LAB-ON-A-CHIP DEVICES WITH PATTERNED HYDROGELS
ENGINEERED MICROARRAYS FOR BIOMOLECULE FRACTIONATION, ORGAN-ON-CHIP AND DESALINATION

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LAB-ON-A-CHIP DEVICES WITH PATTERNED HYDROGELS: ENGINEERED MICROARRAYS FOR BIOMOLECULE FRACTIONATION, ORGAN-ON-CHIP AND DESALINATION

DISSERTATION

to obtain the degree of doctor at the University of Twente, on the authority of the rector magnificus, Prof. dr. H. Brinksma, on account of the decision of the graduation committee to be publicly defended on Thursday 15 September 2016 at 16:45

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Hydrogels are considered to be in the class of smart materials that find application in diagnostic, therapeutic, and fundamental science tools for miniaturized total analysis systems. In this thesis, the focus is on three major applications of patterned hydrogels, which are explored as an alternative strategy to expensive and low throughput systems for preparative DNA fractionation, in vitro compartmentalization of human gut epithelium, and desalination by microelectrodialysis.

The use of patterned hydrogels in closed fluidic microchips for different research fields depends crucially on the ease and accessibility of their fabrication technology. In this work, two simple fabrication procedures are developed to pattern hydrogel microarrays. First, intermittent illumination is applied on mechanically polished microchips for the photopatterning of hydrogels. Second, capillary pressure barriers are used for controlling the position of the liquid-air meniscus in microchip channels, allowing the subsequent patterning of hydrogels by photopolymerization and thermo-gelation. Both fabrication techniques differ from previous studies in terms of versatility and high reproducibility.

Preparative fractionation and purification of small-sized DNA fragments play an important role for second-generation sequencing and personalized medicine, and it is the first major application of hydrogels explored. We describe a novel method for concurrent continuous flow fractionation and purification of DNA fragments in a microfluidic device filled with agarose gel. The innovation of this work is twofold. Firstly, a new principle for continuous flow DNA fractionation is demonstrated. We exploit the variation in the field-dependent mobility of DNA molecules with DNA length for the fractionation, which is a separation mechanism that has hitherto gone unnoticed. Secondly, since this new mechanism can be applied using agarose gel, it provides a low-cost, robust, and versatile separation matrix. The theoretical advancement in combination with the practical advantages can lead to new developments in the field of sample preparation of biological samples. Baseline fractionation of a 0.5-10 kbp
DNA ladder is achieved within 2 minutes, which is ~15 times faster than in commercially available devices. Furthermore, the gel technology is easily adaptable; for example, changing the gel type can enable the fractionation of protein molecules. Thus, the microfluidic device is of broad interest for second-generation sequencing and clinical diagnosis applications.

The second major application of hydrogels reported in this thesis is the use of multicompartamental hydrogel arrays for 3D culturing of human intestine epithelial cells. Engineering in vitro microenvironments that mimic in vivo tissue systems is crucial for improving our understanding of tissue physiology, as well as curtailing the high costs and complexities associated with the existing techniques. We propose and demonstrate an in vitro microfluidic cell culture platform that consists of periodic 3D hydrogel structures. The compartmentalized nature of the microchip architecture and fluid delivery enable culturing of human intestine cells which spontaneously grow into 3D structures on the 3rd day of cell culturing. On the 8th day of culture, Caco-2 cells are co-cultured for 36 hours with intestinal bacteria E.coli, which adhered to the cells without affecting the cell viability. Continuous fluidic perfusion also enables the preliminary screening of chloramphenicol treatment on the intestinal epithelial cells. Finally, we find that different compartment geometries with large and small hydrogel interfaces lead to a difference in the proliferation and cell spread profile of Caco-2 cells. The microchip enables facile fluidic control that allows dynamic regulation of culture conditions.

Microelectrodialysis is explored as the last major application of hydrogels in this thesis. Common methods used to construct microelectrodialysis devices rely on incorporation of membranes into microchips, which is challenging in terms of robustness, consistency, and ease of fabrication. Hydrogels are more promising candidates for desalination by electrodialysis, than membranes due to their ion selective and hydrophilic matrix, which is also versatile, inexpensive, and easily tailorable. Patterning ion selective hydrogels at small scales is therefore used to miniaturize the electrodialysis process in microfluidic devices, and subsequently provides more insight into the ion transport phenomena. In this work, we firstly show that parallel streams of concentrated and ion-depleted water are formed in continuous flow when a potential difference is applied across the microchip containing alternating rows of patterned cation- and anion-selective hydrogels. The device could remove approximately 75% of the 1 mM sodium chloride salt introduced via the inlet streams. We demonstrate different currents and flow rates in the microchip for desalination purposes. Secondly, the microchip enables ion transport visualization in the ion selective hydrogels and microchannels when a charged fluorescent dye is utilized. For sufficiently high potential differences, vortex formation is observed near the hydrogel-liquid interfaces, contributing to an enhanced convective transport towards the hydrogels in the overlimiting current regime.
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A brief introduction to the basics of preparative DNA fractionation, recapitulation of a human gut microsystem, and the basics of electrodialysis in fluidic microchips is given. Advances in lab-on-a-chip technology within each project's scope are briefly discussed, and an overview of the subjects addressed in this thesis is presented.
1.1 | Introduction

More than 35 years ago, a gas chromatographic analyzer—the first miniaturized analytical device—was fabricated on a silicon substrate. It was used to separate a simple mixture of different compounds in a couple of seconds. This device was fabricated to enhance analytical performance and reduce sample consumption. In the 1990s, this development led to further advances in the field of science and technology, as it inspired new research lines. Since then, for example, hydrogels and ion filters have been integrated into miniaturized devices to perform in vitro analyses, enabling a wide range of applications such as preparative DNA fractionation, miniaturized cell culturing, and water desalination (Figure 1.1). These miniaturized total analysis systems (microTAS), or lab-on-a-chip devices, raised expectations that they could one day replace larger equipment currently used to perform biomedical, chemical, and physical analyses. The main advantages of these systems are their ability to use small sample volumes, perform fast analyses, provide high throughput, allow for optimization on the small scale, and enable integration into portable readout systems. The applications of lab-on-a-chip technology are very broad, and in this thesis we will investigate three specific areas: preparative fractionation of DNA molecules, an organ-on-chip platform to culture human intestine epithelial cells, and microelectrodialysis. In the following sections, the advancements and bottlenecks within the field of each application will be addressed.

Figure 1.1. Flow diagram highlighting crucial developments in specific microfluidic applications. Beginning in the 1960s, seminal microfluidic applications enabled fast analyses, high throughput, optimization on the small scale, and compatibility with portable readout systems. Product development started in the 1990s and contributed to crucial functional developments in the technology. In the past decade, these functional developments have contributed to the development of a new generation of research and technology tools, from DNA sequencing to organ-on-chip systems to microdesalination platforms.

1.2 | Preparative DNA fractionation for biomedical applications

The identification and analysis of biomolecules is of utmost importance in bioengineering sciences. After the human genome project was initiated, a new
understanding of the genome's primary structure was reached. The development of DNA electrophoresis, using both slab gels and capillaries, enabled the first-generation DNA sequencing and genotyping technologies required for the human genome project. Only minimal sample numbers were separated and identified at very high cost using these technologies. In recent years, many efforts have been directed towards reducing the cost and analysis time of DNA genotyping in second- and later-generation sequencing tools. To do this, sample preparation methods such as electrophoresis are optimized to increase the throughput and efficiency of the analyses with user-friendly, small, portable, and functional platforms. In some devices, traditional DNA separation gels have also been replaced by microfabricated post arrays.

The first example of an artificial (gel-replacing) electrophoresis platform was presented by Volkmuth and Austin in a study of DNA fractionation: the authors built a two-dimensional array of symmetrical nano-structured obstacles using photolithographic techniques. In this device, DNA fragments were observed to follow the applied electric field's direction while changing their conformation by stretching or folding, depending on the fragment size (larger than 100 kbp). Another device was fabricated with precisely defined nanoslit post arrays, providing an opportunity to investigate the movement of smaller DNA fragments (smaller than 43 kbp) together with circular DNA fragments. In another study, it was found that a nanopillar array allowed rapid DNA separation, and an approximately logarithmic relationship between the observed mobility and the length of DNA fragments in a range of 1 to 25 kbp was established.

A rapid fractionation of 1-15 kbp DNA was also accomplished by Tabuchi et al. In this work, band broadening remained quite low because the samples were loaded into a core-shell nanosphere suspension, which served as the sieving matrix. In 2002, for the first time, an electrophoretic chip was designed without lithography: instead, the formation of superparamagnetic bead columns by an external magnetic field was used. Increasing the electric field was shown to decrease DNA mobility and DNA stretching in that device. Another lithography-free manufacturing approach has been demonstrated to construct a separation matrix, where self-assembled colloidal structures of differently-sized beads were used to tailor a three-dimensional sieving structure with several different pore sizes. The separation of denatured proteins and 0.5 to 50 kbp DNA fragments was achieved in this platform in continuous flow (Figure 1.2a).

Continuous flow is the most preferred method for increasing sample throughput in preparative separations. Such continuous flow separations can only occur when different-sized DNA fragments migrate through a sieving matrix at different angles, driven by applied electric fields. Pulsed electric fields applied at different angles were used in references 8 and 9, where continuous flow separation of differently-sized DNA fragments was obtained. Using a pulsed and angled field approach with a different micropillar
separation matrix, Huang et al. separated 100-200 kbp DNA fragments with good resolution and reasonably high throughput. In another separation matrix—namely a two-dimensional anisotropic nanofluidic filter array—a similar pulsed field protocol enabled continuous flow separation via biased reptation and entropic trapping in the same sieving platform, as shown by Fu et al. (Figure 1.2b). Since the DNA fragments follow different trajectories in these devices, samples can be collected easily for further characterization purposes.

The devices briefly reviewed above allowed for optimization of the separation process in spatially controlled sieving matrices, exploiting the basic physical principles of the separation. However, defect-free fabrication of these micro- and nano-fabricated devices has often been an issue, in addition to their low sample throughput due to dimensional constraints, particularly in nano-fabricated devices. An ideal sieving matrix should thus have simple design and facile fabrication steps, yet should provide high-resolution and high-throughput separation.

In this thesis, we present a new and simple approach for preparative purification and fractionation of sub-10-kbp DNA molecules in a microfluidic device filled with agarose gel as the separation matrix. DNA fragments and other ionic species are separated from each other in continuous flow within 2 minutes when electrical fields with different magnitudes are periodically applied. The high-resolution separation is based on the differing field-dependent mobilities of differently sized DNA fragments, which is used for the

*Figure 1.2. (a) Colloidal nanoarrays fabricated in a PDMS microdevice for (b) continuous flow separation of DNA molecules of 2-10 kbp range. (c) Continuous flow separation of the Hind-III digest λ-DNA. (d) The device proposed for DNA fractionation by Fu et al.*
first time for continuous flow DNA fractionation. As this technique is based on agarose gel technology, the microfluidic device is easy to fabricate and operate, and offers great promise for addressing second-generation sequencing challenges—including low-cost and high-resolution purification and fractionation of DNA sizes of interest.

1.3 In vitro compartmentalization of human gut epithelium and microbiota

Human in vitro cell cultures are widely used to study biological and biochemical changes in tissue constructs, primarily for initial screening of drugs and disease modeling. Traditionally these cultures are performed in Transwell plates; however, this process is time consuming. Furthermore, the conditions in the plates are not always physiologically relevant: many of the metabolites are released by cells in minute amounts within the large volumes of Transwells; and 2D cultures cannot accurately mimic the 3D cell environment in human body. Over the last decades, in vitro microchip platforms, that simulate miniaturized human tissue systems, have been developed as an important advancement in modeling tissues in vitro. Performing experiments in microchips in a continuous, stepwise manner has shortened the analysis duration and offered automation opportunities. The mass transport rates of nutrients and metabolites around the tissue constructs are also improved, owing to the short distances and high surface-to-volume ratios of microchannels. Miniaturization has often been accompanied by compartmentalization, in which the tissue culture is separated into parts. This approach creates well-defined microenvironments, where cells can interact with each other via several modalities to generate tissue function in the compartments. The most frequently used techniques to fabricate compartmentalized 3D cell culture environments have been membranes, pillars, microdroplets, and phaseguides.

Miniaturization of human gut in vitro models (and the associated microbiota) began with static monolayer cultures. The two major pioneering in vitro methods were developed to study drug metabolism. First, a monolayer culture of human intestine epithelial cells (Caco-2) under static conditions in Transwell plate. Second, a parallelized version of the monolayer static culture was performed in a Transwell plate and was called as the parallel membrane permeability assay. Transwell plate studies aside, static 3D cultures have been conducted in microengineered platforms, where hydrogel scaffolds were designed to mimic the microscale geometries of biological tissues, and therefore improve the physiological relevance. For example, drug transport across an epithelial layer was mimicked in a 3D static culture by fabricating villi-like structures on PDMS and culturing the Caco-2 cells on it. Further advances were made by integrating microporous polymeric membranes made of SU-8 into a microchip platform with a supporting post array, which promoted cell
differentiation.\textsuperscript{23} Similarly, Yu et al. reported the use of hydrogel scaffolds to build a well-controlled microenvironment with a strong physiological resemblance to the \textit{in vivo} systems.\textsuperscript{24} In addition to these advanced 2D and 3D microcultures, fluidic platforms have been applied to facilitate long-term cultures. Mahler \textit{et al.} cultured monolayers of multiple cell types in separate compartments of a large-scale fluidic culture platform using a recirculating culture medium.\textsuperscript{25} Following that study, Kimura \textit{et al.} recreated an intestinal 2D model with a PDMS microchip that was separated into two independent channels by a semipermeable membrane, on which Caco-2 cells were inoculated and cultured.\textsuperscript{26} In another pioneering study, Kim \textit{et al.}\textsuperscript{27} constructed a compartmentalized PDMS microchip in which cyclic strain and fluid flow could be applied to a monolayer Caco-2 culture, modulating the cell differentiation and villi formation (Figure 1.3a). The same platform has also been used for microengineered lung-on-chip studies (Figure 1.3b).\textsuperscript{28} Next, on-chip fixation and long-term culture of intact mouse mesenteric artery segments were demonstrated in a microfluidic platform by Günther \textit{et al.} (Figure 1.3c).\textsuperscript{29}

The microfluidic devices presented above typically lack complete fluidic control, which is required to enable on-demand manipulation of the outer cell microenvironment in well-defined structures, as well as to maintain long-term

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.3.png}
\caption{(a) Schematic of the human gut-on-chip platform. (b) Schematic of the lung-on-chip platform. (c) Microfluidic on-chip vessel fixation platform. Scale bars are 500 \(\mu\text{m}\) (left) and 100 \(\mu\text{m}\) (middle and right). The figure was modified from References 22 and 30.}
\end{figure}
cultures of tissue models. The aforementioned platforms do not support compartmentalization, which would be an important advance for spatio-temporal controlling of the microenvironments and monitoring intercellular activity in a high-throughput manner.

