilicity was assessed by measuring the contact angle of water on the surface (angle of liquid-to-solid surface).[18] Hydrophobic recoveries were also measured one and four weeks after treatment. The adsorption of 7-ethoxycoumarin (7-EC) and testosterone (TT) and their liver specific metabolites were measured after treatment. 7-EC and TT are often used in cell culture experiments as model drugs to assess phase I metabolism, and testosterone and its metabolite androstenedione are relatively hydrophobic compounds which adsorb onto PDMS. Finally, as UV ozone appeared somewhat superior to plasma oxygen treatment, the toxicity of the plastics after UV ozone treatment was assessed by the cultivation of liver carcinoma cell line (HepG2) on the substrates for 24 hours. The toxicity was measured by incubating cells after 24 hours with propidium iodide and acridine orange, which stains the dead cells red and the viable cells green, respectively.

In this report it is described how plastic surfaces can be treated to prevent adsorption of hydrophobic compounds, and which plastic is suitable as replacement for PDMS as material to fabricate microfluidic chips for cell or tissue culture.

Materials and Methods

Chemicals and materials

Polycarbonate and polymethylmethacrylate, both 2 mm thick, were obtained from ERIKS Kunststoffen (Leek, The Netherlands). Polystyrene (2 mm thick) was purchased from Kunststofshop (Zevenaar, The Netherlands), and cyclic olefin copolymer (1-2 mm thick, Zeonor®) was obtained from microfluidic chipshop (Jena, Germany). All polymers were used as received and sawn to desired dimensions in-house. 7-Ethoxycoumarin, testosterone, propidium iodide, acridine orange and penicillin/streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum was obtained from Gibco (Paisley, UK). Polydimethylsiloxane was obtained as Sylgard 184 pre-polymer and curing agent from Dow Corning (Mavom BV, The Netherlands).

Surface treatment

The hydrophobicity of native plastic surfaces was altered by oxidizing the surface using UV ozone or oxygen plasma. The UV ozone exposure of substrates was performed using an ultraviolet ozone cleaning system (UVOCS, Lansdale, PA, USA). The apparatus contained a low-pressure mercury UV-light, generating UV-emissions at 185 and 254 nm wavelengths. Samples of PMMA, PC, PS, COC, and PDMS with dimensions of 15 mm x 30 mm (L x W) were exposed to UV ozone for 15, 30, 45, and 60 minutes. In addition, samples of PMMA, PC, PS, COC, and PDMS were treated with oxygen plasma using a PDC-002 Harrick Scientific's Plasma Cleaner (Ithaca, NY, USA) for 30, 60, 90 and 120 seconds. The effect of exposure on hydrophobicity was assessed by measuring the contact angle of water with the surface.

Contact angle measurements

When surface is hydrophilic, a water droplet would occupy as large surface area of the material as possible, resulting in a low contact angle. On a hydrophobic surface a water droplet appears, makes little contact with the surface and has a high contact angle. Contact angles were measured using an OCA 20 video-based contact angle meter from Dataphysics (Filderstadt, Germany). Per experiment, three pieces of the same substrate were used on which three times the contact angle was measured (nine measurements per plastic per treatment method). Native substrates were measured and substrates after UV ozone and oxygen plasma treatment. In both cases contact angles of substrates were also measured one and four weeks after treatment. Nine samples per plastic substrate were oxidized, three pieces were used directly after treatment and the rest was aged in air. These substrates were used to measure the hydrophobic recovery of the plastics. It is known that PDMS shows hydrophobic recovery.[19] However, it is unknown to what extend the plastics exhibit this hydrophobic recovery effect. This effect has been studied for a few plastics after oxygen

plasma treatment,[20, 21] however, to the best of our knowledge the hydrophobic recovery has not been studied for the plastics tested in this study after UV ozone treatment.

PDMS and plastic substrates were only used once for contact angle measurements and disposed after the analysis.

Adsorption of hydrophobic compounds

The adsorption of compounds onto the plastic substrates was measured by flushing medium containing substrate and metabolites through the chip. For this purpose chips were produced using a hot embosser (Dr. Collin GmbH, Ebersberg, Germany). A CNC-milled brass mold with the structure as depicted in Figure 1 was produced in-house (accuracy of < 1 µm). The structure was 200 µm thick, and had a length of 10 mm in total. The channels were 500 µm wide and the chamber had a radius of 2 mm. The total volume of the device was approximately 3.5 µL. The chips were cleaned with isopropanol and ultrapure water before the hot-embossing procedure. The structures were imprinted in the plastics using the parameters given in Table 1. The program started at an initial temperature of 25°C and raised to the hot embossing temperature with a ramp of 5°C/min for all plastics. Subsequently, pressure (see Table 1) is applied for 10 minutes. Thereafter, the temperature is cooled down to 60°C with a ramp of 12°C/min, and de-embossing occurred at 60°C. Openings of Ø1.6 mm at the in- and outlet were drilled for the insertion of tubing. Thereafter each channel was irreversibly sealed using a cover chip of the same material by thermal bonding using the hot embosser. The temperature and pressure used to bond two pieces of plastics are also given in Table 1. Here the temperature was also elevated with 5°C/min to the bonding temperature, and the pressure was applied for 10 minutes. Afterwards, the substrates were cooled down to 25°C with a ramp of 12°C/min.

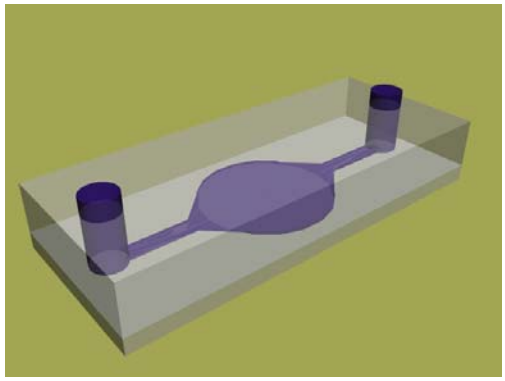

Figure 1. Schematic view of the chip used for adsorption and cell culture studies. The chamber dimension is Ø4 mm x 200 µm (total volume ~3.5 µL) and the flow rate of medium is 5 µL/min.

Table 1. Hot embossing parameters for structure patterning and thermal bonding of plastic substrates.

Plastic	*Glass transition temperature*	*Hot-embossing*		*Bonding*	
		Temperature	Pressure	Temperature	Pressure
PC	145°C[22]	150°C	30 bar	135°C	15 bar
PS	103°C[22]	110°C	20 bar	90°C	15 bar
PMMA	117°C[22]	130°C	10 bar	90°C	10 bar
COC	105°C[23]	110°C	10 bar	95°C	10 bar

Only the production of PDMS devices was different compared to plastic devices. Devices were produced by mixing prepolymer and curing agent in a 10:1 mass ratio, and pouring it over the mold with Ø1.5 mm stainless steel rods placed at the in- and outlet positions for the insertion of tubing during perfusion. The PDMS was left at room temperature for 30 minutes to remove air bubbles which were trapped in PDMS. Thereafter, the temperature was increased to 45°C using a hot plate to cure the PDMS overnight. After curing, the chip was removed from the mold and was bonded to another flat piece of PDMS, which was pretreated in the UV ozone for 60 minutes. An irreversible bond was obtained by placing both substrates in the UVOCS for 15 minutes, and after treatment brought them immediately into contact and left to covalently react with each other, as described before.[6]

Peek tubing (Da Vinci BV, Rotterdam, The Netherlands) were inserted in the chips and connected to syringes filled with substrate and metabolites. 7-Ethoxycoumarin (7-EC) and testosterone (TT) with its metabolites were used as compounds. These solutions were obtained by incubating precision-cut liver slices in well plates with 500 µM 7-EC or 250 µM TT in William's medium E for three hours, as described before.[6] After three hours of incubation, the medium was collected and centrifuged to remove cell debris. Syringes were filled with this medium pre-equilibrated with carbogen gas (95% O_2 / 5% CO_2) at 37°C which contained the substrate with liver specific metabolites. The syringes and the chips were placed in an incubator set at 37°C, and the chips were flushed for two hours with the medium at a flow rate of 5 µL/min. The adsorption experiments were performed in triplicate on three different chips made from the same material. Fractions collected at the outlet were stored at -20°C until analysis. The 7-EC was analyzed using an HPLC with UV-detector as described before.[6] TT with its metabolites were first preconcentrated using liquid-liquid extraction with dichloroform, and the extracted fraction was analyzed using an HPLC with UV detection according to Van 't Klooster *et al.*[24] Samples were also injected onto the HPLC systems before flushing through the devices to measure the concentration in the inflow medium (100% values).

Biocompatibility study

The biocompatibility of the plastics and elastomer was tested by culturing HepG2 cells on the substrates. Cells were cultured in the structure shown in Figure 1, and on flat substrates. The substrates were coated with lyophilized rat-tail collagen (Roche, Basel, Switzerland). As control, the cells were also cultivated in tissue culture-treated polystyrene well plates (Corning Costar, Amsterdam, The Netherlands). Cell suspensions containing approximately 120,000 cells were seeded onto the substrates and in the wells. The cells were cultivated for 24 hours in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. After 24 hours, the medium was removed and cells were stained with propidium iodide (2 µg/mL) and acridine orange (1 µg/mL) for 4 minutes. Propidium iodide is a membrane impermeable compound which can only enter and stain dead cells by binding to cell-interior compounds, resulting in a red color. Acridine orange can diffuse freely across membranes and stains viable cells green upon exposure to the proper wavelength. After staining, the solution was removed and pictures were taken immediately using a Leica Orthoplan fluorescence microscope (Leica, Wetzlar, Germany) equipped with two filter sets (Ex 465-495, Em 512-558 and Ex 560-590, Em >630). The HepG2 cell experiment was performed in triplicate on three different chips made from the same material.

