Microfluidic Digestive Systems for Drug Analysis

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DOI:
10.33612/diss.190919502

Chapter 9
General Discussion

9.1. Miniaturizing the Human Intestine

The work in this thesis revolves around *in vitro* models of the human gastrointestinal (GI) tract. In the pharmaceutical, as well as the toxicological and nutritional fields, there is an urgent demand for more and better models of human tissues and organs, because the currently available models suffer from a number of shortcomings. First, new drugs have to be thoroughly tested including on animals, and this encounters major ethical objections. Second, interspecies differences make it hard to extrapolate results obtained in animals to the human situation, leading to less reliable results. Third, the process of drug development is hugely demanding in terms of both time and money, and most drug candidates fail somewhere along the way in this process. Therefore, the sooner a candidate drug fails, the better – it saves a lot of time and costs (and the cost of innovative drug therapies has risen to almost unaffordable levels over the last decade). We envision that the work described in this thesis can contribute as a more robust and reliable tool in future drug development.

We gave an overview of currently available model systems of the human intestine in Chapter 2, with a focus on miniaturized models inspired by both conventionally used transwell systems and *in vivo* human physiology. We identified that all model systems have shortcomings, as compromises have to be made to balance the degree of complexity of the model system with its technical feasibility and applicability for end-users. None of the gut-chip models reviewed in this chapter incorporated the full process of digestion, which after all is one of the key functions of the digestive tract (the other key function, absorption of nutrients or other compounds, was included in most models). We developed a digestion-on-a-chip system, incorporating the biochemical processes of the mouth, stomach, and intestine into one continuously flowing microsystem (Chapter 3). Each compartment consisted of a single micromixer, in which sample solutions were mixed with artificial versions of digestive juices, producing a digested material termed chyme. We measured enzymatic activity in each of the compounds, to ascertain that the digestive processes are occurring inside the system. One unique feature of this system is the translation of a batch process to a continuous process: all digestive steps and reactions are occurring at the same time, but at different places throughout the system. The traveled distance can therefore be transposed to time, and samples can be taken

at different locations to study reaction progress. The digestion-on-a-chip was benchmarked with the digestion of milk protein, lactoferrin, to establish its performance. The digestion of this protein was found to be faster using the digestion-on-chip than with a conventional batch-wise digestion, besides saving significantly on chemical consumption and labor.

The work in Chapter 3 was based on *in vivo* average compositions of digestive juices and residence times in the respective compartments. A new system based on similar working components was devised specifically for infantile digestion, as their gastrointestinal physiology is markedly different from adults (Chapter 4). The enzymatic load in infantile digestive juices is lower and their gastric content is far less acidic (at pH 5.3 instead of 1–3). The performance of this infantile digestive system-on-a-chip was studied by again using milk protein, lactoferrin. The digestion of lactoferrin had a similar pattern in the chip-based system and in the conventional batch-wise digestion, as indicated by both high-performance liquid chromatography (HPLC) analysis of the lactoferrin content and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the digested peptide fragments. Next, the adult digestion-on-a-chip system was coupled in-line with a downstream barrier model of the human intestine to study both digestion and absorption of chemical compounds in one hyphenated system (Chapter 5). We found that even though intestinal epithelial cells and goblet cells were co-cultured, this was not sufficient to protect the cell layer from harmful effects of the chyme. It is thought that cell damage is induced by the presence of bile salts (which may dissolve cell membranes) and digestive enzymes (digesting cellular materials). The chyme was diluted in a cell medium matrix (1:7) to prevent this, and the subsequent uptake of verapamil and omeprazole was studied using online ultrahigh performance liquid chromatography-quadrupole time-of-flight-mass spectrometry (UPLC-QTOF-MS). We found that it was possible to combine these three components (digestion, absorption, and analysis) in one hyphenated system, while overcoming the inherent incompatibilities of the individual components. Flow rates were different in each of the components, but the addition of dilution buffer to chyme helped to make up for the intestinal barrier model, and only small volumes of apical and basolateral effluent were sampled using an intricate loop system coupled to the MS (simply discarding the surplus). The obtained permeability results for the two model drugs seemed to be in accordance with the literature. More research and finetuning of the system are necessary to validate its relevance for other model samples, however, the developed system has potential for future application in the pharmaceutical and toxicological fields.