In this thesis, we overcome this challenge by combining microfluidics and compartmentalization via capillary line pinning. We present a new approach to building in vitro cell culture platforms for tissue mimicry, using compartmentalized and periodic 3D hydrogel structures inside closed microfluidic chips. The design concept is based on selectively trapping mixtures of collagen pre-gel and cells in compartments via capillary line pinning. The architecture of the microchip and continuous fluid delivery enable long-term and in-parallel culturing of Caco-2 cells that undergo differentiation and spontaneously grow into 3D folds on the 8th day of cell culturing. Caco-2 cells were also co-cultured with an intestinal bacterium (E.coli) which adhered to the cells without affecting their viability, showing cell-bacteria interaction. This microfluidic engineering approach offers great promise both for building next generation organotypic in vitro platforms, and for addressing drug screening and toxicology testing challenges by enabling compartmentalized 3D cell culturing in a microfluidic environment.

1.4 Microfluidic electrodialysis

Integration of ion-selective filter materials with controllable geometries into microfluidic devices is crucial for both applied and fundamental research. Applications include water desalination and pretreatment of sub-nanoliter biological samples, necessary for noise reduction and reproducibility of mass spectroscopy measurements in medical research and clinical applications. Ion-selective filter materials integrated into microfluidic devices can also serve as an ideal tool for studying the fundamentals of ion concentration polarization phenomena, since the processes can easily be controlled and visualized in those devices.

Much investment has been made in the engineering and development of microfluidic desalination systems, including microelectrodialysis, ion concentration polarization techniques (ICP), capacitive deionization, and electrochemical deionization. It has been recognized that filter materials play an important role in efficiency and throughput of the desalination process. In this thesis, we have focused on desalination by microelectrodialysis. This technology is often applied in large-scale systems; however, optimization of the operation parameters on the microscale and the usage of alternative filter materials, such as hydrogels, may improve the overall process. Particularly, microfluidics has been used for downscaling the electrodialysis process to increase energy efficiency and water recovery. Building hybrid membrane
microsystems has been a special focus, as such systems can reduce the energy consumption by decreasing the membrane resistance.\textsuperscript{37}

Recently, the integration of oppositely charged membranes into microdevices was demonstrated for water splitting and pH regulation.\textsuperscript{34} Desalination of an electrolyte solution was also realized by Kim \textit{et al.} (Figure 1.3a-c).\textsuperscript{37} In this approach, two microchannels were connected via a Nafion membrane, creating an ion depletion region, which extended into a side channel via an aqueous solution flow that subsequently becomes desalinated. Kwak \textit{et al.} used a PDMS microchip to perform electrodialysis and desalinate a 10 mM NaCl solution with commercial ion exchange membranes.\textsuperscript{38} The results of this study suggested that the optimal operation parameters in terms of energy efficiency would be close to the end of the limiting current regime. In another study, the overlimiting current region was eliminated by using a polyvinyl alcohol coating layer on top of a membrane surface.\textsuperscript{39} \textit{In situ} fabrication of hybrid membranes by photolithography was demonstrated for studying the physical mechanisms behind the charge-based separations of 1 mM NaCl solutions in a glass microchip.\textsuperscript{39} Aside from membranes, nanochannels have also been used for electrodialysis. Chang \textit{et al.} showed that nanochannels can be ion selective at ion concentrations smaller than 10 mM; in this case, ions in the electrical double layers significantly contribute to ionic transport under an applied electric field.\textsuperscript{40} The nanochannels are often fabricated using glass or silicon

\textbf{Figure 1.3.} (a) Schematic of the desalination platform reported by Kim \textit{et al.}\textsuperscript{37} (b) Fluorescent image of desalination process with an external flow rate of 20 $\mu$L min$^{-1}$ at 75 V cm$^{-1}$. (c) Desalted and concentrated streams of a sea water after a time span. “Gnd” represents the grounded electrode and the grey line represents the charged electrode. (d) pH gradient regulation in a microchip with bipolar membrane system.\textsuperscript{34} (e) The bipolar membrane junction with a thickness of 20 $\mu$m in a microchannel and ion transport to opposite sides of the membrane.\textsuperscript{40} (f) Ion dynamics at 20 V applied voltage, which is beyond the limiting current regime for this system.
materials and have a negative zeta potential at pH > 3, allowing predominantly cationic species to pass through (Figure 1.3b). The methods described above bring experimental challenges. Microdevices consisting of a microchannel sandwiched between ion exchange membranes provide limited operating conditions due to the fluid leakage. Microdevices connected by nanochannels can only operate at low salt concentrations that give rise to electrical double layer overlap when the channel dimensions are in the order of the Debye length.

The desalination study presented in this thesis overcomes these complications by combining microfluidics and charged hydrogels patterned by capillary line pinning. We use a stack of periodic hydrogel structures in a microfluidic platform. Alternating anion- and cation-exchange hydrogels are locally fabricated in confined compartments by capillary line pinning. Parallel streams of concentrated and ion-depleted water are formed in continuous flow when a potential difference is applied across the microchip at different fluid flow rates. The throughput of the desalination process can be increased with this approach, owing to its highly-parallelized nature. This development may lead to low-cost and hybrid hydrogel systems, for use in sample pretreatment and studying fundamentals of charge based separations.

1.5 Scope of thesis

This thesis reports several methods investigated to pattern hydrogels on the microscale in closed microfluidic structures, and presents applications of these methods to three different fields: namely, preparative fractionation of DNA fragments in continuous flow, a compartmentalized human gut epithelium on a chip, and microscale electrodialysis for desalination. In chapter 2, the reader will be introduced in the main principles of hydrogels, DNA fractionation, co-culturing of human intestine epithelial cells, and electrodialysis. Chapter 3 and chapter 4 present two different approaches for patterning periodic hydrogel structures in closed fluidic microchips. In chapter 5, a novel approach for preparative purification and separation of sub-10-kbp DNA fragments in a microfluidic device is introduced. Chapter 6 presents a new approach to building an in vitro cell culture platform for tissue mimicry, using compartmentalized and periodic 3D hydrogel structures inside closed microfluidic chips. In chapter 7, a simple approach for desalinating salty water is introduced, using a stack of oppositely charged periodic hydrogel structures in a microfluidic platform. Chapter 8 provides a self-reflective point of view on the research and development process for all previously discussed applications, by means of their societal perspective. Chapter 9 finally presents a summary and suggestions for further research. An appendix is also added to provide details of the setup, fabrication, and measurement processes.
1.6 References


This chapter introduces the main aspects of hydrogels that are relevant to the applications presented in this thesis. Background information is also given for the three application areas: preparative DNA fractionation in continuous flow, human intestine epithelial cells and bacterial adherence, and desalination of electrolyte solutions by electrodialysis.
2.1 Hydrogels in lab-on-a-chip technology

Hydrogels are a class of crosslinked polymers, which can absorb large quantities of water due to their hydrophilic nature. As hydrogels can be synthesized from a wide variety of natural and synthetic polymers, they have a highly tunable nature. Different material properties enable the transport of molecular and ionic species through the material, and provide tailorable matrices to accommodate cells in microscale environments. Hydrogels are also capable of responding to their surrounding environment, with tunable sensitivities to pH, ionic strength, temperature, electric field, and light. They have therefore been used in various biological and electrochemical applications in lab-on-a-chip technology. Research on hydrogels started in 1960 with the pioneering work of Wichterle and Lim, who studied hydroxyethyl methacrylate for biological applications. After this work, hydrogels based on synthetic and natural polymers with specific material properties (including variations in molecular weight, chain functionality, and charge density) were developed. Dušek, in 1968, demonstrated that net repulsion between the polymer chains in a poor solvent causes sudden changes in the degree of swelling due to the phase transition. In 1980, Lim and Sun showed cell encapsulation by calcium alginate microcapsules. Yannas et al. reported on the use of natural hydrogels in artificial burn dressings in 1989. In the late 1990s, Vacanti and Langer proposed the integration of hydrogels into the tissue engineering field.

Reddy et al. used hydrogels for protein crystallization, evaluating their selectivity for different biomolecules, while Paustian et al. fabricated micro-window hydrogels and used their local electric permeability to sculpt electric fields in a microfluidic chip. In another study, macrophage cells were encapsulated in hydrogel patterns in order to detect enzymatic reactions. Ashley et al. demonstrated that patterned hydrogels can be used as tunable drug release tools and Byun et al. studied integration of three-dimensional protein arrays into a hydrogel matrix. Suzuki et al. reported that the UV light sensitivity of hydrogel structures can be used to make photo-responsive artificial muscles and memory devices.

![Figure 2.1. Molecular structure of agarose.](image)

2.1.1 Synthesis of hydrogels

Hydrogels can be synthesized via physical and chemical crosslinking methods. In this synthesis, a crosslinked polymer chain is formed by covalent
or non-covalent bonding of monomers and copolymers. Bonding through non-
covalent interactions result in physical crosslinking, while bonding by covalent
bonds between polymer chains results in chemical crosslinking.

**Physical crosslinking**

Formation of a polymer network can occur via non-covalent bonds such as ionic interactions, hydrophobic interactions, and hydrogen bonds. These interactions are strong enough to hold a polymer network together, despite the fact that the polymerization process remains reversible.

In ionic interactions, charged polymers attract other ionic polymers or multivalent counter-ions in the solution. This attraction can be influenced by the pH, temperature and salt concentration. For instance, calcium alginate is an example of physically crosslinked hydrogels by ionic interactions.

In hydrophobic interactions, polymers with both hydrophilic and hydrophobic domains (so called amphiphilic polymers) crosslink in water. Here, the hydrophobic domains are coupled to other hydrophobic domains and form aggregates; however, the hydrophilic domains still remain exposed to the water. Temperature, salt concentration, and the nature of the hydrophobic domain have a big impact on this type of physical crosslinking due to the phase change in polymers. Chitin and chitosan are synthesized via hydrophobic interactions.

In hydrogen bonding, loose dipolar interactions take place between hydrogen atoms and electronegative atoms located in inter- and intra-chain polymer networks. These dipolar interactions are formed, for example, by

![Molecular structure of polyacrylamide polymer.](image)

*Figure 2.2. Molecular structure of polyacrylamide polymer.*
decreasing the temperature of the hydrogel precursor.\textsuperscript{23} Agarose is one example of hydrogels which are gelated via hydrogen bonding. It is a linear polymer consisting of alternating copolymers of 1,4-linked 3,6-anhydro-α-l-galactose and 1,3-linked β-d-galactose. The molecular structure of agarose is shown in Figure 2.1. Agarose hydrogels are prepared by heating a mixture of water and agarose to above 65°C to dissolve the agarose, and then cooling the mixture to 17-40°C to form the polymer network.\textsuperscript{24} Agarose hydrogels are of zero net charge and have a typical pore size in the range of 200-500 nm, depending on the agarose concentration.\textsuperscript{25}

**Chemical crosslinking**

Crosslinking has a chemical character when the polymers or monomers are covalently bonded, providing a higher mechanical strength in the hydrogel backbone. The polymerization process, in this case, is irreversible due to the high level of structural integrity. Enzymatic reactions, radical polymerization, and irradiation are common methods for chemical crosslinking.\textsuperscript{26}

Enzymatic reactions provide a high degree of control on the polymerization reaction, avoiding side reactions due to the specificity of enzymes. For example, transglutaminase is a calcium-dependent enzyme that catalyzes crosslinking reactions of polypeptide hydrogels.\textsuperscript{27}

Radical and irradiation polymerizations are frequently used crosslinking strategies. Initiators start the reaction by generating free radicals, which create active sites in the growing polymer chain and add monomers. Polymer chain growth consists of three distinct steps: initiation, propagation, and termination. An active polymer site is created by free radicals during the initiation step. The free radicals can be generated by either oxidation upon

![Figure 2.3. Molecular structure of PEG diacrylate polymer.](image-url)
radical polymerization or UV exposure. The propagation step involves chain growth; during this step, initiation and termination rates are in equilibrium. Termination occurs when two growing chains, one growing polymer chain with one free radical, or two free radicals meet (see section 3.3.3 for more discussion). Polyacrylamide, polyethylene glycol (PEG), and collagen can be polymerized via both radical and irradiation polymerization techniques.28,29

Polyacrylamide is a non-linear polymer consisting of acrylamide monomers and N,N-methylene-bis-acrylamide crosslinker units. The molecular structure of polyacrylamide is shown in Figure 2.2. Polyacrylamide hydrogels are synthesized by either radical or irradiation polymerization, depending on the photoinitiator type used. The resultant polymer is of zero net charge, and has a typical pore size in the range of 5-100 nm.30

PEG is also a non-linear polymer consisting of ethylene glycol monomers. Figure 2.3 depicts the molecular structure of PEG. Similarly, PEG hydrogels with acrylate terminated groups can be chemically crosslinked using radical or irradiation polymerization reactions. The charge of PEG hydrogels is dependent on their functional groups and PEG can be prepared with a wide range of molecular weights ranging from a few thousands to hundreds of thousands g mol⁻¹.31

Collagen is a natural hydrogel which shows excellent biocompatibility and biodegradability. Certain cells in the body, such as fibroblasts, are capable of synthesizing collagen. Since the fast biodegradation and low mechanical strength of the untreated collagen matrices create problems in biological applications, chemical crosslinking is preferred to tailor the hydrogel to fulfill the needs of stability.32 Inter- and intra-molecular bonds form between alpha-chains of the tropocollagen structures during the crosslinking process, which typically occurs by self-assembly at room temperature.33 The triple collagen matrix is kept together by both hydrogen bonds and dipole-dipole

---

**Figure 2.4.** Molecular structure of collagen's triple helix. Dashed lines show the intermolecular covalent bonds between tropocollagen structures. Modified from reference 34.
interactions. Intermolecular covalent bonds result in mechanical stiffness. The molecular structure of a triple collagen helix is shown in Figure 2.4. Pore size of the collagen hydrogel is in the range of 5-20 µm.