Results and Discussion

Surface treatment

The treatment of plastic substrates by oxidizing the surface results in a more hydrophilic surface. Due to the treatment, oxygen-containing functional groups are formed onto the surface layer, which results in higher surface free energy and a lower hydrophobicity.[25] This will prevent the adsorption of hydrophobic compounds. However, when the exposure time of ozone is too long, many oxygen containing groups are formed on the surface resulting in a charged surface. This extremely hydrophilic surface will not adsorb hydrophobic compounds, however, electrostatic interaction might occur with the compounds present in the medium. Therefore there is a turn-over point in ozone exposure. The surface should not be exposed too long to ozone, resulting in charged surface, however, too short results in hydrophobic interactions. The surface energy can be measured using contact angle measurements, which give a good indication of the hydrophobicity of a substrate.[18] The preferable contact angle is 40-50°. Substrates with this contact angle will hardly adsorb hydrophobic compounds and will not have electrostatic interactions. The surface of well plates which are tissue-culture treated also has a contact angle of 40-50° (data not shown).

UV ozone

The treatment of the plastics with UV ozone is given in Figure 2. Contact angles were measured 2 hours after treatment for the first set of samples. All substrates show a decrease in contact angle after UV ozone irradiation, indicating increased hydrophilicity. All plastic substrates showed a tremendous decrease in contact angle, and very hydrophilic surfaces were obtained after 60 minutes of treatment. A contact

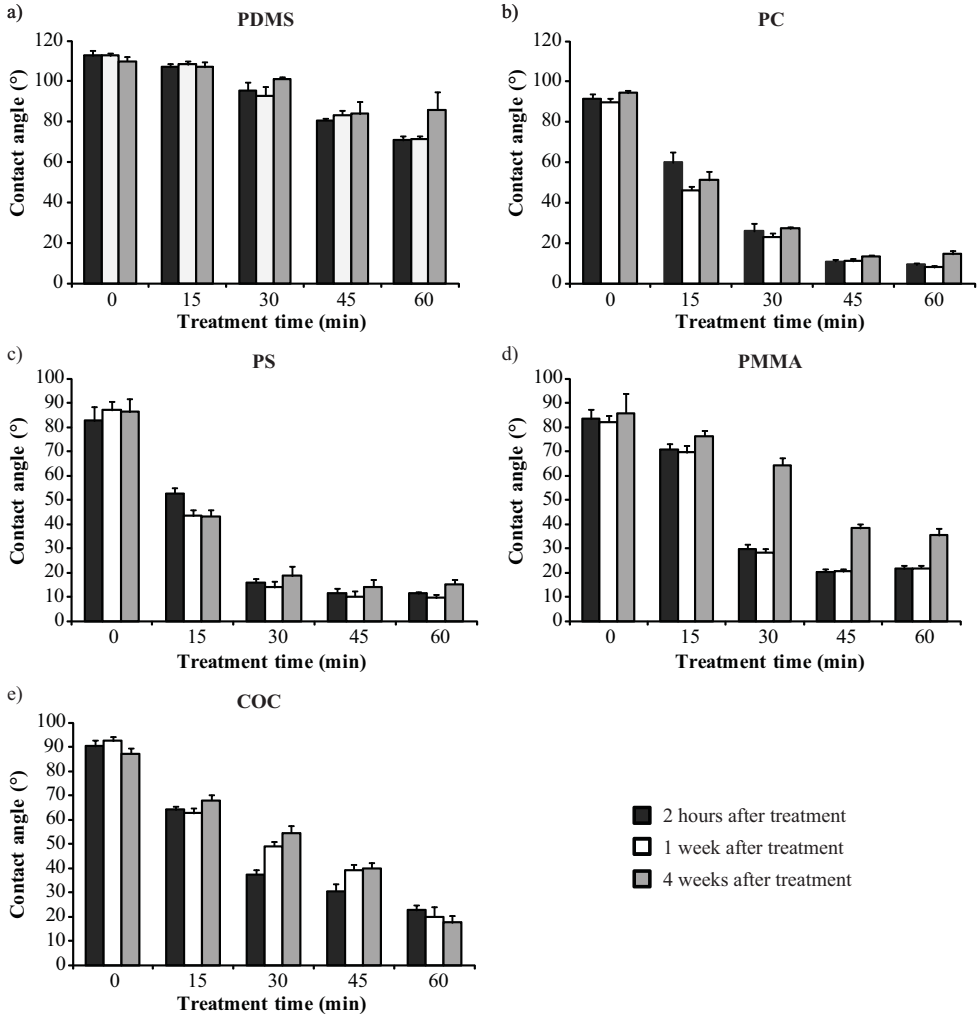

Figure 2. Contact angles of UV ozone-treated (a) PDMS, (b) PC, (c) PS, (d) PMMA, and (e) COC as a function of treatment time. The hydrophobic recovery was measured after one week (white bars) and four weeks (gray bars). Black bars represent the contact angle of the substrates two hours after treatment. Results are average ± standard deviation of 3 separate measurements on three pieces of substrate (nine measurements in total for each type of substrate).

angle of 10° for PC and PS means that many functional oxygen-containing groups are formed, resulting in charged surface which is not beneficial as mentioned above. However, the contact angle of PDMS decreased only from 110° to approximately 70° after 60 minutes of treatment. Probably the contact angle is low directly after treatment as mentioned by others,[14, 15] however, rapid hydrophobic recovery occurs as shown before.[15] It is believed that free siloxanes from the bulk migrate to the surface to recover the surface characteristics to its native form.[19]

PC and PS on the other hand had a contact angle of ~50° already after 15 minutes of treatment. PMMA and COC needed a treatment of 30 minutes to decrease the contact angle to ~50°, as observed before by others.[23] For PC, PS and COC hydrophobic recovery was hardly observed during the 4 weeks measured. However, a relatively high recovery occurred for PMMA 4 weeks after treatment. The surface treated for 30 minutes was almost fully recovered after 4 weeks. Therefore, this substrate can only be used one week after treatment. With the substrates PC and PS hydrophobic recovery was minimal and these substrates can be used for at least four weeks after treatment of 15 minutes with UVOCS. COC does show hydrophobic recovery after treatment for 30 minutes, although after four weeks the contact angle is still ~50°, and therefore COC can also be used for four weeks.

Contact angle measurements after oxygen plasma treatment

Oxygen plasma treatment is harsher compared to UV ozone. Oxygen plasma will produce far more ozone in a given space of time compared to UV ozone, since it contains particles with a high kinetic energy like photons, electrons, ions, radicals, and excited species.[19] Therefore, the exposure time can be much shorter compared to UV ozone treatment to achieve the same contact angles. The exposure time is in seconds compared to minutes with UV ozone. Figure 3 shows the contact angles of the different plastic substrates and PDMS after oxygen plasma treatment. The measurements were performed one hour after treatment for the first set of samples. The contact angles of all substrates decreased also here upon exposure to ozone. PDMS showed a very low contact angle after treatment (<20°). This effect was more pronounced compared to UV ozone, however, this might be due to the fact that contact angles were measured one hour after treatment instead of two hours compared to UV ozone, due to logistic limitations. However, within a week the contact angle of the substrate aged in air almost returned completely to its initial contact angle. This recovery can be reduced when placing the substrates in water after treatment.[26] The hydrophobic recovery was less apparent for the plastics tested. For all four plastic tested, the contact angle decreased to approximately 50° after 30-60 seconds of oxygen exposure. The hydrophobic recovery was comparable to substrates treated with UV ozone, except for polystyrene where the recovery was much higher compared to UV ozone treatment. One week after treatment the contact angle increased by 20°. For

PDMS it was also shown before that hydrophobic recovery was slower when the surface was treated with UV ozone instead of oxygen plasma.[15] According to Berdichevsky *et al.*[15] UV ozone enables much deeper oxidation compared to oxygen plasma. Therefore further studies were performed with UV ozone treatment, where the contact angles were stable for all substrates for at least one week.

