An alternative approach based on microfluidics to study drug metabolism and toxicity using liver and intestinal tissue
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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. 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. 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. 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. 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, and stellate cells are involved in the development of fibroses. 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
organ, cut into slices with a very reproducible thickness. The first example of the use of liver slices was in 1923 by Otto Warburg. 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. 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. 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. 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. Precision-cut liver slices were successfully used to study many liver-specific processes, for example the metabolic pathways and rates of metabolism of xenobiotics, xenobiotic-induced toxicity, mechanism of fibrogenesis, or gene transfection. 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. 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...
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 intestine21, 22, kidney15, lung15, brain23, heart24, prostate25, spleen26 and tumors27. 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. 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
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. 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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