2.1.2 Characterization of the hydrogel structure

Crosslinked polymer networks are generally characterized by analyzing several structural properties, including molecular weight, charge density, porosity, mechanical strength, volume fraction of water, and swelling behavior. Reaction kinetics and double bond conversion are the major factors affecting these structural properties. Other factors include crosslinking time, crosslinking temperature, type of crosslinker, and type of monomer.

Molecular weight and fixed charge density are determined by the monomers or polymers joined to the hydrogel’s molecular structure. While the molecular weight affects the porosity of the hydrogel, the fixed charge density is determined by the charged groups at the backbone. These fixed charges attract oppositely-charged free ions from the solution. Hydrogels can be non-ionic or ionic. Non-ionic hydrogels exhibit water-polymer chain interactions while ionic hydrogels exhibit ion-ion interactions and can be anionic, cationic or amphoteric (possessing both positive and negative charges) depending on the fixed charged groups found in their molecular backbone. A detailed study on charge density of hydrogels was previously reported by Peppas et al.

Porosity is an important parameter especially for physical studies and is determined by crosslink density and molar fraction of crosslinkers, monomers, and initiators. For example, in polyacrylamide polymerization, free radicals generated by the initiator link bis groups and acrylamide monomers to form a polymer network with a certain pore size. The pore size is proportional to the concentrations of crosslinker (C%) and monomer (T%), which are calculated as follows:

\[
C% = \frac{CL}{M+CL} \cdot 100
\]
\[
T% = \frac{M+CL}{V} \cdot 100
\]  

where \( CL \) (g) is the crosslinker amount in grams, \( M \) (g) is the monomer amount in grams, and \( V \) (l) is the total volume.

The mechanical strength determines how well the hydrogel maintains its shape under pressure and it is measured for the swollen state of the hydrogel. In general, a high crosslinking density gives brittleness and a low crosslinking density results in more flexibility. For the measurement of the brittleness, a static compressor is utilized to apply a gradually increasing
pressure until the hydrogel start to deform, and then breaks apart. The breaking point determines the mechanical strength of the hydrogel.

The volume fraction of water (or the amount of water in the hydrogel), determines the diffusion rate and absorption of particles or ionic species in the hydrogel. This water volume fraction includes both bound and free water. When a dry hydrogel starts to absorb water, the most hydrophilic or polar groups will initially be hydrated, followed by the less hydrophilic or more hydrophobic groups. In both cases, water will be bound inside the hydrogel matrix and form ‘bound water’. The hydrogel will still be able to imbibe the “free water” by the osmotic driving force of the polymer chains until the chemical and physical crosslinked groups oppose the additional uptake, leading to an equilibrium state in swelling.\textsuperscript{36}

The swelling behavior of hydrogels has been previously discussed by several reports in the literature.\textsuperscript{7,37,38} Many hydrogels have a high affinity for water which penetrates in the crosslinked polymer chains and causes swelling. The hydrogel porosity and the crosslink density are the most dominant factors in the swelling behavior. The degree of swelling can be described by the equilibrium weight swelling ratio. This ratio is gravimetrically determined,\textsuperscript{38} for which the dry weight ($W_d$, g) and the swollen state weight ($W_s$, g) of the hydrogel are measured. Equation 2.3 then gives the equilibrium swelling ratio ($S$, \%):\textsuperscript{37,39,40}

$$S = \frac{W_s}{W_d} \cdot 100$$  \hspace{1cm} \text{Equation 2.3}

In the following sections, we will discuss the application areas of hydrogels, namely preparative DNA fractionation, gut-on-chip and microelectrodialysis.

2.2 | Preparative DNA fractionation

Here we provide an overview of the characteristics of the deoxyribonucleic acid (DNA) molecule, electrophoresis and DNA fractionation to serve as background for chapter 5.

2.2.1 | Physical and chemical properties of DNA

DNA is a biopolymer, consisting of purine (adenine and guanine) and pyrimidine (cytosine and thymine) nucleotides attached separately to deoxyribose sugars, which are linked together by phosphate residues (Figure 2.5). Located at the backbone of double stranded DNA, the phosphate residues provide -2 charges per nucleotide unit at physiological pH (pH 7.4). Covalent bonds join the nucleotide units to form a single strand, while double stranded DNA is held together by hydrogen bonds between the bases: two between
adenine and thymine, three between guanine and cytosine. In double stranded DNA, the axis of the helix passes through the center of the base pairs. The distance between two base pairs measured along the helix is 0.35 nm.\textsuperscript{41}

The DNA double helix can be treated as a semi-flexible polymer chain, which has a number of repeating units \((N)\) with a length \(d\) \(\left(3.5 \times 10^{-10}\right)\) m. This model represents a chain of linked monomers, reaching a contour length \((L, m)\) as a result.

\[ L = Nd \quad \text{Equation 2.4} \]

The flexibility or mechanical stiffness of the double helix of the DNA molecule is quantified by the persistence length \((p, m)\). It is typically measured as 50 nm or 150 base pairs.\textsuperscript{42} In polymer physics, the persistence length is replaced by the Kuhn length to avoid a numerical prefactor since \(l_{\text{Kuhn}} = 2p\). The contour length divided by the Kuhn length equals the number of DNA Kuhn units that can perform a random walk in the surrounding environment: \(N_{\text{Kuhn}} = L / l_{\text{Kuhn}}\).\textsuperscript{43} Due to the random walk of the Kuhn units, the DNA is found as coiled assemblies in aqueous environments, and the DNA molecule thus occupies a certain volume that affects its movement through a hydrogel matrix. The size of the coiled assembly is characterized by its radius of gyration \((R_g, m)\), which is the root mean square of the distance between the Kuhn units and the center of mass of the chain in its random conformation.

\[ \text{Figure 2.5. Double stranded structure of DNA.}^{42} \]
The radius of gyration increases with the square root of the number of Kuhn units, $R_g = l_{\text{Kuhn}} N_{\text{Kuhn}}^{1/2}$.\textsuperscript{44}

### 2.2.2| The basics of electrophoresis

Electrophoresis is the movement of charged particles in a solution under an imposed electric field. Particles with different charges and/or sizes can be separated in a solution, and the velocity of the particles is quantified by their mobility, $\mu$, which depends on the characteristics of the particle. When the Debye length is larger than the size of the particle, the velocity depends on the charge per size ratio of the particle.\textsuperscript{45} The Debye length will be discussed in the following sections, namely in Electric double layer.

#### Movement of charged particles

Under the application of an electric field, electrically charged particles migrate due to an electric force ($F_E$, N), which is the result of the electric field strength ($E$, V m$^{-1}$), and the charge of the particle ($q$), quantified by the charge number ($z$) times the electronic charge ($e_0$, 1.60x10$^{-19}$ C).\textsuperscript{46}

$$F_E = ze_0 E$$  \hspace{1cm} \text{Equation 2.5}

The electric force leads anions (negatively charged ions) and cations (positively charged ions) to move in opposite directions. This electric force is opposed by the drag force ($F_{\text{drag}}$, N), stemming from the friction of the charged particle with its surrounding matrix. For a homogeneous viscous medium with a viscosity ($\eta$, Pa s), the drag force is linearly proportional to the particles’ velocity ($V$, m s$^{-1}$) through the medium. For spherical particles with a radius $r$ (m), the drag force is given by Stoke’s law:\textsuperscript{46}

$$F_{\text{drag}} = 6\pi\eta r V$$  \hspace{1cm} \text{Equation 2.6}

The migration velocity of ionic species can be derived from the above equations:

$$qE = 6\pi\eta r V$$  \hspace{1cm} \text{Equation 2.7}

$$V = \frac{q}{6\pi\eta r} E$$  \hspace{1cm} \text{Equation 2.8}

or

$$V = \mu E$$  \hspace{1cm} \text{Equation 2.9}

where $\mu$ (m$^2$V$^{-1}$ s$^{-1}$) is the electrophoretic mobility.
For protonatable analytes, the pH of the solution affects the dissociation degree of the analyte, which will in turn affect its mobility. The effective mobility \( \mu_{\text{eff}} \), m\(^2\) V\(^{-1}\) s\(^{-1}\), is the electrophoretic mobility of the fully ionized analyte, modified by its degree of ionization. For a monovalent acid with a dissociation constant \( K_a \), the effective mobility as a function of pH is\(^{47} \)

\[
\mu_{\text{eff}} = \mu \frac{K_a}{[H^+] + K_a}
\]

Equation 2.10

The electrophoretic mobility can be influenced by numerous factors, such as temperature, ionic strength, and pH of the solution. The temperature affects the mobility since the viscosity \( \eta \) is temperature dependent.

\[
\eta = \eta_0 e^{E_A/R T}
\]

Equation 2.11

Here, \( E_A \) is the activity energy in viscous media, \( R \) is the molar gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), and \( T \) the absolute temperature (K).

Current passing through a conductive matrix raises the temperature, also known as Joule heating. Cooling occurs at the walls, and a temperature gradient is formed in the separation matrix. This, in turn, leads to a viscosity gradient throughout the matrix. As a further consequence of the heating, the viscosity decreases and the current increases even more, causing further heating in the matrix. A poorer fractionation is likely to occur due to increased diffusion rates of DNA fragments and unequal mobilities across the matrix. Decreasing the electric field strength or lowering the buffer conductivity is one possible solution; however, these actions may also result in longer separation times and band broadening. Hydrogel matrices tend to dissipate the generated heat, and help minimizing the band broadening.\(^{47} \) Using low channel heights also effectively reduces the current and the heat production.

**Electric double layer**

When glass is immersed in an aqueous solution of pH > 4, the silane terminal groups (Si-OH) located at the glass surface become deprotonated and create a negatively charged surface. The electrical potential of this charged surface is in the order of -100 mV at pH 7. Due to the fixed charges located on the surface, counter-ions will be attracted, which gives rise to the so-called electric double layer near the surface. The first layer of ions close to the charged surface, or Stern layer, consists of immobilized counter-ions and solvent molecules. These counter-ions are fixed to the glass surface due to the strength of the electrostatic interactions. A linear drop in the potential occurs in the Stern layer. The counter-ions in the layer further away from the surface experience less electrical attraction and as a result, they remain relatively
mobile. The ionic concentration in this diffuse layer results from an equilibrium between electrostatic forces and thermal movement (Brownian motion) of the ions. The diffuse layer thus has a lower charge density than the Stern layer. The thickness of the diffuse layer depends on the ionic strength of the solution $I$ (mol l$^{-1}$) which is a function of the concentrations ($c_i$, mol l$^{-1}$) and charge numbers $z$ of all ions in the solution.

$$I = \frac{1}{2} \sum z_i c_i$$  \hspace{1cm} \text{Equation 2.12}

In the diffuse layer the electrical potential drops exponentially with a decay length called the Debye length ($\lambda_D$):

$$\lambda_D = \frac{1}{\lambda} = \sqrt{\frac{\varepsilon \varepsilon_0 k_B T}{2e^2 I}}$$  \hspace{1cm} \text{Equation 2.13}

where $\varepsilon$ the dielectric constant of the medium, $\varepsilon_0$ is the dielectric constant of vacuum, $k_B T$ (J K$^{-1}$) the Boltzmann factor, $e$ the elementary charge (C), $I$ the ionic strength of the solution, $z_i$ the valence electron of the ion (C), and $c_i$ the concentration. The Debye length is, for example, 1 nm in a solution of 100 mM concentration.

Electroosmotic flow (EOF) occurs in a fluid when an electric field is applied parallel to a surface with an established double layer. The mobile ions in the double layer will migrate towards the oppositely charged electrode by the effect of Coulomb forces. The migration of the mobile ions then drags the solvent along, leading to a bulk flow of solvent. In this situation, the flow velocity is assumed to be zero at the shear plane (located approximately at the Stern layer), and reaches its maximum level a few Debye lengths away from the surface. The velocity of EOF is then determined by the zeta potential at the shear plane, the dielectric constant of the solution, and the applied electric field:

$$V_{EOF} = \frac{\varepsilon \varepsilon_0 \zeta E}{4\pi \eta}$$  \hspace{1cm} \text{Equation 2.14}

$$V_{EOF} = \mu_{EOF} E$$  \hspace{1cm} \text{Equation 2.15}

where $V_{EOF}$ is the EOF velocity, $\mu_{EOF}$ the electroosmotic mobility, $\varepsilon$ the dielectric constant of the solution, $\zeta$ the zeta potential, $E$ the electric field, and $\eta$ the solution viscosity. The electroosmotic mobility is positive for a glass slab.
immersed in water, and the flow will be in the direction of the electric field (towards the negative electrode).

The velocity of the ionic species in an electrophoretic separation is equal to the sum of the electrophoretic velocity and the EOF velocity. EOF can be decreased via several approaches, including adjusting the system to low pH values, coating the surrounding walls with an EOF suppressor agent, and changing the viscosity of the solution or zeta potential of the surroundings. Due to the high viscosity, EOF is generally suppressed in gel electrophoresis.

2.2.3 DNA electrophoresis

The aromatic components (purines and pyrimidines) of the DNA molecule are buried inside the double helix, and negatively charged phosphate groups remain at the outer part. This structure enables the electromigration of DNA fragments in a medium when applying an electrical potential gradient.

In free solution electrophoresis, when an electric field is applied in an aqueous solution, DNA molecules and the counter-ion cloud surrounding the molecule move in opposite directions. The thickness of the counter-ion cloud scales with the Debye length, $\lambda_D$. In case $\lambda_D$ is thinner than the molecule size, the hydrodynamic interactions between the different parts of the DNA molecule are screened over the distances larger than $\lambda_D$. The friction of the molecule with the surrounding environment now scales with the molecular length owing to the screening of hydrodynamic interactions. The electric force also scales with the length of the DNA molecule. Since the mobility $\mu$ is proportional to the electric force divided by the frictional force, the resulting mobility becomes independent of the molecule size. Thus, DNA fragments of different length cannot be separated by electrophoresis in an aqueous solution.

To perform size-based electrophoretic DNA separation, hydrogels are used as they provide a sieve-like matrix that impedes DNA movement in a size-dependent manner. The principles of DNA electrophoresis in hydrogels are explained in detail by Viovy et al.45

2.2.4 Physics of DNA separation in porous matrices

When modeling the DNA separation mechanisms, the hydrogel structure is often regarded as a large network of nanopores with a certain pore size. Thus, porous micromachined matrices can serve as tools for DNA separation. The ratio of pore size to DNA size is one of the major parameters determining the separation, having a direct effect on the mobility of DNA fragments. On the other hand, DNA fractionation in entangled polymers, where the porous matrix is absent, has also been demonstrated.31,52 Excellent reviews on the topic of DNA separation were written by Viovy,45 Heller53 and Slater54. In this section, we will focus on the DNA separation mechanisms in
systems with inherent nanopore networks. Four main separation mechanisms have been described for size-based DNA fractionation in such systems.