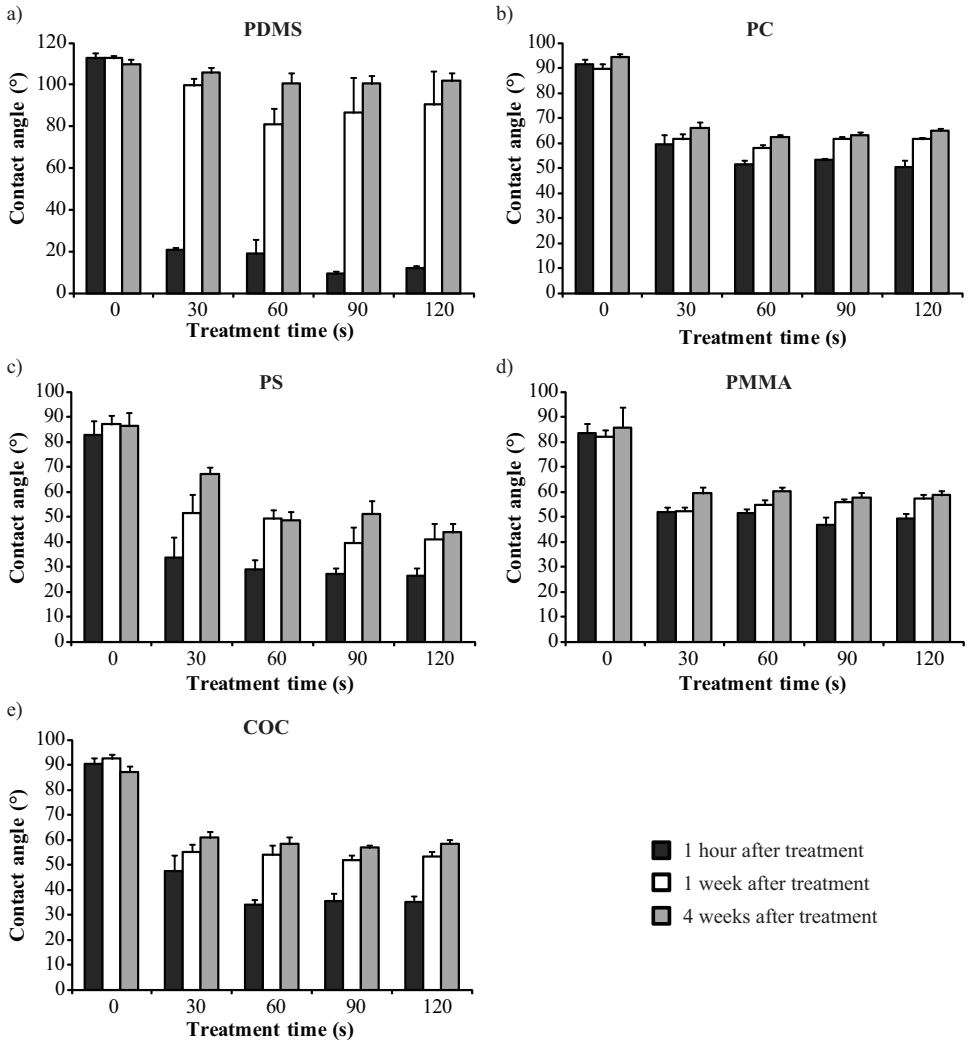

Figure 3. Contact angles of oxygen plasma-treated (a) PDMS, (b) PC, (c) PS, (d) PMMA, and (e) COC as a function of treatment time. The hydrophobic recovery was measured after one week (white bars) and four weeks (gray bars). Black bars represent the contact angle of the substrates one hour after treatment. Results are average ± standard deviation of 3 separate measurements on three pieces of substrate (nine measurements in total for each type of substrate).

Adsorption study

Microfluidic chips were developed using a hot embosser as described above. The structure, given in Figure 1, consisted of an inlet channel, outlet channel, and a chamber for the hepatocyte culture. The structure was patterned in plastic using a brass mold. Thereafter the pieces of plastics with structure and flat pieces to seal the device were placed in the UV ozone to oxidize the surfaces. PS and PC were treated for 15 minutes, and COC and PMMA were treated for 30 minutes. Thereafter the plastic pieces were again placed in the hot embosser for thermal bonding. Due to the UV ozone treatment, the glass transition temperature (T_g) of the plastics decreased at the surface layer, while the bulk polymer retained the T_g.[25, 27, 28] This resulted in thermal bonding at temperatures below the glass transition point (see Table 1) without deformation of the structure.

The chips were placed under a microscope and perfused with medium with a flow rate up to 10 µL/min, and no leakage was observed for at least 24 hours (data not shown). Thermally bonded chips were placed in a humidified incubator set at 37°C, and flushed with syringes filled with medium containing 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin glucuronide (7-HC-G) and 7-hydroxycoumarin sulfate (7-HC-S) at a flow rate of 5 µL/min. Medium was collected during two hours of perfusion and the concentration of 7-EC and its metabolites was measured in the 600 µL collected fraction. Recoveries of the compounds were calculated from the initial concentrations of 7-EC and its metabolites present in the

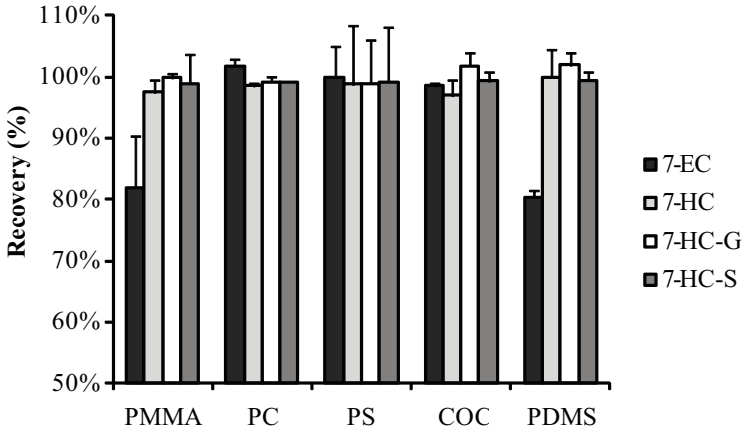

Figure 4. Recovery of 7-ethoxycoumarin (7-EC) and its metabolites 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin glucuronide (7-HC-G), and 7-hydroxycoumarin sulfate (7-EC-S) measured at the outlet as a percentage of the initial concentration after 2 hours flushing through the device at 5 µL/min. Substrates were pretreated with UV ozone (15 minutes for PS, PC and PDMS; 30 minutes for PMMA and COC). Results are average ± standard deviation of 3 separate devices for each substrate.

medium before perfusion. The recoveries of the metabolites and substrate during 2 hours of perfusion are given in Figure 4. For all plastics and PDMS no adsorption of the relatively hydrophilic 7-hydroxycoumarin and the charged metabolites 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate was observed. However, the recovery of 7-ethoxycoumarin was lower in PMMA and PDMS after perfusion. Only 80% of the initial value was found at the outlet of the device. This indicated adsorption of 7-ethoxycoumarin onto the wall of the PMMA and PDMS structures, as observed before for PDMS.[6]

The chips were also flushed with medium containing testosterone with its metabolites for two hours, similar as for 7-ethoxycoumarin. Recoveries were calculated from the 600 µL collected medium and the concentration of testosterone and it metabolites present in the medium before perfusion (Figure 5). Again the substrates PC, PS, and COC did not adsorb the compound and its metabolites. One hundred percent recovery was obtained for all compounds. In case of PMMA, the amount of androstenedione and the hydroxytestosterone metabolites at the outlet of the chip were determined at a concentration similar as the initial value, however, testosterone had a lower recovery. Only 85% of testosterone was found back at the outlet of the chip. Again adsorption of hydrophobic compounds at the channel wall of PMMA was found, as described for 7-ethoxycoumarin. PDMS showed also adsorption of testosterone and its metabolites. Only 6β-hydroxytestosterone, the most hydrophilic metabolite, had a recovery of 100%. 16α and 2α had a recovery of only

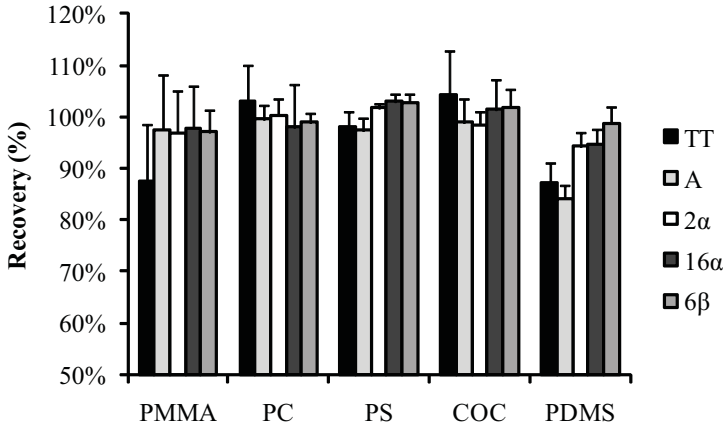

Figure 5. Recovery of testosterone (TT) and its metabolites androstenedione (A), 2α-hydroxytestoterone, 16α-hydroxytestosterone, and 6β-hydroxytestosterone measured at the outlet as a percentage of the initial concentration after 2 hours flushing through the device at 5 µL/min. Substrates were pretreated with UV ozone (15 minutes for PS, PC and PDMS; 30 minutes for PMMA and COC). Results are average ± standard deviation of 3 separate devices for each substrate.

95%, and around 15% of testosterone and androstenedione were adsorbed onto the PDMS, or maybe absorbed into PDMS.[8] These results indicate that only PC, PS, and COC from the tested substrates are resistant to adsorption of hydrophobic compounds after UV ozone treatment.

Biocompatibility study

Toxicity can be caused by the peroxides formed by UV ozone treatment, which remain present for several days.[29] Previously it was shown that PMMA[30], PS[31], PC[32], COC[33], and PDMS[34] are biocompatible for HepG2 cells. However, most of these studies were performed without surface treatment, and few experiments have been reported in which different substrates were compared with each other.

PC and PS substrates were treated for 15 minutes with UV ozone, and PMMA and COC for 30 minutes, similar as for the adsorption experiment. HepG2-cells were cultured on substrates without collagen coating, and adhered to the plastic. Many cells were adhered to COC, PC and PS, and also grow on the surface (data not shown). On PMMA less cells were present after 24 h, and ca 25% of the cells appeared non viable (results not shown). This may indicate that either the cells do not adhere or they do not proliferate on PMMA and/or they were released from the surface due to the loss of viability. On PDMS hardly any cells were attached, although the few cells that did adhere to the surface were all viable.

Normally, cell culture plates are coated with collagen to improve the initial adhesion of HepG2 cells on the substrates. Collagen is a natural extracellular matrix component with low immunogenicity, promoting cell adhesion and proliferation.[35] Figure 6 shows the HepG2 cells cultured on the different plastics, on PDMS and in well plates after collagen coating. As can be seen in the figures, many intact cells are attached to the surface. With collagen coating also many cells attached to the hydrophobic PDMS surface, and hardly any dead cells were visible. Again very similar results were obtained for cells cultured on PC, PS, COC and in well plates (control), with only a few dead cells (~2%). On the other hand, many cells adhered to PMMA after collagen coating, however, again many dead cells were visible. Around 11% of the cells were not viable (see Figure 6g). Therefore the treatment of PMMA with UV ozone does not result in a biocompatible surface to cultivate liver cells. The other plastics were all biocompatible after treatment.