9.2. Methods, Tools, and Equipment for Organ-Chips

The second part of the work in this thesis focuses on the development of methods, tools, and equipment to support the work with organ-on-a-chip devices. Many quips have been made about miniaturized lab-on-a-chip devices requiring a lab bench of full-size supportive equipment, and the there is no denying this – good surrounding equipment is absolutely essential for microfluidic experiments, and most stable pieces of equipment are fairly large next to a microfluidic chip. The most common type of equipment is the syringe pump, used to deliver a flow of liquid to microfluidic chips by positive displacement. The mechanics of syringe pumps cause unstable flows, which becomes apparent when flow patterns are visualized in microfluidic channels. We developed a far more stable liquid delivery system for organs-on-chips and other microfluidic experiments using gas-pressurized liquid containers and Coriolis-based mass flow sensors to regulate microflows (Chapter 6). These Coriolis sensors measure mass flows through minute changes in vibrations of an internal loop, and this sensing principle is independent of the flow rate (over a very wide range), temperature, and the density of the liquid that is being displaced. This ensures that accurate flow rates are delivered to the microfluidic systems, which is important for the stability of the living cells inside the gut-on-a-chip and to accurately perform analyses. The stability of the flow control system was demonstrated in three scenarios: the mixing of ethanol and water at different ratios (with density and viscosity of the mixture varying as a function of the ratio), the long-term supply of medium to a gut-on-a-chip, and the regulation of four flows of complex, biologically relevant liquid matrices in on-chip digestions.

Chapter 7 describes a different aspect of supportive methods, namely the fabrication of thin, flexible membranes with massive arrays of through-holes to serve as basis for the gut-on-a-chip barrier. Such membranes are usually fabricated by using microstructured silicon molds, but the fabrication of these silicon molds is complex and requires specific dry-etching machinery. We describe a method to fabricate porous membranes by molding using photoresist-based molds. The procedure to produce micromolds with photoresist layers is well-known, but it takes specific strategies to produce 30-µm-thick membranes with thousands of pre-defined pores, as described in this chapter. This method can be used to produce flexible membranes for gut-on-a-chip or other organ-on-chip devices with barrier functions.

Finally, Chapter 8 includes our efforts to date to fabricate microfluidic (and millifluidic) components for analytical systems by stereolithography, a specific version of 3D-printing. By selectively curing resin on a print platform, three-dimensional objects such as static mixers, microreactors, and extractor columns could be produced. The printed objects were functionalized chemically in various ways, in order to make them more resistant to solvents or to introduce surface functionalities. In one example, these surface modifications were used as anchors for the immobilization of an enzyme in a flow-through microreactor. In this way, the unique features of microchannels such as a high surface-to-volume ratio can be utilized.

9.3. Relevance of Model Systems

In the introduction of this thesis, we discussed the compromises that have to be made to balance the degree of complexity (to incorporate as many physiologically relevant elements as necessary) with the technological feasibility of the new model system; and in the end its robustness and reliability, since a more complex system has more components that may break or fail. A program of requirements with minimal performance criteria is thereby a useful tool. In the case of the gut-on-a-chip described in this work, the digestive functions of the different gastrointestinal organs and the barrier function of the intestinal wall were the main components of the new model system. The chemical analysis of processes occurring inside these devices was to be done by high-end analytical systems. We chose to recreate the digestive processes in a cell-free model, with three microreactors as miniaturized versions of the mouth, stomach, and intestine. Recipes for artificial digestive juices were optimized to create the exact right conditions *in situ* for the relevant digestive enzymes to work, and the absence of living materials such as cells makes this digestive model quite robust. The potential influence of the epithelial lining of the mouth, esophagus, and stomach are not included in this model, even though it is known that some drugs may have oromucosal absorbance. Since by far most drugs are absorbed through the wall of the small intestine, this is a defensible compromise. The digestive enzymes used are therefore not freshly secreted by glands, but pre-made in the lab and from animal origin. This, too, is a compromise, but the activity of the animal enzymes used is comparable to those of humans, and they are more readily available.