**Brownian ratchet, Ogston sieving, entropic trapping, and biased reptation**

Brownian motion of particles is random and omnipresent. Brownian ratchet devices have been constructed to use the differences in Brownian motion of large and small DNA molecules for separation. In such a ratchet, DNA fragments follow a size-dependent path as depicted in Figure 2.6 when they are electrokinetically driven towards the micrometer-sized, tilted obstacles. The small fragments will diffuse over larger distances during transport by the electric field, and as a result, many of them are blocked and deflected through the gaps in the obstacle-rich area (Figure 2.6). This mechanism thus leads small fragments to migrate at a different angle with the horizontal axis than large fragments. In this method, DNA fragments are not stretched, but the movement actually is based on the diffusion coefficients. The disadvantage of this technique is that the fractionation takes a long time (in order of a few hours), since the difference in diffusion coefficient for large and small DNA fragments is small.

In Ogston sieving, DNA fragments have a radius of gyration that is smaller than the pore size. In this case, they are assumed to retain their approximately spherical (‘blob’) shapes. The fractionation of different-sized DNA molecules occurs due to the steric interactions between the fragments and the sieving matrix, assuming the fragments are migrating in the matrix without perturbation (Figure 2.7). The mobility is proportional to the fractional hydrogel volume available to the DNA molecule. The Ogston

![Figure 2.6. Schematic diagram of (a) the working mechanism of the Brownian ratchet and (b) the device.](image)
sieving mechanism has been proven to work well in both polyacrylamide and agarose hydrogels for DNA fragments larger than 2-3 kbp and 50-300 bp, respectively.

![Diagram of molecular sieving mechanisms](image)

**Figure 2.7.** Molecular sieving mechanisms. Yellow lines and blue dots illustrate DNA fragments and the hydrogel matrix, respectively. Rectangles are the post arrays and dashed lines are the migration trajectories of DNA fragments.

Entropic trapping mechanism can only be used when the radius of gyration of DNA fragments is comparable to the pore size in the hydrogel matrix. DNA fragments, which are contained within a large pore, need to pay an entropic penalty to escape and move through small pores, until finding a new large pore where the entropy loss is minimal again (Figure 2.8). The bigger the DNA fragment is, the higher the entropic penalty; thus, they tend to spend more time in the hydrogel’s large pores even in the presence of an electric field (Figure 2.7). The size-dependent migration thus occurs between the entropic barriers via thermally activated jumps of DNA fragments.\(^{56,61}\). When the applied electric field energy is in the order of the required entropic trapping energy, the traps reduce the mobility of DNA fragments in a size-dependent fashion. Entropic trapping becomes a negligible factor in determining DNA mobility at higher electric fields. Although this regime can be applied to flexible biomolecule chain separations, band broadening is a major limitation especially for the combination of high concentration hydrogels and low electric fields.\(^{59,60}\)

Biased reptation occurs when the radius of gyration is much larger than the pore size of the hydrogel. It is driven by electric forces that cause a preferential motion of the fragment in the direction of the field. The DNA needs to uncoil to move through the pores and the movement can be visualized as the migration of an uncoiled DNA fragment head-first through a porous medium. The fragments travel through a sequence of many hydrogel pores with a snake-like motion (Figure 2.7). The friction of the DNA fragment...
with the hydrogel strands hinders the center-of-mass movement. The fragment therefore moves slower than in free solution. The mobility of the DNA during biased reptation can be calculated with the equation given by Viovy:

\[
\frac{\mu}{\mu_0} = \left[ \frac{(b/l)^2}{3N_{Kuhn}} \right]^{\frac{1}{2}} + \left[ \frac{2\varepsilon_K(b/l)^2}{5 + 2\alpha\varepsilon_K(b/l)^2} \right]^{\frac{1}{2}}
\]

Equation 2.16

where, \(\mu\) is the mobility, \(\mu_0\) the free solution mobility, \(b\) the pore size of the gel (m), \(l\) the Kuhn length of DNA molecule (m), \(N_{Kuhn}\) the number of DNA Kuhn units, \(\varepsilon_K\) the reduced electric field per Kuhn length (where \(\varepsilon_K = \eta l^2 \mu_0 E/k_B T\)), \(\alpha\) the mobility reduction factor for gel electrophoresis in strong fields (\(\alpha = 3\)). This model captures the DNA mobility over a wide range of molecular weights, gel concentrations, and electric fields. During its movement through the gel, the DNA fragment may change the sieving regime from biased reptation to entropic trapping or Ogston sieving, depending on variations in the pore size in the hydrogel matrix.

2.3 | *In vitro* compartmentalization of human gut epithelium

We will provide an overview of the characteristics of human gastrointestinal system to serve as background for chapter 6.

2.3.1 | Gastrointestinal tract microbiota

The environment in the human gastrointestinal tract (GI) is generally anaerobic, however the physiological conditions alter throughout different parts of the GI tract due to the changes in pH, peristaltic movements, transit time, nutrient availability, or dietary substrates. The GI tract has a surface area of approximately 300 m\(^2\), on which \(10^{14}\) external microorganisms are harbored, including yeasts, fungi, archaea, viruses, and bacteria which account for 75 percent of the microbe content in terms of abundance. This complex system is termed the gut microbiota. The large capacity to host different species is due to the presence of finger-like microscopic structures, particularly in the small intestine. These structures are called villi, and are typically 0.5 to 1.5 mm in
length. Villi contain blood capillaries to maintain nutrient transport from the small intestine to the blood stream. The outer layer of the villi consists of epithelial cells, which also have 100 nm long finger-like structures on the apical side, being called microvilli.

Despite the fact that humans are born without any microorganisms in their body, bacterial colonization on the exposed body surfaces starts immediately at birth. The first bacteria that appear in the human gut include *Escherichia coli*, *Lactobacillus spp.*, *Clostridium spp.*, *Streptococcus spp.*, and *Bifidobacterium spp.*, deriving from the birth canal and the mother’s milk. The diverse community of bacteria is considered as an organ-in-organ system, which plays an important role in human health and psychology.

The nutrient balance in the gut lumen is maintained by the host cells, and it serves as a reliable selection factor for certain bacteria species. The gradients of pH, oxygen, and antimicrobial peptides strongly affect the microbiota in different parts of the GI tract (Figure 2.9). Bacteria that are resident in gut microbiota are termed indigenous, as they are distinct from transient bacteria that are passing through the gut lumen without any specific interactions with other gut components. Transient bacteria can colonize the gut under abnormal conditions such as infections, while indigenous bacteria typically colonize particular habitats of the GI tract, depending on pH, oxygen concentration, peristalsis, mucin secretion, nutrient availability, diet and bacterial antagonism. Compared to the other parts of the GI tract, microbial colonization primarily occurs in the colon due to the slow peristaltic motion. Figure 2.10 shows a schematic overview the mucus thickness, which is affected by the abundance of the microbial habitats in the gastrointestinal tract. The
abundance of the dominant phyla is also highly dependent on the diet, age, and living environment of the individuals, and may therefore change accordingly.\textsuperscript{64,66}

2.3.2 The intestinal epithelial cells and the Caco-2 cell line

The epithelial layer is the outer layer of the intestinal wall and is composed of polarized cells with an apical surface towards the intestinal lumen. Intestinal epithelial cells have microvilli on their apical surface and tight junctions with the adjacent cells on their basal surface. The epithelial layer consists of several cell types, including enterocytes, goblet cells, microfold cells, and Paneth cells. Enterocytes constitute about 80 percent of the epithelial layer and are responsible for maintaining nutrient absorption and bacteria-cell attachment.\textsuperscript{67}

Being one of the most extensively studied enterocytes, Caco-2 spontaneously displays typical differentiation and polarization phases. The differentiated and polarized Caco-2 cells develop characteristics of the intestinal epithelium such as brush-border microvilli at the apical surface, tight junctions at the basolateral surface, and vectorial transport of anions and cations through the basolateral surface.\textsuperscript{67} Differentiated cells express brush border enzymes including sucrose, alkaline phosphatase, and aminopeptidase.\textsuperscript{68}

Caco-2 cells are seeded in a glutamine rich medium because glutamine is the most abundant amino acid in the blood and it is required for the production of the growth factors of enterocytes.\textsuperscript{69} Glutamine has also been reported to affect the production of tight junction proteins and the localization

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{gastrointestinal_tract_architecture.png}
\caption{Schematic of the gastrointestinal tract's architecture. Modified from reference 70.}
\end{figure}
of the Caco-2 cells. The cells start to differentiate when they reach confluence. Tight junctions are developed from 5-21 days of culturing, and during this time, the length and density of villi increases. After 21 days, the Caco-2 cells form a monolayer and achieve a full structural polarization. Seeding cell densities influence the cell growth cycle and differentiation and they, therefore, need to be optimized in order to obtain reliable and comparable experimental results in microchip platforms. In addition, polarization and differentiation processes occur in every individual cell in an independent manner. Cell maturation occurs in a stepwise pattern, including homogenously undifferentiated cells when they are sub-confluent, heterogeneously polarized & differentiated cells when they are within 0-20 days after confluence, and homogenously polarized & differentiated cells when they are in more than 30 days of confluence.

2.3.3 | In vitro growth conditions of Caco-2 cells

The platforms used to culture Caco-2 cells may affect the cell response to physiological changes. The material of the platform, its pore size, and the dimensions of the channels define the cell attachment to the surface, and therefore can limit or enhance the growth. In traditional cultures, Transwells made of poly(ethylene-terephthalate) are preferred due to the accelerated cell differentiation. In miniaturized platforms, PDMS is mostly preferred due to its oxygen permeability.

Transwells or microchips are often combined with an extracellular matrix material to provide mechanical support for the cells and promote growth. Matrix proteins modulate both the rates of cell spreading and migration, and the production of brush border specific proteins. Collagen type I and IV are commonly used natural extracellular matrix materials because of their metabolite-rich content and adjustable pore size. The difference between both types of collagen is the peptide composition of the triple helix structure of the collagen fibers. Being composed of collagen fibers, elastin fibers, glycoproteins, and polysaccharides, the collagen matrices readily provide native adhesion sites for cells. Alternatively, synthetic hydrogels are used as extracellular matrices, including polyacrylamide and polyethylene glycol diacrylate. The pore size of the synthetic hydrogels is more controllable than the natural hydrogels; however, these materials must be supplemented with adhesion molecules to ensure the cell attachment. Controllable pore size is important when it comes to oxygen and nutrient transport through the extracellular matrix material. The pore size also determines the stiffness of the hydrogel (or extracellular matrix) and it is specific to each tissue, determining the cell migration, differentiation, and adherence.

In 2D cultures, cells are seeded on the hydrogel matrix to form monolayers, enabling control over the polarization of single cells. Cells are in
contact with substrates and culture media on their basolateral and apical surfaces, while the intercellular interactions occur at the basal surface. 2D cultures are applicable on both conventional large scale platforms and custom-made microchips. As most of the cells reside in 3D microenvironments in the human body, 2D cultures may result in non-natural cell behavior and morphology. 3D cultures have therefore been suggested to generate *in vitro* tissues with a structure very similar to the *in vivo* tissues. In 3D cultures, cells are seeded in the hydrogel matrix to form various spatial configurations. This can be achieved using 3D printing, soft lithography, and hydrogel patterning. Owing to these fabrication methods, long-term cultures from primary adult tissues was achieved by organoid technology without inducing genetic transformation. In a recent study, 3D intestinal epithelial organoids were grown for periods greater than 1.5 years in an *in vitro* culture system. In these cultures, it was found that the success rate of establishing the cultures from individual patient samples, or “living biobanks” is near almost 100%, which is promising to bridge the experimental gap between deep-sequencing efforts in human colon cancer and patient samples.

### 2.3.4 Host-bacteria commensalism

**Dietary substrates**

Host-bacteria commensalism is needed for maintaining the health of a person. For example, fermentable carbohydrates and proteins are processed via bacteria native to the intestinal environment by saccharolytic and proteolytic degradation, respectively, to provide processable forms to human body. *Escherichia coli*, *Bacteroides* spp., *Ruminococcus* spp., *Clostridium* spp., *Bifidobacterium* spp., *Eubacterium* spp., and *Fusobacterium* spp. conduct saccharolytic fermentation by glycosidase enzymes. These bacteria help their host to ferment the dietary nondigestable polysaccharides, such as plant cell walls, resistant starch molecules, short chain fatty acids (i.e. butyrate and acetate), and gases (i.e. hydrogen and carbon dioxide). Certain Firmicutes, such as *Eubacterium* spp. and *Lactobacillus* spp., conduct proteolytic fermentation to degrade lactate into acetate and butyrate, aiding in the maintenance of the host’s lactate metabolism.

**Bacterial attachment**

Bacterial attachment has been extensively studied on Caco-2 cells. Figure 2.11 illustrates the morphology of a Caco-2 cell and bacteria attachment. A mucus layer covers the entire apical surface of the intestine, and creates a boundary between the gut lumen and the host tissue. Mucus glycoproteins, or mucins, are composed of glycoprotein monomers linked with disulfide bridges. The side chains of these substances serve as the substrata for the
attachment of bacteria to the mucosal surface. The attachment requires rigid stereochemical constraints, such as hydrogen bonding, ion-ion pairing, or hydrophobic interactions. These bonds form between a receptor on the human cell surface and an adhesin on the cell surface of the bacteria. The adhesins include deploying outer membrane proteins, capsules, lectins, and fimbriae. For example, *Escherichia coli* has type I fimbriae, which recognize D-mannose as a receptor site on the mucosal surface of human intestinal cells.

One of the benefits afforded to the host by the indigenous bacteria is the colonization resistance against exogenous species. Indigenous bacteria create a barrier by occupying the available attachment sites on the mucus layer with a strong foothold, inhibiting the exogenous species from colonizing the gut. In colonization resistance, indigenous bacteria either secrete growth inhibitors such as bacteriocins, or compete with the exogenous species for metabolic substrates such as fatty acids, which play an important role in the specific cell-bacteria attachment. Several factors limiting the direct access of the bacterial community to host cells are: the mucus layer secreted by goblet cells, antimicrobial peptides secreted by Paneth cells, immunoglobulin A secreted in the small intestine, detection of exogenous species by microfold cells, and available spatial surface on enterocytes.