Conclusion

Culturing cells in microfluidic devices is a powerful tool to be able to study cellular functions under conditions that mimic the *in vivo* situation. Many substrates can be used to fabricate microfluidic devices; however, not all of them are biocompatible. The main question of this study was to find out which plastics were suitable for the cultivation of cells or tissue in a microfluidic device with low toxicity and low

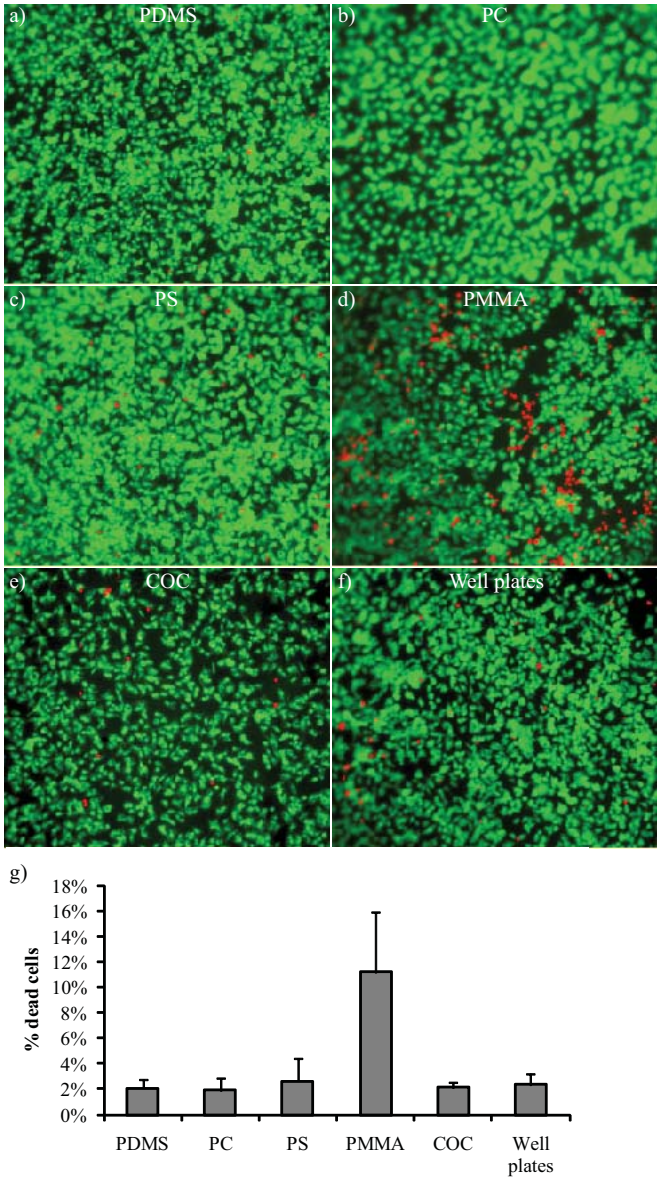

Figure 6. Viability of HepG2 cells cultured on polymers after UV ozone. Life cells are stained green (by acridine orange) and dead cells are red (by propidium iodide). The polymers tested are (a) PDMS, (b) PC, (c) PS, (d) PMMA, (e) COC, and (f) well plates (polystyrene). (g) The amount of dead cells as percentage of the total amount of cells present. Results are average ± standard deviation of 3 separate devices for each substrate measured for three different HepG2 passages (nine measurements in total for each type of substrate). PC and PS were treated with UV ozone for 15 minutes, and PMMA, COC and PDMS were treated for 30 minutes.

adsorption of substrates and metabolites. Here the use of polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), polystyrene (PS), polycarbonate (PC), and cyclic olefin copolymer (COC) was tested for suitability in cell and tissue applications. The native surface of these materials is relatively hydrophobic; however, surfaces can be easily altered through oxidation to become more hydrophilic. It was shown that both UV ozone and oxygen plasma can be used to increase the hydrophilicity of the materials tested. An additional advantage of oxidizing the plastic surfaces appeared the lowered glass transition temperature of the surface, which facilitated the bonding of the substrates at temperatures below the glass transition point of the bulk of the device, preventing deformation of the microfluidic structure.

UV ozone treatment is preferred over oxygen plasma treatment because the hydrophilic surface was more stable after treatment compared to oxygen plasma. After UV ozone treatment of the materials which creates a contact angle of ~50°, hydrophobic substrates were hardly adsorbed onto the materials. Solutions containing 7-ethoxycoumarin or testosterone and their metabolites were flushed through devices made of PS, PC and COC without measurable absorption, whereas PDMS and PMMA exhibited a lower recovery of the hydrophobic compounds.

After collagen coating, HepG2 cells adhered to the surface of all materials with high viability, except for PMMA. The UV ozone treatment of PDMS, PC, PS and COC did not result in toxicity, and showed a dense network of viable cells on the surface 24 hours after seeding. However, on surfaces made of UV ozone treated and untreated PMMA a considerable amount of dead cells were visible.

The chemical resistance of PC and COC is far better than PS.[28] When devices are flushed with organic solvents, which are used for on-line introduction of liquid chromatography on chip for example, the use of PC or COC is recommended. COC has the additional advantage compared to PC of exhibiting a lower autofluorescence, which is beneficial for optical imaging.[36] In conclusion, microfluidic devices made from PC after oxidizing the surface for 15 minutes, or COC after 30 minutes of oxidation, are suitable for the incorporation of cells or tissue and will allow the low-cost production of a biocompatible device with low-adsorption profile for both hydrophobic and hydrophilic compounds. Structures can be easily patterned by using a hot embosser or by injection molding.[37]

Acknowledgements

The authors would like to thank Hella Logtenberg and Dr. Wesley R. Browne (Stratingh Institute for Chemistry, University of Groningen) for the use of their apparatus to measure contact angles. We also would like to thank Marieke van Beuge and Catharina Reker-Smit for their help with the HepG2 cells.

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9

Summary, Discussion and Perspectives

It is of crucial importance to detect the kinetic, metabolic and toxicity (ADME-Tox) profiles of new drug candidates in an early stage of drug development. The costs and the use of animals can be significantly reduced when the *in vivo* behavior of new chemical entities is accurately predicted by *in vitro* methods earlier on in the development process. Therefore, the role of *in vitro* systems to predict these profiles is essential. Many *in vitro* systems have been developed the last decades,[1] however, a significant number of drugs is still withdrawn in a late stage of drug development.[2, 3] As mentioned in **Chapter 1**, the aim of this thesis was to develop a new *in vitro* system which can be used to gain more information about the ADME-Tox properties of new drug candidates in an early stage. The *in vitro* system which has been realized incorporates precision-cut tissue slices in a microfluidic-based biochip. The use of microfluidic technology for the development of *in vitro* systems has many advantages compared to conventional incubation systems.[4] The main advantage is the ability to control the cell's microenvironment by introducing a continuous flow which enables the establishment of steady-state conditions.

Various microfluidic systems have been developed with liver cells incorporated on chip, with the aim to measure the biotransformation of drugs in the human liver. **Chapter 2** reviews the currently available microfluidic-based *in vitro* models for liver metabolism and toxicity profiling and discusses their respective advantages and disadvantages. From this literature review, it became clear that only two of these models, primary hepatocytes and precision-cut liver slices (PCLS), can adequately represent liver metabolism *in vivo*. As PCLS retain an intact architecture with all cell types present in their native configuration and intact cell-cell contacts, they seem eminently suitable for toxicity studies.

The first microfluidic-based system incorporating rat PCLS is presented in **Chapter 3**. A polydimethylsiloxane device is described, allowing continuous supply of medium by perfusion to rat liver slices for 24-h incubation. Care was taken that the pH, oxygen concentration, and temperature remain constant during the incubation. For this reason aeration windows were integrated into the chip to act as "breathing" membranes. As it was crucial to first verify the performance of the newly developed system by comparison with validated conventional systems, we compared the viability and metabolic function of PCLS in the biochip with PCLS incubated in a conventional well-plate system. The viability and metabolic activity for the tested substrate were not significantly different in both systems, indicating that PCLS remain functionally intact in the biochip for at least 24 h.

An advantage of the continuous flow is that the outflow can be directly and continuously analyzed for metabolite formation. The development of an on-line analysis system by coupling the biochip with an HPLC with ultraviolet detection is demonstrated in **Chapter 4**. The design of the device results in a low medium-volume-to-slice-volume ratio, thereby resulting in high concentrations of metabolites

in the outflow. This automated system makes it possible to repeatedly expose a slice to substrates. For example, PCLS were incubated four times with 7-hydroxycoumarin for 2-h periods, with intervals of 4 hours between incubations. An immediate production of metabolites was observed, and the maintenance of the viability and metabolic activity of the slice was demonstrated. It will be interesting to use this system in the future to try to measure metabolic circadian rhythms. In this chapter, the on-line system was also used to study inhibitory drug-drug interaction. The concentration of inhibitor in this case was increased in a stepwise fashion. Only three slices were used in one experiment, allowing the determination of the dissociation constant for inhibitor binding using a minimal amount of tissue. In the future, the time-dependent induction of metabolism may be studied in this device. The system is also ideal for studies on scarce tissue, like human material. Another great potential of this system is the ability to detect unstable metabolites. This was demonstrated by the measurement of the formation of diclofenac acyl glucuronide, an unstable metabolite formed by the liver from the drug, diclofenac. Acyl glucuronide metabolites are considered to be reactive metabolites and thus cause toxicity. These reactive metabolites are difficult to detect with conventional systems due to their chemical instability. It was shown that the on-line analysis system is able to measure these metabolites directly after their formation at a relatively high concentration, which may be useful for the prediction of the formation of other reactive metabolites as well. In future, it would be interesting to couple this on-line analysis system directly to a mass spectrometer, allowing the simultaneous on-line analysis and identification of metabolites.