For the barrier model, initial experiments focused on the use of Caco-2 human intestinal cells. This cell line was derived from a colonic adenocarcinoma (large intestine), but the cells are robust and easy to work with, and since they show a more small-intestine-like phenotype when cultured, they are used as the gold-standard cell model for intestinal epithelium. Again, primary

intestinal cells, or even induced pluripotent stem cell-derived epithelium would be closer to the *in vivo* physiology, but this would introduce too much complexity for use as a "simple" intestinal barrier model. For the absorption studies in Chapter 5, a co-culture with intestinal goblet cells was used to increase the physiological relevance with mucus-secreting cells, which may help to protect the barrier from harmful effects of the chyme. Other potentially relevant *in vivo* physiological elements were not incorporated into this intestinal model either. Peristaltic action of the intestine, which helps to mix ingested food with digestive juices and propels food forward through the GI tract, have been shown to have a positive contribution to the functions of intestinal epithelium. The introduction of peristalsis requires the use of even more supportive equipment (*e.g.* inducing lateral stretching by applying cyclic vacuums to specific regions of the device), so the disadvantages outweighed the benefits in this case – the barrier model is fully functional even without peristalsis.

The drug absorption studies performed in the same chapter were done over a time of three hours. This is enough to model the initial drug absorbance kinetics of an orally administered drug, monitored by highly sensitive analytical equipment such as mass spectrometry. We did find, however, that especially basolateral concentrations may be very low after pretreatment (dilution) with digestive juices, and translocation through a cell layer. For some drugs, the limit of detection of these analytical methods may not be sufficient for online measurements. A possible solution would be to accumulate more volume of basolateral effluent, and analyze the content after appropriate sample preparation (concentration of analytes by solvent evaporation, solid-phase extraction, etc.).

Summarizing, we can state that a number of compromises had to be made in order to achieve both physiological relevance of the new intestinal model and technological feasibility (with a healthy dose of pragmatism). The models were shown to be relevant, and their performance was benchmarked against commonly used techniques.

9.4. Conclusions and Future Perspectives

I envision that the work described in this thesis finds applications in the fields of the pharmaceutical sciences, toxicology, and nutrition. As alluded to above, the systems have been benchmarked against commonly used techniques in these respective fields, including *in vitro* digestions and Transwell permeability assays. The continuous-flow design of our systems gives

them great potential for automation: digestions of samples may be performed continuously, with possible distribution of the resulting chyme over multiple intestinal barrier models (to obtain more statistical power in biological assays) and several different analytical tools coupled inline to do sampling and analysis of pharmacokinetic processes while they are happening.

Offline analysis with accompanying sample preparation may be used in the future to further increase the detection limit of analyte molecules. Longer-term dosing studies and intermittent administration of drugs and toxicants would be of great interest for further research – in this way, longer-term processes like accumulation of compounds in the body or multi-dosage drug regimens can be modeled better. Combining these systems with a (miniaturized) liver model would complete the entire process to study the bioavailability of orally ingested compounds. A further expansion into different target organs, reached *via* some sort of central circulation and incorporating (renal) drug elimination would make a resulting "body-on-a-chip" an entirely new pharmacokinetic model.

Together, these pragmatically designed systems may be a valuable contribution for the drug development process, after a successful further development and optimization of these methods for applicability in the field. Toxicological and nutritional research may also find them valuable for better, more realistic *in vitro* testing.

Reflecting on the multidisciplinary character of this research, as first described in Chapter 1, I conclude this is fully a blessing, a *sine qua non*. The work described in this thesis is only possible by combining the best of the individual fields of biology, physics, chemistry, and engineering. As a matter of fact, these fields are inseparably intertwined. The best inventions can only be achieved by taking the essential elements of each of the fields, considering the desired scientific functionalities, and engineering the technological feasibility.