Figure 2.11. Schematic of the apical and basolateral surfaces of Caco-2 cells. Adapted from reference 66.
2.4 | Desalination by microelectrodialysis

We will provide an overview of the most important characteristics of the electrodialysis process to serve as background for chapter 7.

2.4.1 | Ion exchange hydrogels and electrodialysis

Ion exchange hydrogels are materials that are charge selective for positively or negatively charged ions, or ion-selective. According to the sign of selectivity, hydrogels are either anion-exchange hydrogels (AEH) or cation-exchange hydrogels (CEH). The sign of the charge selectivity is determined by the charged groups attached to the polymer backbone. In CEH, almost all mobile anions (co-ions) are excluded by fixed anionic groups while cations are enriched; the reverse case occurs in AEH (Figure 2.12). These charge selective hydrogels can be used to remove ions from electrolyte solutions by applying an electric field via a process called electrodialysis. In this process, the current in the hydrogels is carried by the abundant counter-ions. In principle, the concentrations of counter-ions and fixed ions are equal; however, the mobilities of counter-ions depend on the hydration radius of the ion and the chemistry of the hydrogel (i.e. crosslinking density). Counter-ions with higher valence and smaller hydrated radius therefore have a higher mobility in an ion exchange hydrogel when compared to counter-ions with lower valence and larger hydration radius.

In the electrodialysis process, multiple AEH and CEH are placed between two electrodes. Each of the elements are separated from each other via channels filled with electrolyte solution. Upon applying an electric field across the system, the electric potential difference drives a mass transfer of

![Figure 2.12. Schematic illustration of an anion exchange membrane with fixed anions on a polymer matrix.](image-url)
ions: anions move towards the cathode while cations move towards the anode. The ions in the electrolyte solution are transported through the membranes: cations through the CEH and anions through the AEH, due to the Donnan exclusion. Thus, only counter-ions can pass ion-exchange hydrogels. As a result, alternating concentrated and depleted streams are obtained. Juxtaposed AEH and CEH thus enable the scalability and controllability of electrodialysis. The desalination process is highly dependent on the strength of the applied electric field, input concentration, and the flow rate of the electrolyte solution.

2.4.2 Ion transport

During electrodialysis, ion transport in the electrolyte solution is caused by diffusion, electromigration, and convection, as described by the Nernst-Planck equation.

\[
J_i = -D_i \nabla C_i - \frac{z_i F}{RT} D_i C_i E + C_i \nu
\]  

Equation 2.17

Here, \( J_i \) is the flux of ions \( i \) (mol m\(^{-1}\) s\(^{-1}\)). The contribution of diffusion is given by Fick’s equation, where \( D_i \) is the diffusion coefficient (m\(^2\) s\(^{-1}\)) and \( C_i \) is the concentration (mol m\(^{-3}\)) of ions \( i \); the contribution of electromigration is given by the electromigration equation where \( z_i \) is the ionic valence, \( F \) the Faraday constant (C mol\(^{-1}\)), \( R \) the gas constant (J K\(^{-1}\) mol\(^{-1}\)), \( T \) the temperature (K), and \( E \) the electric field (V m\(^{-1}\)); the contribution by convection is given by the convection equation where \( \nu \) is the solution flow speed. The mass balance (continuity) equation should also be taken into account:

\[
\frac{\partial C_i}{\partial t} = -\nabla J_i
\]  

Equation 2.18

Combining the equations above with an additional chemical reaction term, and rewriting it in a one-dimensional form, results in:

\[
\frac{\partial C_i}{\partial t} = -\frac{\partial J_i}{\partial x} + \sum r_j = \frac{\partial}{\partial x} \left( D_i \left( \frac{\partial C_i}{\partial x} + C_i \frac{Z_i F}{RT} E \right) \right) - \frac{\partial}{\partial x} (C_i \nu)
\]  

Equation 2.19

where \( r_j \) is the chemical reaction rate (mol m\(^{-3}\) s\(^{-1}\)).

The ionic fluxes can give rise to the accumulation or depletion of ionic charges. During electrodialysis, an electric double layer is formed at the hydrogel/solution interface (similar to the diffuse layers at the glass/solution
interface discussed in section *Electric double layer*). To describe the resulting electrical potential changes, the charge density $\rho = \sum z_i F C_i$ (C m$^{-3}$) is related to the electric field through the Poisson equation:

$$\varepsilon \nabla^2 E = -\rho$$

Equation 2.20

where $\varepsilon$ is the electrical permittivity of the solution (F m$^{-1}$). The combination of the above equations in the Nernst-Planck-Poisson model is often used for the description of ion transport in membrane systems.

### 2.4.3 Ion concentration polarization

Ion concentration polarization is related to diffusion-limited charge transport near ion-selective boundaries, including ion-exchange hydrogels. The electric current in an electrodialysis system is carried by the migration of counter-ions through the permselective membranes or hydrogels under an applied electric field. The electric current increases proportionally with the applied field, and concentration gradients develop at each side of the membrane or hydrogel. The reason for this phenomenon is the difference in ion transport numbers between the electrolyte solution and the ion exchange hydrogel. This difference leads to the depletion of ions in the solution at the hydrogel boundary where the counter-ions are supplied, while ions accumulate at the other boundary and increase in the concentration.$^{86}$ Salt concentration gradients, which are formed in the diffusion layers (Figure 2.13), can supply extra ions by a diffusional flux. When the applied field is too large, however, the conductivity of the electrolyte solution eventually becomes

![Figure 2.13. Schematic representation of ion concentration polarization at an anion exchange hydrogel. Modified from reference 86.](image)
infinitely small at the diluted side of the hydrogel boundary. In this case, increasing the field will no longer lead to an increase in ion transport, and the electric current reaches a diffusion-limited state (or the limiting current region). If higher voltages are applied to the system, the transport of extra ions can occur due to the generation of convection rolls in the electrolyte solution. This regime is termed the overlimiting current regime. More discussion on ion concentration polarization can be found in Mani, Zaltzman, and Yossifon et al.

A nonlinear current-voltage characteristic thus results due to ion concentration polarization, and includes three current regimes that are (1) Ohmic, (2) limiting, and (3) overlimiting current (Figure 2.14). In the Ohmic current regime, the applied electric field and the resulting current are linearly related. The limiting current regime is reached upon complete depletion of ions at the hydrodynamic boundary layer. In the overlimiting current regime, the current is transported by (1) protons and hydroxyl ions generated at the membrane-electrolyte solution boundary due to the dissociation of water molecules, or (2) through coupled convection, which is defined as a secondary liquid flow. The cause of the coupled convection can be due to gravitational convection or temperature gradients, or electroconvection or electric space charge localized near the electrolyte solution-membrane boundary.

The observation of a limiting current in the current-voltage characteristic implies that the system resistance approaches infinity, and thus the point of the maximum ion flux has been reached. Therefore, the observation of a limiting current provides the standard condition for optimum electrodialysis performance.

Figure 2.14. Schematic drawing of a typical current density-voltage curve of a nonpolar ion exchange membrane. Modified from reference 85.
2.5 References

16. Paustian, J.S.; Azevedo, R.N.; Lundin, S.T.B.; Gilkey, M.J.; Squires, T.M. Microfluidic Microdialysis: Spatiotemporal Control over Solution


van den Abbeele, P.; Grootaert, C.; Marzorati, M.; Possemiers, S.; Verstraete, W.; Gérard, P.; Rabot, S.; Bruneau, A.; Aidy, S.E.; Derrien, M.; Zoetendal, E. Microbial Community Development in a Dynamic Gut Model is


To date, optical lithography has been extensively used for *in situ* patterning of hydrogel structures in a scale range from hundreds of microns to a few millimeters. The two main limitations which prevent smaller feature sizes of hydrogel structures are: (1) the upper glass layer of a microchip maintains a large spacing (typically 525 μm) between the photomask and hydrogel precursor, leading to diffraction of UV light at the edges of mask patterns, (2) diffusion of free radicals and monomers results in irregular polymerization near the illumination interface. In this work, we present a simple approach to enable the use of optical lithography to fabricate hydrogel arrays with a minimum feature size of 4 μm inside closed microchips. To achieve this, we combined two different techniques. First, the upper glass layer of the microchip was thinned by mechanical grinding and polishing to reduce the spacing between the photomask and hydrogel precursor, and thereby the diffraction of UV light at the edges of mask patterns. The grinding process reduces the upper layer...
thickness from ~525 μm to ~100 μm, and the polishing reduced the mean surface roughness from 20 nm to 3 nm. Second, we developed an intermittent illumination technique consisting of short illumination periods followed by relatively longer dark periods, which decrease the diffusion of monomers. Combination of these two methods allows for fabrication of $0.4 \times 10^6$ sub-10-micron-sized hydrogel patterns over large areas (cm$^2$) with high reproducibility (~98.5% patterning success). The patterning method is tested with two different types of photopolymerizing hydrogels: polyacrylamide and polyethylene glycol diacrylate. This method enables in situ fabrication of well-defined hydrogel patterns, and presents a simple approach to fabricate 3D hydrogel matrices for biomolecule separation, biosensing, tissue engineering, and immobilized protein microarray applications.

3.1 Introduction

Integrating hydrogel arrays into closed microchips is beneficial for numerous microfluidic applications owing to their capacity to handle small sample volumes and to perform in-parallel analyses.\textsuperscript{15-17} Hydrogel arrays consisting of small-volume subunits enable faster mass transport and provide higher surface-to-volume ratios, which are essential to increase the sample throughput and number of analyses. Although the microfabrication of hydrogel arrays on rigid substrates has been extensively investigated, the ability to do the same in closed microchips has not been sufficiently examined. For the fabrication of patterned hydrogels on the microscale on top of rigid substrates, a variety of techniques have been used, including 3D printing,\textsuperscript{18} soft-lithography,\textsuperscript{19} multiphoton lithography,\textsuperscript{20} and optical lithography\textsuperscript{21}. In 3D printing, hydrogel structures are built up by layer-by-layer deposition via consecutive lithographic steps.\textsuperscript{22} Despite the promise of accurate and fast fabrication, 3D printing provides a poor degree of control over the size distribution of hydrogel structures, which are typically in a scale range from hundreds of microns to tens of millimeters.\textsuperscript{22} Soft lithography techniques, including microcontact printing\textsuperscript{23} and micromolding,\textsuperscript{24} offer inexpensive, convenient, and scalable templates for patterning. However, the usage of polymer molds is unsuitable to pattern hydrogels in closed microchips.\textsuperscript{25} Multiphoton lithography\textsuperscript{20} is known for providing high resolution patterns; on the other hand, complexity of the setup and low area coverage are the main drawbacks of this technique. Masked photolithography\textsuperscript{7,26,27} and laser patterning\textsuperscript{28} are the most preferred optical lithography techniques, which are well-established and have proven to be reliable for patterning hydrogel arrays. When it comes to \textit{in situ} patterning of very small feature sizes using optical lithography in closed microfluidic chips; however, the resolution turns out to be rather poor compared to the resolution obtained on open rigid surfaces. One problem with the resolution is the UV diffraction caused by the thickness of the upper glass layer (typically 525 μm), which maintains a large spacing between the photomask and hydrogel precursor. A second problem is the high diffusion rate of monomers and free radicals under flood illumination, leading to structures with indistinct edges.\textsuperscript{29} As a result, the current state of these techniques cannot satisfy the requirements of emerging applications which call for the use of \textit{in situ} patterned hydrogel arrays with feature size of a few microns.

Previously, our group has introduced the capillary pinning technique for autonomous fabrication of picoliter-volume microarrays of both photopolymerizing and thermo-gelling hydrogels in closed microchips.\textsuperscript{30} Glass obstacles (capillary barriers) were fabricated in microchannels for local pinning of the hydrogel precursor, yielding periodic hydrogel patterns over a 1 cm\(^2\) area. Even though the capillary pinning technique is promising, alternative and
simple fabrication processes to integrate hydrogel microarrays in closed microchips would still be beneficial. Here we report the fabrication of periodic hydrogel structures in a fused silica microchip enabled by optical lithography. After bonding two glass layers of the microchip, the upper layer was ground and polished to reduce its thickness and roughness. This, in combination with an illumination recipe developed to enhance hydrogel boundary definition, allowed us to control precisely the photopatterning of hydrogel microarrays over large areas (cm$^2$) with sub-10 µm feature size and up to 98.5 percent success. For proof of concept demonstration, we fabricated a closed microchip with an array of $0.4 \cdot 10^6$ hydrogel patterns sandwiched between glass pillars using 0.5 µl of hydrogel precursor. Our approach greatly simplifies hydrogel integration into microchips, as the microarrays are fabricated in situ, and can be used with multiple types of photopolymerizing hydrogels.

3.2 Methods

3.2.1 Microchip fabrication

The microchips were fabricated in the MESA+ cleanroom facility at the University of Twente and consisted of two 525 µm thick layers of fused silica. The upper layer contained glass pillar array, microfluidic channels, fluidic inlets and fluidic outlets, while the lower layer was not processed. Figure 3.1 shows an assembled microchip containing a glass pillar array.

![Figure 3.1. (a) An image of the microchip with glass pillars, microfluidic inlets and outlets. (b) Optical microscopy image of glass pillar array (light colored rectangles) and air filled channels (darker colored areas). (c) Tilted top view of glass pillars under SEM. The depth, width, and length of pillars are 20 x 20 x 5 µm, respectively.](image)

We used standard reactive ion etching (RIE) for the fabrication of the glass pillars and microfluidic channels on the upper layer (Figure 3.2). Before the RIE process, the upper layer was spin-coated with a negative photoresist (SU-8 2050, MicroChem) to protect the underlying layer from etching. An optical lithography step was then performed to pattern the pillars and microfluidic channels, which were subsequently postbaked and developed before etching by RIE. The SU-8 layer was then stripped in a piranha bath. Etched channels were ~20 µm deep, as measured using the Dektak 150 Surface Profiler (Bruker). Scanning electron microscopy (SEM) images of the etched structures were taken using a JEOL JSM 5610 (Jeol) field emission scanning electron microscope.
Fluidic inlets and outlets were powderblasted on the backside of the upper layer using an Ordyl tape resist (BF410; Tokyo Ohka Kogyo) for masking. After lamination and optical lithography, the tape resist was developed using 1% of NaHCO$_3$ and subsequently removed by acetone once the powderblasting process was completed. Finally, the processed layer was cleaned and thermally bonded with a plain fused silica layer at 1080°C.