In the first chapters, the maximum slice incubation time was 24 hours. In **Chapter 5** this incubation time was extended to 72 hours for rat PCLS by embedding slices in a hydrogel matrix. When slices were suspended in solution in the biochip, the viability was well maintained for 72 hours; however, the metabolic capacity of the slices both for phase I and phase II metabolism decreased over time. It was found that PCLS had attached themselves to the upper filter of the microchamber after prolonged incubations (>24 hours). The embedding of PCLS in a hydrogel significantly improved the maintenance of metabolism. Matrigel was used as hydrogel, which is a basement membrane matrix. The embedding prevented the attachment of the PCLS onto the polycarbonate filter at the outflow of the chamber. In addition, by supplementing the WME medium with bovine serum, dexamethasone and insulin, the capacity for phase II metabolism was fully maintained at the level of fresh slices for 72 hours. However, phase I metabolism of 7-ethoxycoumarin still decreased over time in a manner analogous to that in well plates, as was also found by others.[5-7] This is probably due to an unoptimized medium composition and the lack of endogenous stimuli to activate transcription factors involved in the expression of the phase I metabolizing enzymes, rather than damage incurred in the incubation system. For

further research, the addition of a low concentration of cytochrome P450 inducers might result in a more stable phase I metabolism.[8]

It is now generally accepted that for proper prediction of drug metabolism in man, human *in vitro* preparations are preferred, because of the well-known species differences in drug metabolism.[9] PCLS can be prepared from surgical liver waste tissue obtained after liver transplantation, or after partial hepatectomy (resection of liver parts to remove tumors).[10] In addition, it is also possible to prepare slices from diseased tissue, thereby enabling the study of pathophysiological processes. In **Chapter 6** the successful incorporation of healthy human liver slices in the microchip was demonstrated. The viability was determined by morphological evaluation and enzyme leakage tests. The metabolic activity was assessed by incubating the slices with four different substrates, testosterone, lidocaine, 7-ethoxycoumarin and 7-hydroxycoumarin, covering a wide range of phase I and phase II metabolism pathways. The results showed that human PCLS function similarly to those incubated in the conventional well plate system, which also for human PCLS correlates well with the *in vivo* situation.[11] The flow introduced in the microfluidic device did not resulted in different metabolic activities or lower viability. We conclude, therefore, that human liver slices can also be incubated in the biochip for metabolism and toxicity studies.

The incorporation of precision-cut rat intestinal slices was also established, as shown in **Chapter 7**. This is the first example of intestinal slices incubated in a microfluidic device. The slices were incubated with three different substrates, and high metabolic activity was observed for 3 hours of incubation, comparable to the activity in well plates. In contrast to liver slices, which remain viable for 72 h, intestinal slices partially lose their metabolic function and ATP content after 24 h of incubation in well plates. Therefore, in future studies, it would be interesting to test if intestinal slices retain their viability during longer perifusion in the biochip. However, it should be noted that the apical and basolateral sides of the intestinal tissue were not perfused separately in our system. It would also be interesting to develop a device which does separate the two sides of the intestine, to be able to perform absorption studies. Conventional system exists in which the two sides can be separated, so-called Ussing chamber.[12] It would be interesting to develop an Ussing chamber on chip to investigate the transport and metabolism of drugs in very specific regions of the intestine.

Due to the continuous flow, the outflow of a chamber containing the metabolites and products formed by a slice can be directed to the inlet of another chamber which also contains a slice. It thus becomes possible to measure interorgan effects, something which is not possible in well plates. Two slices from different organs can be placed in one well; however, mutual interaction can take place, and it is impossible to conclude which organ was responsible for an observed effect. To verify whether

such a sequential perifusion of slices would affect the functioning of the slices, two slices from the same organ were first sequentially perifused with model substrates. No significant differences in the specific metabolic rates resulted when one or two slices were perifused. Thereafter, sequential perifusion of an intestinal slice and a liver slice with model substrates showed that liver slices functioned normally. Apparently, the excreted products from the intestine did not affect the liver metabolism, indicating no interorgan effects for these substrates. Interestingly, interplay between intestine and liver could be demonstrated for the regulation of bile acid synthesis by primary bile acids. After exposure to the bile acid chenodeoxycholic acid, a significant down regulation of CYP7A1 in the liver slice occurred. This could be ascribed to the bile acid-induced release of fibroblast growth factor 15 (FGF15) by the intestine, which was directed by the medium flow towards the liver slice. The enzyme, CYP7A1, is responsible for the synthesis of bile acids in the liver, and is known to be regulated by FGF15.[13] This bile acid regulation pathway showed the potential of the biochip system to investigate interorgan effects.

Until now, only liver and intestinal slices have been incorporated in the biochip. As it is also possible to produce precision-cut slices from other organs such as lung and kidney,[11] this biochip technology offers the potential to study the effect of liver metabolites on these tissues as well, or vice versa. The influence of metabolites excreted into the bile of the liver on the intestine can also be studied with the biochip. By integrating multiple organ slices on chip, the *in vivo* situation is more closely approached than when cell monocultures are employed.

All the experiments were carried out with a biochip made from polydimethylsiloxane (PDMS). This is an ideal elastomer for rapid prototyping during the development of the biochip; however, the adsorption/absorption of hydrophobic compounds is an important disadvantage.[14] This problem was observed with the incubation of PCLS with testosterone in **Chapter 6**. In **Chapter 8**, we set out to investigate whether other materials, such as plastics, could replace PDMS as fabrication material. In this study, the thermoplastic polymers, polystyrene (PS), polycarbonate (PC), polymethylmethacrylate (PMMA), and cyclic olefin copolymer (COC), were tested. These polymers were selected because of their reported biocompatibility and because chips can be fabricated using a hot embosser, which is superior to PDMS casting for producing large numbers of biochips in a shorter period of time. In their native form, these plastic substrates are also hydrophobic. However, the surface could be modified by oxidation using UV ozone or oxygen plasma treatment, resulting in more hydrophilic surfaces which remain stable for up to 4 weeks. PC, PS and COC chips showed no significant adsorption of testosterone and 7-ethoxycoumarin and their metabolites. As a model to test the biocompatibility of these plastics after treatment, a liver cell line (HepG2) was cultured on the UV ozone treated plastics. The cells were able to grow on all treated substrate surfaces after

coating with collagen. Hardly any dead cells were found (~2%) on PC, PS, and COC. This indicates that the plastics are not toxic after treatment. In contrast, 25% of the cells were non-viable on PMMA, indicating a potential effect of the plastic or the UV ozone treatment on cell viability. We concluded that PC, PS and COC are all suitable to fabricate biochips with low adsorptive characteristics and high biocompatibility. Since PS is less resistant to the organic solvents used, for example, for on-line introduction of liquid chromatography on chip, the use of PC or COC is recommended. Of these two, COC has the additional advantage of exhibiting a lower autofluorescence compared to PC,[15] which is beneficial for optical imaging.

A biochip made from COC with aeration windows to control the pH and oxygen concentration in medium would be the best option for further studies. This low-cost device could be produced in batch, would not adsorb hydrophobic compounds if surface-oxidized, and could be used in the on-line analysis system. Multiple chambers containing different slices could be sequentially perfused in a manner similar to the PDMS device. Another interesting aspect for further studies would be the integration of components for the generation of concentration gradients[16] and other components such as a pump[17] for medium flow or detectors for on-chip monitoring of pH[18] or metabolite formation[19].

The successful development of a microfluidic-based *in vitro* system with perifusion of liver and intestinal slices to study drug metabolism has been realized. A stable incubation environment, e.g. pH, oxygen concentration and nutrients, was achieved by introducing a low flow rate. Liver slices could be maintained viable for 72 hours and intestinal slices for at least 3 hours. Although it was previously suggested that the introduction of medium flow in cell or tissue culture would promote longer viability,[20, 21] this has not yet been confirmed in our system. Possible causes for this and their resolution remain to be elucidated. A limitation of the system is the possible adsorption/absorption of hydrophobic compounds onto the PDMS material; however, by producing the chip in COC, this can be prevented. This newly developed *in vitro* system is very promising for gaining better insight into possible interorgan effects. Moreover, earlier detection of unstable metabolites is made feasible with the on-line HPLC system. As an alternative to well plates, this system will contribute to a better prediction of metabolism and toxicity in man with a concomitant reduction in the use of experimental animals. The microfluidic biochip thus has the potential to significantly impact drug metabolism and toxicology studies of novel chemical entities.

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Appendix

Samenvatting

Het ontwikkelen van een nieuw geneesmiddel kost gemiddeld 800 miljoen dollar en kan meer dan 10 jaar duren. Tijdens regulier geneesmiddelonderzoek wordt het geneesmiddel eerst uitvoerig getest tijdens de zogenoemde preklinische fase voordat het toegediend wordt aan gezonde vrijwilligers. Tijdens deze preklinische fase wordt naast de werking onder andere de omzetting (metabolisme) van het geneesmiddel tot afbraakproducten (metabolieten) en de mogelijke toxiciteit getest. Tijdens deze preklinische fase wordt veel gebruik gemaakt van *in vitro* systemen, wat inhoudt dat het metabolisme en toxiciteit van een geneesmiddel wordt getest op gekweekte (lichaams)cellen en/of weefsel. Daarnaast wordt het geneesmiddel getest op proefdieren, *in vivo*. Het verschil tussen *in vivo* en *in vitro* studies is dat tijdens *in vivo* studies het complete organisme wordt gebruikt, terwijl tijdens *in vitro* studies cellen of weefsel van een of enkele organen worden gebruikt. Daarbij is het natuurlijk essentieel dat de *in vitro* systemen de omzettingen van geneesmiddelen *in vivo* correct voorspellen. Vele *in vitro* systemen zijn de laatste decennia ontwikkeld,[1] maar helaas falen veel potentiële medicijnen alsnog in een laat stadium van de ontwikkeling door onacceptabele toxische eigenschappen.[2, 3] Ondanks het vele onderzoek met die ontwikkelde *in vitro* systemen is het nog steeds lastig om met de huidige *in vitro* methoden de *in vivo* situatie goed te voorspellen.

Het doel van het onderzoek beschreven in dit proefschrift is het ontwikkelen van een nieuw *in vitro* systeem dat gebruikt kan worden om meer en betere informatie over het metabolisme en de toxische eigenschappen van potentiële medicijnen te verkrijgen in een vroeg stadium van geneesmiddelontwikkeling. Hierdoor kan het gebruik van proefdieren en de totale kosten tijdens medicijnontwikkeling verminderd worden. Het streven was om de *in vivo* situatie zo goed mogelijk na te bootsen. Daarom is er gekozen voor het gebruik van weefselplakjes (slices) van een intact orgaan, waarvan in eerder onderzoek is aangetoond dat ze de eigenschappen van het orgaan *in vivo* goed weergeven. Het ontwerp van het hier beschreven incubatiesysteem maakt het mogelijk dat de slices continue kunnen worden voorzien van vers medium, dit in tegenstelling tot bestaande incubatiesystemen waar de slices gedurende 24 uur in hetzelfde medium worden geïncubeerd. Hierdoor krijgen de cellen in de slices continue nieuwe voedingsstoffen en worden de afvalstoffen afgevoerd, net zoals dat in het menselijk lichaam gebeurt. Omdat de omzetting van medicijnen voornamelijk plaats vindt in de lever is het systeem in eerste instantie ontwikkeld voor leverslices. Om de *in vivo* situatie nog beter na te bootsen werd er een geminiaturiseerd systeem ontwikkeld voor de incubatie van slices met behulp van microfluïdische technieken. Hierdoor is de verhouding incubatiemedium ten opzichte van slice oppervlakte vele malen kleiner dan in huidige *in vitro* systemen. Het toepassen van microfluïdische technologie in het systeem heeft vele voordelen in vergelijking met conventionele incubatiesystemen.[4] Het belangrijkste voordeel is de

mogelijkheid om de incubatieomgeving waarin de cellen zich bevinden precies te controleren en om de evenwichtstoestand van voedingsstoffen en afvalstoffen te handhaven door een continue stroom van vers medium.

Er zijn in de afgelopen jaren vele microfluïdische systemen ontwikkeld voor de incubatie van geïsoleerde levercellen met als doel om de omzetting van medicijnen in de menselijke lever te bepalen. **Hoofdstuk 2** behandelt de verschillende op microfluïdica gebaseerde *in vitro* systemen die zijn beschreven in de literatuur voor het bepalen van levermetabolisme en toxiciteit, en de bijbehorende voor- en nadelen ervan. Uit dit literatuuroverzicht kan worden geconcludeerd dat slechts twee van deze modellen in staat zijn om het *in vivo* levermetabolisme goed na te bootsen, en dat zijn primaire (vers geïsoleerde) hepatocyten (de belangrijkste cellen voor metabolisme in de lever) en leverslices. Leverslices zijn dunne plakjes gemaakt van het intacte weefsel. Aangezien in slices de architectuur van het weefsel met alle verschillende celtypes, inclusief cel-cel en cel-matrix contacten, intact blijft, lijken deze uitermate geschikt voor metabolisme- en toxiciteitsstudies. Met name omdat alle celtypes aanwezig zijn is het ook mogelijk om met dit model multicellulaire toxiciteitsstudies uit te voeren.[5]

Het eerst prototype van een door ons ontwikkelde, op microfluïdica gebaseerde *in vitro* systeem waarin leverslices geïncubeerd kunnen worden, wordt beschreven in **Hoofdstuk 3**. Hiervoor werd er een chip ontwikkeld van het elastomeer polydimethylsiloxaan (PDMS). De slice in deze chip wordt continue voorzien van vers medium door het introduceren van een vloeistofstroom (flow). Om ervoor te zorgen dat de pH, de zuurstofconcentratie en de temperatuur constant blijven in het incubatiemedium zijn er twee dunne gas-doorlaatbare membranen geïntegreerd en wordt de chip in een temperatuur gecontroleerde omgeving geplaatst welke 5% koolstofdioxide (voor pH regulatie) en 95% zuurstof bevat. De gassen passeren de membranen en houden de pH en zuurstofconcentratie constant gedurende tenminste 24 uur. Aangezien het van belang is om de prestaties van een nieuw ontwikkeld systeem te verifiëren met een conventioneel systeem, zijn de resultaten van rattenleverslices die werden geïncubeerd in de chip vergeleken met slices geïncubeerd in well platen. De levensvatbaarheid en metabolisme waren in beide systemen niet significant verschillend, wat erop wijst dat de leverslices intact en functioneel blijven in de chip gedurende tenminste 24 uur.

In **Hoofdstuk 5** staat beschreven dat wanneer de slices ingebed zijn in een hydrogel, Matrigel, en het medium wordt aangevuld met runderserum, dexamethason en insuline de rattenlever slices zelfs gedurende 72 uur intact blijven. In deze opzet blijft het fase II metabolisme constant gedurende 72 uur, maar neemt het fase I metabolisme nog wel af net zoals ook is beschreven in de literatuur voor slices die worden geïncubeerd in conventionele systemen.[6-8]

Een voordeel van de continue vloeistofstroom is dat de vorming van metabolieten

en producten door de slice direct en continue gemeten kan worden in het medium dat de chip verlaat. De ontwikkeling van een on-line analyse systeem waarbij de chip is gekoppeld aan een HPLC met ultraviolet lichtdetectie wordt in **Hoofdstuk 4** beschreven. Door het volume van de chip zo klein mogelijk te houden, is de metaboliet concentratie in het medium hoog, en makkelijk te detecteren. Met het ontwikkelde systeem is het mogelijk om de slices volledig geautomatiseerd herhaaldelijk bloot te stellen aan substraten. In de beschreven experimenten werden de slices gedurende 24 uur vier keer gedurende twee uur blootgesteld aan 7-hydroxycoumarine met intervallen van vier uur. De vorming van metabolieten werd continu gemeten. In deze opstelling blijft de metabolietvorming constant tijdens de elk van de vier periodes van blootstelling. Tevens is het mogelijk om met het ontwikkelde systeem automatisch een concentratiegradiënt van een te onderzoeken substraat aan te brengen. In **Hoofdstuk 4** zijn slices geïncubeerd met een inhibitor die het metabolisme van een modelverbinding remt, waarbij de concentratie van de inhibitor tijdens het experiment varieerde van 0 tot 200 µM. Een volledige remmingscurve kan zo worden verkregen met slechts drie slices per experiment. Dit is dus erg geschikt voor weefsel dat in geringe hoeveelheid beschikbaar is, zoals humaan materiaal. Met dit on-line systeem is het mogelijk om geneesmiddel-interacties te meten en vast te stellen welke concentratie van inhibitor een remming vertoont op de enzymactiviteit van de slices. Een ander groot voordeel van dit systeem is de capaciteit om chemisch instabiele metabolieten te meten. Dit werd aangetoond door diclofenac acyl-glucuronide te meten, een instabiele metaboliet van diclofenac die door de lever wordt gevormd. Acyl-glucuronides zijn reactieve metabolieten en kunnen daardoor toxiciteit veroorzaken. Het is lastig om deze metabolieten te meten in conventionele systemen, doordat ze al tijdens de incubatie uiteenvallen vanwege de chemische instabiliteit. Met het on-line systeem kunnen deze metabolieten direct na vorming in een vrij hoge concentratie gemeten worden.

Om de juiste voorspelling te maken van *in vivo* geneesmiddelmetabolisme in de mens, is het beter om menselijk materiaal te gebruiken in *in vitro* studies dan dierlijk weefsel, omdat metabolisme in de mens en dier vaak sterk van elkaar verschilt.[9] Levermateriaal kan verkregen worden als “chirurgisch restmateriaal” na operaties, bijvoorbeeld bij verwijdering van een tumor.[10] In **Hoofdstuk 6** wordt het gebruik van slices van gezonde humane levers in het nieuw ontwikkelde *in vitro* systeem beschreven. Ook van humane leverslices blijft de levensvatbaarheid, gemeten aan de hand van morfologie en de lekkage van lever specifieke enzymen, gedurende 24 uur intact. Er werden geen significante verschillen gevonden tussen leverslices geïncubeerd in de chip en in well platen. Ook de fase I en fase II metabole omzetting van vier model geneesmiddelen, testosteron, lidocaïne, ethoxycoumarine en hydroxycoumarine, zijn goed vergelijkbaar met resultaten behaald in well platen, waarvan al eerder was aangetoond dat die goed correleert met de *in vivo* situatie.[11]

Daarom concluderen wij dat de ontwikkelde chip ook geschikt is om humaan materiaal te incuberen voor metabolisme- en toxiciteitsstudies.

Gezien het feit dat metabolisme niet alleen in de lever plaats vindt, maar dat ook de darm een belangrijke rol speelt, is in **Hoofdstuk 7** ook de incubatie van darmslices in de chip beschreven. Dit is het eerste voorbeeld van darmslices in een microfluïdische chip. De slices vertonen gedurende tenminste 3 uur een hoge metabole activiteit met drie testgeneesmiddelen. Vervolg experimenten zullen moeten uitwijzen hoe lang de darmslices levensvatbaar en functioneel kunnen blijven.