3.2.2 | Grinding and polishing

The upper layer of the microchip was laminated with a protection tape to avoid clogging of microchannels during the grinding and polishing processes. The microchip was placed in an Engis 15 grinding and polishing tool consisting of diamond cup wheels, a porous ceramic chuck, and a vacuum holder. Silicon carbide particles (particle sizes: 280, 400, 800 and 1000 μm) were mixed with deionized water for the grinding process. Surface roughness was reduced by polishing the lower layer using “Kemet Vloeistof, Type K” solution (Kemet), which consisted of water-treated light oil and surface-modified silica particles. The microchip was subsequently cleaned with deionized water and the protection tape was removed.

3.2.3 | Fabrication of hydrogel structures

Surface silanization

Microchannels were silanized to enable the formation of covalent bonds between the glass layer and the hydrogel. Firstly, the microchip was cleaned in 0.1 M NaOH for 30 min in an ultrasonic bath. Microchannels were then rinsed with deionized water and placed in a solution of 2: 3: 5 (v/v/v) 3-trimethoxysilylpropyl methacrylate (Sigma): glacial acetic acid (Sigma): deionized water for 60 min. Finally, the microchip was rinsed with acetone for 1 min, then with deionized water for 2 min, and dried at 110°C for 7 min to promote covalent siloxane bond formation on the glass layer.

Preparation of hydrogels

It is crucial to take the next experimental step immediately after finishing the previous one during preparation and polymerization of hydrogel precursors, since > 1 min waiting times can influence the degree of oxygenation and, therefore, polymerization. Milli-Q water was used in all experiments. All solutions were degassed under 92 kPa vacuum for an hour immediately prior to use. Polyacrylamide precursor was prepared in a > 99% N$_2$ containing glovebox by blending 20% v/v of acrylamide/bis (19:1) (BioRad), 2% w/v of 2,2-dimethoxy-2-phenylacetophenone (DMPA, Invitrogen) and 2% w/v of ammonium persulfate (Invitrogen) solutions in a fume hood to avoid dust particles.
Patterning and polymerization were also performed in a > 99% N\(_2\) containing glovebox, because the crosslinking reaction is quenched by O\(_2\). Concentration of O\(_2\) was measured using an oximeter (GMH 3691, Greisinger) and > 1% O\(_2\) was observed to inhibit the polymerization reaction. It is possible to replace N\(_2\) with Ar when polymerizing polyacrylamide. Polymerization did not occur when 1 ml of each solution was not degassed for less than 1 hour.

Polyethylene glycol diacrylate (PEG DA) (MW 3400; Laysan Bio) precursor was prepared in a fume hood by dissolving the PEG DA powder in 15% w/v of PBS solution and blending the final mixture with 10% w/v of 2-hydroxy-1[4-(2-hydroxyethoxy)-phenyl]-2-methyl-1-propane (Irgacure 2959; Sigma) in ethanol solution. The molar fraction of the PEG DA monomer was 15%, while that of the Irgacure was 85%. A high molar fraction of the photoinitiator was used to maintain the polymerization yield at 365 nm wavelength. Possible contact with light was avoided during the preparation of PEG DA precursor solution.

All precursor solutions were sonicated with a VWR Ultrasonic Bath USC300D at full power for 1 hour and then degassed under 7 kPa vacuum prior to use, as void-free hydrogels are critical. Figure 3.3 outlines the hydrogel fabrication process. Immediately after preparation, 0.5 μl of polyacrylamide or PEG DA precursor was quickly pipetted into the microchip via the inlets. The silanized microchannels and void space in the microchip were then filled by capillary forces.
Illumination procedure

The microchip was carefully aligned and brought in direct contact with a chromium photomask consisting of periodic rectangular- or square-shaped patterns (Figure 3.3, Figure 3.4, and Figure 3.5). An optical mask alignment system (EVG 620) or transparent tape was then used to hold the photomask and microchip together. Hydrogel precursor solution was exposed to UV light through the photomask using a mercury arc lamp with a light uniformity of

**Figure 3.4.** Phase contrast microscopy image of photopolymerized polyacrylamide hydrogel. Grey squares are glass pillars and blue rectangles are hydrogels. (a) Photopolymerization by 10 min UV exposure with 2 sec on 4 sec off periods using an illumination intensity of 12 mW cm⁻² and (b) photopolymerization by 10 min flood exposure.
100 mm ± 2% for 365 nm wavelength and with a spectrum range of 350 to 450 nm. The UV lamp was modulated in a mask alignment system and had a software controlled mechanical shutter. The illumination intensity of the UV light source was 12 mW cm\(^{-2}\), which was measured using a UV intensity meter. The total exposure duration was 10 min with intermittent illumination cycles (2 sec on followed by 4 sec off). After exposure, the non-crosslinked hydrogel precursor was removed from the main channels by vacuum suction. Images of patterned hydrogels were recorded immediately after the patterning process and processed microchips were kept in deionized water at room temperature.

### 3.3 Results and discussion

Our approach consists of polishing the upper glass layer of the microchip, injecting hydrogel precursor into the microchannels, applying optical lithography with intermittent illumination to pattern spatially defined hydrogel microstructures, and removing non-crosslinked hydrogel precursor by vacuum suction. Inside the microchip, rectangular- or square-shaped glass pillars provide mechanical stabilization of microchannels and hydrogel structures, while microfluidic inlets and outlets facilitate precise control of the fluids.

#### 3.3.1 Grinding and polishing

The minimum resolution of the hydrogel structures has been shown to be directly proportional to the square root of the spacing between hydrogel precursor and photomask.\(^{32}\) In our case, the spacing is determined by the upper glass layer thickness, which has to be minimized in order to improve the structure resolution. To create structures with a minimum feature size of 4 μm, it is essential to grind and polish the upper glass layer. We ground the upper glass layer to reduce its thickness from ~525 μm to ~100 μm, hence, the overall thickness of the microchip was reduced from 1050 μm to ~600 μm. It is also possible to combine a 525 μm-thick upper layer with a 1000 μm-thick lower layer to increase the stability of the microchip after the grinding process.
During grinding, the surface roughness increased from ~20 nm to 1200 nm due to the size of the silicon carbide particles. Since this roughness will cause UV light diffraction, the surface was subsequently polished to reduce the mean surface roughness to ~3 nm. Polishing improved the fidelity with which the pattern of the photomask is transferred to the polymerized hydrogel. Fidelity was determined by counting the hydrogel structures with widths that showed 90-110% similarity to the patterns on the photomask. When compared to the patterning results of a non-polished surface, the polished surface increased the fidelity by ~20%. The integrity of the pattern transfer was characterized by optical microscopy and SEM. Figure 3.5 contains examples of fabricated hydrogel structures (a) with indistinct edges when an unpolished upper layer was used, and (b) with high fidelity pattern transfer when a polished upper layer was used in the patterning process.

### 3.3.2 Surface functionalization

In early experiments, we fabricated microarrays of polyacrylamide and PEG DA hydrogels in microchannels that had not been functionalized with an adhesion-promoting silane layer. While we could successfully pattern hydrogel microarrays using this approach, individual hydrogel structures easily detached from the glass surface when excess precursor solution was removed via vacuum suction, due to the weak surface attachment. To prevent detachment, 3-trimethoxysilylpropyl methacrylate was coated on the inner walls of the microchip. The methacrylate contains unsaturated C=C functional groups which attached to the glass surface during surface functionalization. When exposed to UV light, these functional groups react with radical species of the hydrogel precursor, and form covalent bonds between hydrogel and the glass surface. A detailed explanation of the silanization process and its optimization was reported by Vidić et al. Surface functionalization allowed the hydrogels to attach to the glass surface during photopolymerization, eliminated the detachment problems, and increased the mechanical stability of the hydrogel structures in the chip. Figure 3.6 shows the cross section of a microchannel where a polyacrylamide hydrogel block was photopolymerized between two glass pillars. It can be seen that the polymerized hydrogel is in contact with both the top and bottom surfaces of the microchannel.

### 3.3.3 Optimization of patterning resolution

**Polymerization process and the effect of diffusion**

Both polyacrylamide and PEG DA precursors create negative patterns upon exposure to UV light; in other words, the regions of the hydrogel precursor exposed to UV light form a crosslinked polymer network while masked regions stay non-crosslinked. In the illuminated region, photoactivation of the
photoinitiator leads to the generation of free radicals in non-crosslinked hydrogel precursor.\textsuperscript{35} Figures 3.7 and 3.8 give an overview of the polymerization reactions for polyacrylamide and PEG DA hydrogels. Free radicals start the crosslinking process by randomly associating with monomers and growing polymer chains, which eventually become polymerized in the illuminated region. Free radicals also diffuse from the illuminated region to the masked region due to the concentration gradient formed (Figure 3.9). Diffusing free radicals will lead to irregular polymerization by extending the polymerized area towards the masked region.\textsuperscript{36} Similarly, monomers will diffuse from the masked region to the illuminated region, where they are consumed by the crosslinking reaction. Fuxman\textsuperscript{37} and Vergote\textsuperscript{38} showed that diffusion of monomers creates an edge overshoot at the illumination interface, by modeling hydrogel-based dosimeters with high intensity radiation doses. Since both processes are diffusion-driven, they are affected by a number of factors: (1) illumination intensity, (2) illumination wavelength, (3) the rate of production of free radicals, (4) the rate of consumption of free radicals and monomers by polymerization, (5) the diffusion rate of the free radicals towards the masked region, (6) the diffusion rate of monomers towards the illuminated region, and (7) the shape and dimensions of the photomask.\textsuperscript{39,40} All these effects make accurate control of the structure resolution difficult.

**Polymerization propagation and diffusion rates**

We optimized the polymerization rate for both polyacrylamide and PEG DA by tuning the monomer concentration, photoinitiator concentration, and diffusion rates of both monomers and crosslinkers. Two different photoinitiators with different molar absorptivities at 365 nm wavelength were also used for the two hydrogels. Polymerization and diffusion rates of polyacrylamide and PEG DA will be calculated and compared in this section.
The initiation rate for chain polymerization, \( R_i \), is given by \(^{41-43}\)

\[
R_i = \frac{2\varnothing \varepsilon f I C_i}{N_A h \nu}
\]

Equation 3.1

where \( I \) is the incident light intensity (a constant 12 mW cm\(^{-2}\) is taken), \( C_i \) is the photoinitiator concentration, \( \varepsilon \) is the molar absorptivity, \( \varnothing \) is the quantum yield, \( f \) is the photoinitiator efficiency, \( N_A \) is Avogadro’s number, \( h \) is Planck’s constant and \( \nu \) is the frequency of the initiating light. Table 3.1 shows the parameters used for calculations in this study. \(^{42-46}\) The molar absorptivity of DMPA, the photoinitiator used for polyacrylamide, was reported as 15.000 M\(^{-1}\)m\(^{-1}\) at 365 nm wavelength under a 50 mW cm\(^{-2}\) of illumination intensity. \(^{42}\) We find the initiation rate of DMPA as 0.05 mol m\(^{-3}\) s\(^{-1}\) from Equation 3.1. For polyacrylamide, the polymerization proceeds by forming a linear network of a crosslinker and a monomer with occasional interchain crosslinks (Figure 3.7). The rate of chain propagation is the result of the initiation rate and the chain termination rate and in the steady state approximation is given by

\[
\nu_p = \frac{k_p}{\sqrt{k_t}} \sqrt{\frac{R_i}{2}[M]}
\]

Equation 3.2

where \( k_p \) is the propagation rate of the polymer, \( k_t \) its termination rate, and \([M]\) the initial monomer concentration. From Equation 3.2, using the value for \( k_p / \sqrt{k_t} \) of 3.3 (at 25°C) reported by Curry et al., \(^{41}\) we obtain the chain propagation rate of polyacrylamide, \( \nu_{p,PA} = 105 \) mol m\(^{-3}\) s\(^{-1}\).

In PEG DA, every monomer is a crosslinker; however, the unsaturated C=C functional groups, which propagate the crosslinking reaction, are sterically hindered due to the neighboring methyl group (Figure 3.8). Therefore, \( k_p \) of PEG DA is slightly lower than \( k_p \) of polyacrylamide. Irgacure 2959 was used as the photoinitiator of PEG DA, having a molar absorptivity of 400 M\(^{-1}\)m\(^{-1}\) at 365 nm wavelength under 10 mW cm\(^{-2}\) illumination intensity. \(^{43}\) From Equation 3.1 the reaction rate initiated by Irgacure 2959 was found as 0.02 mol m\(^{-3}\) s\(^{-1}\). The chain propagation rate of PEG DA is then calculated as \( \nu_{p,PEG} = 33 \) mol m\(^{-3}\) s\(^{-1}\) using Equation 3.2, \( k_p \) (25 m\(^3\) mol\(^{-1}\) s\(^{-1}\)), and \( k_t \) (2520 m\(^3\) mol\(^{-1}\) s\(^{-1}\)) reported by Dendukiri et al. \(^{47}\) In water, the diffusion coefficients for both Irgacure 2959 and DMPA are given as 3·10\(^{-10}\) m\(^2\) s\(^{-1}\) in Fang et al., \(^{35}\) while those for both acrylamide and bis are given as 4·10\(^{-10}\) m\(^2\) s\(^{-1}\) in Fuxman et al., \(^{37}\) and the diffusion coefficient for PEG DA monomer is given as 1.5·10\(^{-11}\) m\(^2\) s\(^{-1}\) in Harada et al. \(^{48}\)
When monomers and free radicals are consumed by the polymerization reaction in the illuminated region, a concentration gradient is formed. Fick’s second law gives the resulting concentration changes:

\[
\frac{dC}{dt} = D \frac{d^2C}{dx^2}
\]

Equation 3.3

where \(D\) is the diffusion coefficient, \(C\) is the concentration of monomers, \(t\) is the time, \(x\) is the direction normal to the illuminated region. From Equation 3.3, we can calculate the concentration difference over a distance of 10 μm (that is the distance between the midpoint of the illuminated region and the border of the masked region), when \(\frac{dC}{dt} = 0\) (i.e. when diffusional monomer supply to the illuminated region is in balance with the monomer consumption in this region). We find that the generated concentration difference is 26 mol m\(^{-3}\) and 33 mol m\(^{-3}\) for polyacrylamide and PEG DA, respectively. Both values are comparable to the initial monomer concentrations, which are 400 mol m\(^{-3}\) and 100 mol m\(^{-3}\) for polyacrylamide and PEG DA, respectively. For both polymers a continuous 12 mW cm\(^{-2}\) illumination intensity is therefore too high as it depletes monomers and will cause diffusion of free radicals to the masked region.