Doordat er een continue vloeistofstroom aanwezig is, worden de producten gevormd door de slices (waaronder de metabolieten) direct afgevoerd. Door deze stroom is het ook mogelijk om twee kamers die elk een orgaanslice bevatten aan elkaar te koppelen om interacties tussen twee organen te onderzoeken. In **Hoofdstuk 7** zijn op deze manier een darmslice en een leverslice achter elkaar gekoppeld en doorstroomd. Uit eerder gepubliceerd onderzoek is bekend dat blootstelling van de darm aan CDCA ervoor zorgt dat de darm een eiwit, genaamd fibroblast groeifactor 15 (FGF15) produceert. Vervolgens gaat dit eiwit *in vivo* via de leverpoortader naar de lever, waar het de expressie van het enzym CYP7A1 onderdrukt. CYP7A1 is verantwoordelijk voor de productie van galzouten, en door deze remming worden er minder galzouten gesynthetiseerd. Dit proces bleek ook op te treden in een leverslice wanneer een darm en een leverslice achter elkaar doorstroomd werden met het primaire galzout, chenodeoxycholzuur (CDCA). Door de sequentiële doorstroming van de kamers is het exact te bepalen welk orgaan het effect veroorzaakt. Twee slices kunnen ook in één well of kamer geïncubeerd worden, maar dan is het niet duidelijk welk orgaan een product vormt dat effect heeft op het functioneren van het andere orgaan.

Alle experimenten werden uitgevoerd met een chip gemaakt van polydimethylsiloxaan (PDMS). Dit is een ideaal polymeer om snel prototypes te maken van nieuw ontwikkelde chips. Dit polymeer heeft echter als nadeel dat het stoffen die sterk hydrofoob zijn kan adsorberen of zelfs kan absorberen.[12] In **Hoofdstuk 6** kwam dit probleem naar boven bij de incubatie met het hydrofobe substraat testosteron. Omdat dit de toepasbaarheid van de chip beperkt tot onderzoek naar niet hydrofobe geneesmiddelen, is er in **Hoofdstuk 8** gezocht naar een ander polymeer dat PDMS eventueel zou kunnen vervangen. In dit hoofdstuk werden de thermoplastische polymeren: polystyreen (PS), polycarbonaat (PC), polymethylmethacrylaat (PMMA), en cycloolefine copolymeer (COC) getest. Deze polymeren werden geselecteerd vanwege de goede biocompatibiliteit en omdat het mogelijk was om met deze polymeren chips te maken met behulp van een hot embosser. Hiermee kunnen in een korte tijdsperiode meer chips gemaakt worden dan van PDMS. In hun originele vorm zijn deze polymeren ook hydrofoob, maar deze kunnen hydrofiel gemaakt worden door het oppervlakte te oxideren met behulp van UV-ozon of zuurstofplasma behandeling.

Met behulp van UV-ozon bleek het mogelijk om het oppervlakte gedurende tenminste vier weken hydrofiel te maken. Chips gemaakt van UV-ozon behandelde PC, PS en COC vertoonden geen adsorptie van testosteron en 7-ethoxycoumarine en hun metabolieten. Om de biocompatibiliteit na UV-ozon behandeling te bepalen zijn er levercellen van een cellijn (HepG2) op platen van met UV-ozon behandeld materiaal geïncubeerd. De cellen bleken goed te groeien op deze platen wanneer deze bedekt werden met een laag collageen. Op PC, PS en COC werden nauwelijks dode cellen gevonden (~2%), wat erop wijst dat het materiaal niet toxisch en dus biocompatibel is. Daarentegen was ongeveer 25% van de cellen op PMMA dood na 24 uur kweken. Dit wijst erop dat de combinatie PMMA en UV-ozon niet geschikt is voor het gebruik in *in vitro* systemen met cellen of weefsels. Het is mogelijk om van PC, PS en COC chips te maken die biocompatibel zijn en een lage adsorptie van hydrofobe en hydrofiele componenten hebben. Gezien het feit dat PS minder resistent is tegen organisch oplosmiddelen heeft het gebruik van PC en COC de voorkeur. Van deze twee heeft COC het extra voordeel dat het een lagere autofluorescentie heeft dan PC, wat weer voordelig is voor experimenten waarin gebruik gemaakt wordt van optische detectie, zoals fluorescentie.[13]

Samenvattend, in dit proefschrift staat de ontwikkeling van een op microfluïdica gebaseerd *in vitro* systeem met continue vloeistofstroom voor lever- en darmslices beschreven. Een stabiele incubatieomgeving werd gerealiseerd (stabiele pH, zuurstofconcentraties en voedingsstoffen) door een lage vloeistofstroom te introduceren. Leverslices blijken levensvatbaar gedurende tenminste 72 uur en darmslices gedurende tenminste 3 uur. Door andere onderzoekers werd er gesuggereerd dat de aanwezigheid van vloeistofstroom een langere levensvatbaarheid tot gevolg zou hebben, maar hiervoor hebben wij nog geen aanwijzingen gevonden.[14, 15] Een beperking van dit systeem is de mogelijke adsorptie/absorptie van hydrofobe componenten aan het materiaal van de chip, maar dit kan verholpen worden door de chip te maken van COC in plaats van PDMS. Dit nieuw ontwikkelde systeem kan gebruikt worden voor de bepaling van metabolisme van geneesmiddelen, van geneesmiddelinteracties en van interorgaan interacties, waarbij slechts een minimale hoeveelheid weefsel nodig is. Tevens is het mogelijk om instabiele metabolieten te bepalen met het ontwikkelde on-line analysesysteem. Samen met het well plaat systeem, zal dit biochip systeem bijdragen tot een betere voorspelling van metabolisme en toxiciteit in de mens en proefdieren *in vivo*. Daardoor heeft het de potentie om een significante bijdrage te leveren aan metabolisme- en toxiciteitsstudies van nieuwe medicijnen en aan de reductie van het proefdiergebruik bij de geneesmiddelontwikkeling en bij toxiciteitstesten van andere chemicaliën.

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List of non-standard abbreviations

ADME-Tox :	Adsorption, distribution, metabolism, excretion and toxicity
AhR :	aryl hydrocarbon receptor
ALAT :	Alanine aminotransferase
ASAT :	Aspartate aminotransferase
CAR :	Constitutive androstane receptor
CDCA :	Chenodeoxycholic acid
CNC :	Computer numerical control
COC :	Cyclic olefin copolymer
Ct :	Cycle number at threshold value
CYP :	Cytochrome P450
DCF :	Diclofenac
DCF-AG :	Diclofenac acyl glucuronide
DMEM :	Dulbecco's modified eagle medium
DOC :	Dynamic organ culture system
FGF :	Fibroblast growth factor
FXR :	Farnesoid X receptor
HE :	Haematoxylin and eosin
LDH :	Lactate dehydrogenase
Li :	Lidocaine
MEGX :	Monoethylglycinexylidide
µCCA :	Microscale cell culture analog
NCE :	New chemical entities
PC :	Polycarbonate
PCLS :	Precision-cut liver slices
PCR :	Polymerase chain reaction
PCTS :	Precision-cut tissue slices
PDMS :	Polydimethylsiloxane
PEEK :	Polyether ether ketone
PMMA :	Polymethyl methacrylate
PTFE :	Polytetrafluoroethylene
PS :	Polystyrene
SHP :	Small heterodimer partner
TT :	Testosterone

TT-A :	Androstenedione
UGT :	Uridine diphosphate glucuronosyltransferase
UVOCS :	Ultraviolet ozone cleaning system
UW :	University of Wisconsin solution
WME :	William's medium E
2β-OH :	2β-Hydroxytestosterone
4′-OH-DCF :	4′-Hydroxydiclofenac
5-OH-DCF :	5-Hydroxydiclofenac
6β-OH :	6β-Hydroxytestosterone
7-HC :	7-Hydroxycoumarin
7-HC-G :	7-Hydroxycoumarin glucuronide
7-HC-S :	7-Hydroxycoumarin sulfate
7-EC :	7-Ethoxycoumarin

Dankwoord

Ondanks alle interessante onderwerpen die in dit proefschrift staan, vermoed ik toch dat dit hoofdstuk, het dankwoord, het best gelezen wordt. En terecht, want zonder de personen die ik graag wil bedanken waren de vorige hoofdstukken er nooit gekomen. Waarschijnlijk ga ik iemand vergeten hier persoonlijk te noemen, maar bij deze wil ik alvast iedereen die op een of andere manier heeft bijgedragen aan de totstandkoming van dit proefschrift bedanken!

Allereerst wil ik mijn beide promotores, Geny en Sabeth, enorm bedanken voor jullie steun en het vertrouwen.
Geny, ik heb erg genoten van onze vrijdagmiddag besprekingen, wat niet altijd werk gerelateerd was. En buiten de besprekingen om kon ik altijd zomaar binnenlopen voor een vraag. De snelheid waarmee documenten weer terug gestuurd werden was ook ongelooflijk. Slaap je wel?
Sabeth, die “hyphens” blijven een kriem, maar dankzij jou is mijn Engels wel enorm verbeterd de afgelopen jaren. Ik ben ook zeer onder de indruk van je algemene kennis over de wereld. De gesprekken tijdens de lunch en borrels waren dan ook erg gezellig, met altijd weer een leuke anekdote.

Tevens wil ik de leden van de beoordelingscommissie, Prof. dr. Bas Blaauboer, Prof. dr. Rainer Bischoff en Prof. dr. Shuichi Takayama, bedanken voor het lezen en beoordelen van het proefschrift.

Gelukkig hoef ik de verdediging niet alleen te doen, en heb ik twee geweldige paranimfen naast me staan! LJ allereerst bedankt dat je mijn paranimf wil zijn, en enorm bedankt voor de sessies, werk gerelateerd en niet. Je bent een ware vriend en ik zal je steun tijdens de afgelopen vier jaar niet vergeten, net zoals de trip met een Ford Mustang door San Diego! Marjolijn, bedankt voor je steun en hulp de afgelopen jaren. Het was altijd weer interessant hoe je het voor elkaar kreeg om mijn geweldig ingewikkelde systemen met een paar kleurtjes weer simpel te maken, zodat iedereen het weer begreep :-s. Je hebt mij enorm geholpen, en je kritische opmerkingen hielden mij scherp.