Table 3.1. Parameters used in this study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for DMPA</th>
<th>Value for Irgacure 2959</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\varepsilon)</td>
<td>15000</td>
<td>400</td>
<td>M(^{-1}) m(^{-1})</td>
<td>42, 43</td>
</tr>
<tr>
<td>(\varnothing)</td>
<td>1</td>
<td>0.3</td>
<td>-</td>
<td>44, 45</td>
</tr>
<tr>
<td>(f)</td>
<td>0.6</td>
<td>0.5</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>(I)</td>
<td>120</td>
<td>120</td>
<td>W cm(^{-2})</td>
<td>this work</td>
</tr>
<tr>
<td>(C_i)</td>
<td>400</td>
<td>100</td>
<td>mol m(^{-3})</td>
<td>this work</td>
</tr>
</tbody>
</table>

\(^a\) These values were used to calculate \(R_i\) and propagation rates. The different sources in the literature used to collect the values are indicated in the rightmost column.

Illumination recipe

An intermittent illumination technique was previously used to fabricate single hydrogel membranes using solid state and pulsed UV laser setups.\(^{49,50}\) In our case, the intermittent illumination strategy was chosen due to the limitations of our equipment that does not allow for using a neutral density filter or changing the light intensity. The dark period of 4 sec was chosen to maintain the diffusional relaxation time over 10 μm distance for PEG DA monomers, which is 3.3 sec. The dark period was then combined with an illumination period of 2 sec since high-resolution patterning results were obtained for both hydrogels in this illumination scheme. In contrast to intermittent illumination, continuous
illumination (see section 3.3.3.6) was found to result in polymerization in the entire chip (Figure 3.9).

We also investigated possible temperature changes during polymerization. Figure 3.10 demonstrates the temperature change of the microchip during the intermittent illumination and flood illumination. The results show that the temperature rise due to UV absorption was 0.5°C after 10 min of intermittent illumination (2 sec on followed by 4 sec off), while it was ~3°C after 10 min of continuous illumination. Since the illumination intensity
was as low as 12 mW cm\(^{-2}\) in our study, only small temperature gradients were apparently formed in the microchip, leading to trivial changes (~5\%) in the diffusion fluxes (Figure 3.10).

Using the intermittent illumination approach, hydrogel patches were successfully patterned between glass pillars and remained separated from each
other in both horizontal and vertical directions. Figure 3.4 illustrates the difference between hydrogel patterns fabricated using intermittent illumination cycles and continuous exposure. Longer UV illumination periods were found to increase the width of the hydrogel features and reduced the patterning resolution. Figure 3.11 shows arrays of polyacrylamide and PEG DA structures fabricated using intermittent illumination.

**Photoinitiator and monomer concentrations**

In addition to polishing the upper glass layer and applying an intermittent illumination recipe, we studied the influence of the initial photoinitiator and monomer concentrations on the patterning success. We used DMPA and Irgacure 2959 photoinitiators to crosslink polyacrylamide and PEG DA hydrogels, respectively. We observed that varying the photoinitiator concentration did not affect the patterning resolution (Table 3.2). Liu et al. reported a similar result, suggesting that the resolution of hydrogel structures is independent of the initial photoinitiator concentration. We also observed that polyacrylamide and PEG DA precursor solutions did not crosslink at concentrations lower than 2% (v/v) of DMPA and 10% (v/v) of Irgacure 2959, respectively.

**Table 3.2.** Comparison of hydrogel polymerization times between 1 ml tubes and microchips. The results present the mean and standard error deviations, n ≥ 3.

<table>
<thead>
<tr>
<th>Monomer concentration (%T)</th>
<th>DMPA (%)</th>
<th>UV Exposure*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Polymerization in tube (V = 1 ml)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>NP</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>1.5 ± 0.5 min</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>20 ± 10.4 sec</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>15 ± 5.6 sec</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>10 ± 4.3 sec</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monomer concentration (%)</th>
<th>Irgacure (%)</th>
<th>UV Exposure*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Polymerization in tube (V = 1 ml)</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>NP</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>6 ± 1.4 min</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>3 ± 0.5 min</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>1 ± 0.2 min</td>
</tr>
</tbody>
</table>

NP: no polymerization or partial polymerization longer than an overnight.

*: UV exposure was applied at 365 nm, with 12 mW cm$^{-2}$ intensity, and using intermittent illumination cycles (2 sec on followed by 4 sec off).
In contrast, photopolymerization time, which is needed to crosslink all the precursor in the illuminated region, and structure resolution were found to be dependent on initial monomer concentration. Under intermittent illumination, we observed that the polymerization time decreases with increasing monomer concentration, resulting in well-defined but highly dense hydrogel structures. Reducing the monomer concentration led to structures with indistinct edges and a softer texture probably due to the formation of the hydrogel with a larger pore size. Table 3.2 summarizes the relation between monomer concentration and polymerization time for both hydrogel types in

**Table 3.3** Patterning success versus upper glass layer thickness. The results present the mean and standard error deviations, n = 2. Approximate values are given for the upper layer thicknesses.

<table>
<thead>
<tr>
<th>Upper layer thickness (μm)</th>
<th>Patterning success (%)</th>
<th>Standard deviation (%)</th>
<th>Polyacrylamide</th>
<th>Patterning success (%)</th>
<th>Standard deviation (%)</th>
<th>Polyacrylamide</th>
<th>Patterning success (%)</th>
<th>Standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>98.5</td>
<td>0.8</td>
<td>98.7</td>
<td>1.0</td>
<td></td>
<td>Polyacrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>94.9</td>
<td>4.5</td>
<td>95.5</td>
<td>4.7</td>
<td></td>
<td>Polyacrylamide</td>
<td>20.0</td>
<td>10.3</td>
</tr>
<tr>
<td>250</td>
<td>37.7</td>
<td>10.6</td>
<td>20.0</td>
<td>10.3</td>
<td></td>
<td>Polyacrylamide</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>400</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td>Polyacrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>525</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td>Polyacrylamide</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 3.9](image-url) **Figure 3.9.** Schematic diagram of the crosslinking reaction in the hydrogel under (a) high and (b) low intensity UV illumination.
Hydrogel Microarray Fabrication by Photopatterning

Eppendorf tubes and microchips. It is important to note that polymerization was observed to take longer time in the microchip (with a volume of 0.5 μl) compared to polymerization in the large volume Eppendorf tube (with a volume of 1 ml). Polyacrylamide has been observed to fail crosslinking below 2.5%T (%T refers to the total mass of monomer) in both Eppendorf tubes and microchips, while PEG DA fails crosslinking at 5% concentration and below.

**Patterning success evaluation**

The patterning success was evaluated based on the similarity between the hydrogel patterns in the microchannels and structures on the photomask. Similarity was defined as actual hydrogel size relative to the structures on the photomask expressed as percentage. Three representative images were
collected from two opposite corners and a middle section of the arrays, each containing 250 hydrogel subunits.

Hydrogel structures with 90-110% similarity were considered successful, while structures with less similarity or indistinct edges were considered failures. Hydrogel patterns larger than 110% and smaller than 90% of the photomask structures were not observed when the intermittent illumination recipe was applied. Patterning success and resolution were found to improve for small feature sizes with a thinner glass cover thickness for both polyacrylamide and PEG DA hydrogels, as shown in Table 3.3. Upper glass layer thicknesses larger than 115 μm were not suitable for patterning feature sizes of 4 μm, hydrogel structures could not be obtained with thicknesses of 250, 400, and 525 μm, regardless of the UV exposure time.

We also investigated the possibility that the size of the features after photopolymerization could be impacted by swelling or shrinking of the hydrogels after evacuating the microchip, or after changing aqueous media conditions. To achieve this, we compared the volume changes of hydrogels just after the fabrication (when the surrounding environment was dry) and after 24 hours in aqueous media. Swelling ratios were found as 1-2% for polyacrylamide and 5-6% for PEG DA. Since both of the hydrogels are of zero net charge, the swelling or shrinking behavior was not substantial. With these fabrication and processing techniques, we successfully patterned 0.4·10⁻⁶ per cm² hydrogel structures, each with ~1.25 pl volume, with a ~98.5% patterning success using both polyacrylamide and PEG DA hydrogels.

**Other illumination strategies**

The hydrogel precursor was exposed to continuous illumination for 1, 3, 5, 7, and 10 min. While partial polymerization (~15% of the precursor volume) was observed after 1 min, a faithful reproduction was obtained by 3 min of exposure. The energy of 3 min exposure is equal to approx. 10 min intermittent...
illumination (2 sec on followed by 4 sec off cycles). In continuous illumination cases, except for 1 min, photomask patterns could not be transferred to the microchip. Figure 3.4a demonstrates the polymerization after 10 min of continuous illumination. In Figure 3.4b, hydrogel arrays contain defect-free subunits with no hydrogel precursor residue remaining in the main channels. The same image shows the polymerization result for intermittent illumination for 10 min, before which hydrogel patterns were not fully polymerized.

A couple of more strategies could further reduce the feature size; i.e. increasing the distance between the hydrogel structures, using a thinner upper layer, reducing the illumination intensity and shortening the exposure times. However, the spatial resolution of the microstructures will be limited by UV light diffraction, diffusion of hydrogel components, and wavelength of the UV light.

3.4 Conclusions

In this work, we demonstrated a simple and reproducible photopatterning process for micron-sized hydrogel arrays inside a closed microchip. Fabricating periodic hydrogel microarrays in polished microchips by optical lithography is preferable to the vast majority of previously demonstrated patterning methods because of the sub-10-micron-sized features, improved resolution, and high reproducibility. However, while capillary pinning method provides the patterning of both photopolymerizing and thermo-gelling hydrogels, only photopolymerizing ones can be patterned using the present technique.

This technique is based on mechanically polishing the top glass layer to reduce the spacing between the photomask and hydrogel precursor, and applying intermittent illumination for enhancing the definition of hydrogel boundaries. Microfluidic networks consisting of $0.4 \times 10^6$ periodic picoliter-volume hydrogel patterns can easily be patterned over 1 cm$^2$ areas with $\sim$1.5% failures. We experimentally showed that upper glass layer thickness, monomer concentration, and different optical lithography recipes have a significant effect on structure resolution. By tuning these parameters, a minimum feature size of 4 μm was patterned using both polyacrylamide and PEG DA 3400 hydrogels. Using this method, various shapes and thicknesses of periodic hydrogel structures can be patterned by adjusting the pillar size, pillar shape, and channel depth. This simple and effective fabrication strategy allows for direct integration of hydrogel microarrays into microfluidic systems. It further holds great potential in the fabrication of 3D hydrogel matrices for elucidation of fundamental structure-function relationships, biomolecule separation, biosensing, tissue engineering, immobilized protein microarrays, subdivided-hydrogel microarrays for bacteria cultures, and 3D cell cultures.
3.5 References


Capillary barriers provide a simple and elegant means for autonomous fluid-flow control in microfluidic systems. In this work, we report on the fabrication of periodic hydrogel microarrays in closed microfluidic systems using non-fluorescent capillary barriers. This design strategy enables fabricating picoliter-volume patterns of photopolymerized and thermogelling hydrogel types without any defects and distortions.

4.1 Introduction

Selective hydrogel patterning offers a novel way to expand the capability of biological and clinical microarrays, gel-based lab-on-a-chip bioassays, cell patterning techniques, and biomolecule separation technologies. A reduction in size to small volumes enables dramatic increases in number of analyses and throughput of hydrogel microarrays due to faster mass transport and increased surface to volume ratios. Despite the large promise of small volume hydrogel microarrays, their fabrication has remained challenging. Here we demonstrate that picoliter volume microarrays of photopolymerizing and thermo-gelling hydrogel types can be robustly and autonomously fabricated by capillary pinning in microfluidic devices. The method enables patterning in closed microfluidic systems entirely manufactured from non-fluorescent materials.

Hydrogels have found widespread use in microfluidic systems due to their unique material properties. They provide excellent sensitivity to chemical and physical stimuli such as pH, ionic strength, temperature, electric field, and light. Hydrogels have been used for electrokinetic biomolecule separations for over a century, as they provide a dense mesh of porous three dimensional matrix without a significant effect on electrolyte composition. These functionalities have brought hydrogel usage to the fore in wireless biomolecule measurements, two dimensional (bio)molecule separation, drug delivery, microdialysis, and biosensing applications with microfluidic devices. The incorporation of hydrogels in microfluidic systems is commonly accomplished by traditional methods such as optical and soft lithography. The majority of optical lithography techniques are based on masked photolithography and laser patterning. Optical lithography has proven to be a well-established and reliable method. However, patterning via conventional photomasks comes at the cost of poor structure resolution in closed platforms due to diffraction of UV light from the microchip walls and uncontrollable free radical diffusion during polymerization. Hence, working with conventional lithography photomasks poses serious challenges when the aim is to fabricate hydrogel microarrays in tens of micrometers scale without any defects. Soft lithography techniques, including microcontact printing and micromolding, offer inexpensive, convenient, and scalable templates for patterning. However, these techniques require polymer (polydimethylsiloxane) molds for patterning and therefore are not suitable for hydrogel fabrication in closed microfluidic systems.

For the hydrogel array applications mentioned above, a high degree of control over the shape and the size distribution of hydrogels down to micrometer scale is needed. In the past, capillary valves (‘phaseguides’) have been successfully implemented in closed microchips to pattern hydrogel structures by local pinning of the hydrogel precursor in a scale range from ~100 of microns to tens of millimeters. Using this method, a maximum number of
~400 pinned liquid patches, each containing a few microliters of liquid, were patterned. In addition, phaseguide arrays presented in the literature were made of SU-8, a photopatternable polymer, that was chosen for its relatively less hydrophilic character ($65 < \theta < 85^\circ$, $\theta$ is the contact angle) in comparison with glass surfaces.\(^2\) This approach hinders fluorescence-based biomedical applications because SU-8 is strongly autofluorescent, overlapping with the emission bands of many fluorescent tagging agents.\(^3\) Both the large size and the fact that structures made of SU-8 present limitations to this approach. Further development of the phaseguide technique is thus warranted for applications where large scale patterning of picoliter volume hydrogel patches in large scale areas and/or the use of non-fluorescent polymers in fabrication are necessary.