Het leuke van werken in twee vakgroepen is het aantal personen dat ik heb ontmoet tijdens het project. En ondanks de relatief kleine vakgroep Farmaceutische Analyse was er wel een enorm leuke sfeer in de groep. Patty en Maurits, tijdens en buiten werktijd was het altijd onmeunig gezellig. Patty succes met die *&^@# Huvec cellen en met golf, ben benieuwd wanneer die auto komt. Maurits bedankt voor de vele “diners voor twee” met “garden view” in het ziekenhuisrestaurant, en sorry voor je lichtknopje. Binnenkort maar weer een biertje drinken? 26 november? Maria, dos

cervezas por favor, thanks for the good time and I wish you all the best. Byeong-Ui, thank you for being my roommate, sorry for the music, and good luck with finishing your thesis! Hopefully we will meet again in South Korea sometime. Ook mijn andere kamergenoot Prof. dr. Jan Piet Franke wil ik bedanken, je was een rustige kamergenoot ;-). Jolanda, bedankt voor het controleren van de galley proofs, voor je hulp met het invullen van formulieren en voor de gezellige gesprekken! “Goedemorgen” Meint en Ilona bedankt en succes.

De andere vakgroep, Farmacokinetiek, Toxicologie en Targeting is veel groter, en ook onmogelijk om iedereen persoonlijk te noemen, maar ik wil bij deze iedereen bedanken voor de gezellige tijd. Er zijn wel een paar personen in het bijzonder die belangrijk waren voor mij: Inge de Graaf, jij was direct vanaf het begin al super enthousiast en geïnteresseerd in dit project. Je bent een enorm goede wetenschapper en ik heb veel van je geleerd. Ook bedankt dat ik in je huis heb mogen bivakkeren toen je op vakantie was. Marieke Elferink, waarom doen die computers toch nooit wat jij wilt? Mackenzie it was always a lot of fun with you, good luck with your project and thanks for the “coupons”. Inge W. schrik niet, maar succes met je project als analist/aio/analist, ik weet het niet meer. Ook alle andere aio’s: Adriana, Ansar (ex-aio), Marike, Marlies, Marianne, Martin, Na, Ruchi, Venkatesh en Xiaoyu enorm bedankt voor de leuke tijd en succes met jullie projecten (Dutch lesson, meaning: thanks a lot for the great time and good luck with your projects). Esther van de Kerkhof, helaas hebben wij maar kort samengewerkt, maar ik heb je proefschrift regelmatig doorgenomen, en daardoor veel over de darm geleerd, bedankt! Rose Hayeshi, it is still a pity that our µUssing chamber did not work, however, it was great working with you. Thank you also for staying in your room when you where in Zimbabwe. Maybe Sanna can take care of the µUssing project. Sanna good luck.

Jan bedankt voor je hulp met de HPLC’s, en ik zal nooit meer vergeten hoe je keek toen ik in het eerste jaar één van je HPLC’s had open gemaakt en aankwam zetten met een seal die vervangen moest worden. Ik zal dat nooit weer doen...

Klaas Poelstra bedankt voor de nuttige opmerkingen tijdens de lijnbesprekingen. Helaas heb ik iets minder contact gehad met de groep van Klaas, maar ik wil iedereen van die groep wel bedanken voor de gezellige sfeer en de leuke koffiepauzes (BMW Alie). Frits, Bert Stok, Peter Olinga, Hans Proost en zijn groep wil ik ook bedanken voor de gezellige tijd.

Bonjour Gillian, mijn uren hoef je niet meer te schrijven, maar enorm bedankt voor het werk wat je voor mij gedaan hebt. Ik heb ook altijd weer genoten van onze gesprekken, maar ik vind wel dat ik weinig post kreeg… ;-).

Verder kon ik de experimenten niet uitvoeren zonder hulp van de personen die de ratten hebben opgeofferd. Anne-miek, Ansar, Catharina, Eduard, Inge W. en Patricia, bedankt. En Sylvia en Patricia ook bedankt voor het maken van coupes en uitvoeren van kleuringen, en Miriam succes met het overnemen!

Dan is er nog een derde vakgroep waar ik ook veel mee te maken heb gehad tijdens mijn project. De vakgroep van Rainer Bischoff. Allereerst wil ik Rainer bedanken voor zijn goede opmerkingen tijdens de woensdagmiddag presentaties en de gezellige tijd tijdens de lunches. Verder wil ik ook de hele vakgroep Analytical Biochemistry bedanken voor de leuke tijd, in het bijzonder Berend, Ishtiaq (Sjoerd), Peter, Jos, Lorenza, Laurette, Therese, Hanna, Christin, Julien, Nicolas, Sara en Jan-Willem.

Ook de studenten die ieder een goede bijdrage hebben geleverd aan het proefschrift wil ik bedanken. Muzikale Niek, "vozer" Joost en keukenprins Arnout (die chocolade koekjes zijn enorm lekker, hint). Zonder jullie hadden er drie hoofdstukken een stuk kaler uit gezien. Bedankt!

Tevens is de support van vrienden buiten het werk om, enorm belangrijk voor mij geweest om zo ver te komen. Arnoud en Karin bedankt dat jullie er altijd voor mij waren, en die kleine spruit van een Elise kreeg mij altijd weer vrolijk. José en (RSV mille) Serge, Martijn en Sandra ook jullie wil ik bedanken voor jullie interesse in mijn project en voor jullie support.

Petra, drie jaar van mijn aio-periode heb je altijd voor mij klaar gestaan en heb je mij enorm geholpen. Ik wil je dan ook enorm bedanken voor alles. Wanneer ik weer vast liep op het werk plande jij weer een dagje weg, zodat ik mijn gedachten weer ergens anders op kon richten.
Ook wil ik Gerrit en Janny en de rest van je familie bedanken voor hun interesse in mijn onderzoek.

Marina, niet alleen heb je veel slices voor mij gemaakt, maar ook na werktijd beteken je veel voor mij. Het laatste jaar van een aio hield in dat ik elke dag aan het werk was, maar jij hebt mij enorm gesteund. Bedankt!

Tenslotte wil ik mijn familie bedanken. Al mijn broers en zussen, Johan en Kirsten, Marjolein en Harald, Annelies en Dion, bedankt voor jullie interesse en hulp in moeilijke tijden! Annelies, ook bedankt voor je hulp met de omslag. En als laatste: pa en ma enorm bedankt voor alles. Jullie stonden altijd achter mij, en zonder jullie vertrouwen had ik dit nooit gehaald. Ik kan niet in woorden uitdrukken hoeveel jullie voor mij betekenen!

Paul.

Curriculum Vitae

Paul van Midwoud werd op 18 juli 1983 geboren te Hoogeveen. Na het behalen van zijn HAVO-diploma aan het OSG De Groenedriehoek te Hoogeveen in 2000 begon hij in hetzelde jaar zijn HLO-opleiding chemie aan de Hogeschool Drenthe te Emmen. Zijn afstudeerproject “Toepasbaarheid van sorbent sampling in proteomics” heeft hij voltooid in 2004 bij Spark Holland BV. Vervolgens is hij in 2004 de masteropleiding Analytical Chemistry begonnen aan de Vrije Universiteit van Amsterdam. Zijn master titel werd met cum laude behaald in 2006 met als titel van het afstudeeronderzoek “Improvement of repeatabiltiy and recovery in peptide analysis”. Vervolgens is Paul zijn promotieonderzoek begonnen aan de Rijksuniversiteit Groningen. Het promotie-onderzoek werd uitgevoerd als een samenwerkingsproject bij de vakgroep Farmacokinetiek, Toxicologie & Targeting als bij Farmaceutische Analyse. Het project is uitgevoerd onder begeleiding van prof. dr. G.M.M. Groothuis en prof. dr. E.M.J. Verpoorte. Het onderzoek richt zich op het ontwikkelen van een nieuw *in vitro* systeem waarin verschillende types orgaanweefsel geïncubeerd kunnen worden in een geminiaturiseerd systeem met een continue aan- en afvoer van voedingsstoffen. Voor de ontwikkeling van het systeem is gebruik gemaakt van microfluïdische technieken zoals beschreven in dit proefschrift.

List of publications

Full papers

Based on research described in this thesis

Van Midwoud, P. M.; Groothuis, G. M. M.; Merema, M. T.; Verpoorte, E., Microfluidic biochip for the perifusion of precision-cut rat liver slices for metabolism and toxicology studies. *Biotechnol. Bioeng.* 2010, **105** (1), 184-194.

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Van Midwoud, P. M.; Groothuis, G. M. M.; Verweij, N.; Merema, M. T.; Verpoorte, E., Hydrogel embedding of precision-cut liver slices in a microfluidic device improves drug metabolic activity. *Submitted.*

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Other publications

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Van Midwoud, P.M.; Merema, M.T.; Koster, S.; Groothuis, G.M.M.; Verpoorte, E., Towards an improved in vitro system for ADME-Tox studies: Development of an intestine-liver biochip, *Gordon Research Conference on the Physics and Chemistry of Microfluidics*, Waterville Valley, NH, USA, July 15-20, **2007**.

Van Midwoud, P.M.; Groothuis, G.M.M.; Merema, M.T.; Verpoorte, E., Towards improved in vitro systems for ADME-Tox studies: Development of an intestine-liver biochip, *The 12th International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS 2008)*, San Diego, CA, USA, October 3-4, **2008**.

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Awards

Best **Poster Presentation Award** at Pharmacy Day 2010, June 8, 2010, Groningen, The Netherlands.

Received the **Tony B. Academic Travel Award** (SLAS) to give an oral presentation at LabAutomation 2011, January 29 - February 2, 2011, Palm Springs, CA, USA**.**