In this work, we fabricated massively parallel hydrogel patterns by capillary pinning followed by photopolymerization or thermo-gelation in closed microfluidic platforms. Capillary pinning barriers were made of fused silica glass, which is a non-fluorescent material. Despite fused silica provides hydrophilic surfaces ($\theta < 25^\circ$) unfavorable for pinning,\(^2\) we show that silanization can enable the production of periodic hydrogel patterns on this material. Capillary barriers allow for autonomous and precisely controlled trapping of the hydrogel precursor solution over large areas (cm\(^2\)), with high reliability and spatial resolution, and without any defects and distortions. For a proof of concept demonstration, we fabricated a closed microchip with an array of ~400k hydrogel patterns, sandwiched between ~400k glass pillars, and ~800k capillary barriers using only 0.5 μl of hydrogel precursor. We showed that large scale microarray patterning by capillary barriers is applicable both to photopolymerized and to thermogelling hydrogel types.

### 4.2 Methods

#### 4.2.1 Microchip fabrication

The microfluidic devices were fabricated in the MESA+ cleanroom facility at the University of Twente. Microchips were structured in fused silica glass wafers and consisted of two plates. The top plate contained capillary barriers, channels, buffer reservoirs, and fluidic inlets and outlets, while the bottom plate remained unprocessed. Figure 4.1a and 4.1b show an assembled microchip with glass pillars and capillary barriers.

This design was fabricated using two consecutive reactive ion etching (RIE) steps to define glass pillar and capillary barrier height for hydrogel containment. Before each RIE process, an SU-8 layer was spin-coated on the wafer. The SU-8 layer served as a mask during the subsequent dry etching process to fabricate the structures in the underlying wafer. A photolithography step was performed to pattern the structure layout in SU-8. After the postbake step, the exposed layer was developed and dry etched. Subsequently, the SU-8
layer was stripped in a piranha bath. The etch depth of the structures was 15 μm measured after the first RIE step. After the second, the etch depth was 20 μm for the channels and 15 μm for the capillary barriers. Final capillary barrier structures were uniform, stable and reproducible. Figure 4.1c depicts glass pillars, capillary barriers, and channels after the second RIE step. Buffer reservoirs together with fluidic inlets and outlets were opened on the back side of the wafer by powder blasting. To pattern the holes, the wafer was laminated with an Ordyl tape resist (BF410; Tokyo Ohka Kogyo). The foil was then developed using 1% of NaHCO₃, powder blasted with Al₂O₃ particles, and subsequently removed by acetone. Finally, the processed wafer was thermally bonded with a plain fused silica wafer. The minimum feature size obtained by the classical lithography and reactive ion etching processes are emerging as physical limitations in the hydrogel patterning by capillary barriers. This limitation can become an issue in the next generation microfluidic devices where less than picoliter volume size hydrogel patterns are needed.

4.2.2 Surface silanization

Silanization allows the hydrogel to covalently bond to the glass surface, increases the mechanical stability of the hydrogel structures in the chip, and decreases the surface wettability of the fused silica. The inner walls of the microchip were therefore silanized to enable the formation of covalent bonds between the glass plate and the hydrogel. To achieve this, the microchip was cleaned in 0.1 M NaOH for 30 min in an ultrasonic bath. The microchip was rinsed with DI water and placed in a solution of 2:3:5 (v/v/v) 3 trimethoxysilyl propyl methacrylate (Sigma): glacial acetic acid (Sigma): DI water for 60 min. After rinsing with DI water and acetone, the microchips were dried at 110°C to promote covalent siloxane bond formation to the glass surface. Figure 4.2a shows a photopolymerized polyacrylamide hydrogel block firmly adhered to the silanized glass plate.
4.2.3 Hydrogel preparation and patterning

Polyacrylamide hydrogel precursor was prepared by blending 20% solution of acrylamide/bis (19:1) (BioRad), 2% 2,2 dimethoxy 2 phenylacetophenone (Invitrogen) and 2% ammonium persulfate (Invitrogen) solutions. A pore size range between 10 and 50 nm was obtained, as shown in Supporting Information, Figure 4.2b. Preparation, patterning and polymerization of precursor solution were performed under N\(_2\) flow due to the oxygen sensitivity of polyacrylamide. Polyethylene glycol diacrylate (PEG DA) (MW 3400) (Laysan Bio) was dissolved in PBS at 15% w/v and combined with 10% 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propane (Irgacure 2959) (Sigma) solution to prepare PEG DA hydrogel precursor. No special equipment was used in preparation, patterning and polymerization of PEG DA precursor solution. Collagen hydrogel precursor was prepared by blending purified collagen solution (Advanced Biomatrix) with 10X PBS, and 1 M NaOH (Collagen: PBS: NaOH ratio of 8:1:1). Agarose hydrogel precursor was prepared by dissolving ultrapure agarose powder (Invitrogen) in 1X TBE buffer at 0.5% w/v in a hot water bath. Preparation and gelation of collagen and agarose precursor solutions were performed in ambient conditions. Patterning of agarose hydrogel was performed on a hot plate adjusted to 45°C.

Figure 4.3 outlines the hydrogel fabrication process. Immediately after the silanization, the precursor solutions were injected into the closed microchip. The entire void space in the chip was then filled by capillary action owing to the positive pressure applied through pipetting. Excess precursor solution was subsequently removed by vacuum suction through the outlets. In this stage, the hydrogel precursor remained contained between the pillars due to capillary pinning at the barriers. No air bubbles were trapped in the microchip during filling and emptying. Patterned polyacrylamide and PEG DA hydrogel precursors were then cured by UV light at 400 mW cm\(^{-1}\) for 3 min, while patterned collagen hydrogel precursor was placed in an incubator at 37°C and patterned agarose hydrogel precursor was cooled to room temperature for
gelation. Processed microchips were kept in DI water at room temperature. In this process, ~1.25 pl hydrogel structures with 4 x 20 x 20 μm dimensions were produced for all of the photopolymerized and thermogelling hydrogel types, confirming the versatility of the method. The final hydrogel patterns were robust with a patterning consistency of more than 99% as seen in Table 4.1, and Figures 4.4 and 4.5.

**Figure 4.3.** Fabrication process flow for custom hydrogel patterning in a closed microfluidic chip. Scale bars indicate 15 μm for all images.

**Figure 4.4.** Comparison of the patterning consistency of microchips with no barriers, parenthesis-shaped barriers and slash-shaped barriers for polyacrylamide, PEG DA, collagen and agarose hydrogels. The images on the right present the patterning overviews of devices without barriers, with parenthesis-shaped, and with slash-shaped capillary barriers (the images are artificially colored on the basis of gray scale differences).
Contact angles of hydrogels on fused silica surface were measured using the Dataphysics OCA-20 contact angle software. Scanning electron microscopy (SEM) images were taken using a JEOL JSM 5610 field emission SEM.

4.3 | Results and discussion

4.3.1 | Capillary barrier operating principle

Hydrogel precursor was pinned by the capillary barriers in the channels during the vacuum suction. Pinning is attributed to the abrupt expansion in the cross sectional geometry of the channel at the barriers, hydrogel meniscus alteration and the local Laplace pressure change (Figure 4.6a). As described in literature, the Laplace pressure is determined by the angle of expansion and constriction at the abrupt expansion point, channel height, capillary barrier height, and the contact angle, \( \theta \), between the channel wall and hydrogel, requiring \( 45^\circ < \theta < 90^\circ \) (Figure 4.6a). The latter requirement is consistent with our measurements of the contact angle at capillary barriers, which was found to be \( 62.9^\circ \pm 8.4^\circ \) after silanization. Figure 4.6a illustrates the top view of the channel structure and hydrogel pinning during the vacuum suction. Hydrogel overflow does not occur at the capillary barriers despite the fact that the hydrogel did not recede with the same speed from neighboring channels during vacuum suction.

![Figure 4.5. Phase contrast microscopy images of (a) polyacrylamide; (b) PEG DA; (c) collagen; (d) agarose hydrogel patterns (the images are artificially colored on the basis of gray-scale differences).](image)
The pinning performance theoretically improves with increasing capillary barrier to channel height ratio. Barriers with one fourth of the channel height were chosen as optimal in this study.

We also studied the effect of the capillary barrier shape on hydrogel pinning. Both slash- and parenthesis-shaped capillary barriers were tested for their pinning performance. As depicted in Figure 4.6b and 4.6c, the barriers in top-view made an acute angle to the pillar walls. A capillary barrier glass pillar wall interface angle of 60° was found to facilitate microchip filling for both parenthesis- and slash-shaped barriers.

Pinning performances of parenthesis-shaped barriers, slash-shaped barriers and no-barrier geometries were evaluated on a pass/fail basis. For this evaluation, three representative images were collected from two opposite corners and the middle part of the arrays. In case of microchips with barriers, full hydrogel

![Figure 4.6. (a) Top and cross sectional schematic views of meniscus pinning at capillary barriers. Direction of hydrogel flow in channels during vacuum suction is indicated by arrows and cross sectional planes are shown by dashed lines. Phase contrast microscopy image of custom patterned polyacrylamide trapped in between glass pillars after photopolymerization using (b) parenthesis-shaped capillary barriers and (c) slash-shaped capillary barriers (the hydrogels are artificially colored on the basis of gray-scale differences). Scale bars indicate 10 μm for all images.](image-url)
occupation between both barriers was counted as pass. In case of microchips without barriers, hydrogel patches covering the entire gap between glass pillars were counted as pass. All other configurations were counted as fail. The pinning performance of the parenthesis shaped barriers during hydrogel emptying was slightly superior to slash-shaped ones (P > 0.9). Both parenthesis- and slash shaped capillary barriers demonstrate statistically significant superiority (P < 0.0001) when compared to the no capillary barrier geometry (Figure 4.4 and Table 4.1). These findings are in accordance with previous reports, describing that an acute angle at both ends of the barrier provides a minimal meniscus stretching and maximal surface wetting area for the hydrogel precursor.23

<table>
<thead>
<tr>
<th>Table 4.1. Comparison of the patterning consistency of microchips with no barriers, parenthesis-shaped barriers and slash-shaped barriers for polyacrylamide, PEG DA, collagen and agarose hydrogels.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patterning success (%)</strong></td>
</tr>
<tr>
<td>Barrier type</td>
</tr>
<tr>
<td>No capillary barrier</td>
</tr>
<tr>
<td>Parenthesis-shaped capillary barrier</td>
</tr>
<tr>
<td>Slash-shaped capillary barrier</td>
</tr>
</tbody>
</table>

4.4 | Conclusions

In summary, non-fluorescent capillary barriers were used to fabricate high resolution hydrogel structures over large areas with neither defects nor distortions in closed microchips. Small volumes of different hydrogel types, fabricated with photopolymerization and thermogelation, were uniformly patterned using this method. This design strategy allows for robust processing of elaborate microfluidic networks consisting of ~400k periodic picoliter-volume hydrogel patterns. Capillary barrier technology is a highly reliable technique, enabling full control over selective hydrogel patterning and allowing for fluorescence based analyses. Picoliter volume hydrogel microarray patterning is promising as a unique method with great potential for biological and clinical applications, such as subdivided-hydrogel microarrays for bacteria cultures31,32 and 3D cell cultures,33 and enclosed hydrogel microarrays for electrokinetic biomolecule separation purposes.34 Although our demonstration is limited to hydrogel formation, patterning of aqueous solutions would be equally possible, for example enabling massively-parallel liquid35 and droplet36 patterning in microfluidic devices, and liquid compartmentalization for sensing and bioassays.3
Chapter 4

4.5 References


A new approach for preparative continuous flow fractionation of sub-10-kbp DNA fragments, which exploits the variation in field-dependent mobility of DNA molecules based on their length, is presented. Orthogonally pulsed electric fields of strongly different magnitudes are applied to a microchip filled with a sieving matrix of 1.2% agarose gel. Using this method, we demonstrate a high-resolution separation of 0.5, 1, 2, 5, and 10 kbp DNA fragments within 2 minutes. During the separation, DNA fragments are also purified from other ionic species. Preparative fractionation of sub-10-kbp DNA molecules plays an important role in second-generation sequencing. The presented device performs rapid high-resolution fractionation while being manufactured with reliable, flexible and simple microfabrication procedures.

*This chapter is in submission as Gumuscu, B.; Bomer, J.G.; Boer, H.L.; van den Berg, A.; Eijkel, J.C.T. “Exploiting Biased Reptation for Continuous Flow Preparative DNA Fractionation in a Versatile Microfluidic Platform”.
5.1 Introduction

Standard gel electrophoresis has been intensively utilized for DNA fractionation in various genotyping and sequencing applications.\textsuperscript{1-3} This method has the advantages of great simplicity, versatility, and reproducibility; however, it suffers from long processing times in the order of a few tens of hours.\textsuperscript{2,3} For instance, the Bio-Rad CHEF-DR device performs fractionation of 5-120 kbp DNA using pulsed-field gel electrophoresis (PFGE) in 25 hours.\textsuperscript{4,5} Trends towards second generation sequencing motivate the replacement of standard gel electrophoresis with microchip-based systems, which could provide efficient platforms to minimize the processing time and to perform optimal DNA fractionation.\textsuperscript{6-8}

Micro- and nano-fabricated post arrays—which resemble a gel matrix with well-ordered and identical pores—have been integrated in microchip-based systems, increasing both the understanding of DNA separation principles and the fractionation's efficiency and speed.\textsuperscript{23} Two decades ago, Volkmuth and Austin introduced a patterned micro-post array in a microfluidic electrophoresis platform, in which DNA fragments were separated by biased reptation under a DC electric field.\textsuperscript{9} Later, Duke et al. separated large fragments in a range of 60-135 kbp in a microfabricated array using DNA reorientation—or the “switchback” principle. Although the switchback principle is similar to what is used in PFGE, the device yielded much faster separations owing to its sparse and regular sieving array.\textsuperscript{10} In a later work, Kaji et al. studied a three-dimensional (3D) nano-post array as an optimal separation matrix for DNA fragments over a few kbps under a DC electric field, resulting in similarly fast separations due to the sparse matrix.\textsuperscript{11}

With the aim of increasing sample throughput and facilitating sample recovery further, continuous flow separation was developed. Huang et al. performed continuous flow PFGE in a micromachined post array by applying pulsed electric fields of slightly unequal strength. DNA fragments ranging between 61 and 209 kbp were separated within 15 seconds in the “DNA prism”.\textsuperscript{12} A comparable device was later incorporated into a gene analysis system and is currently being marketed by the company Pathogenix, to detect the genome of rare pathogenic bacteria from complex mixtures.\textsuperscript{13} The operation time of this device is reported as ~1.5 h to fractionate 60-200 kbp DNA.\textsuperscript{13,14} Another reported variant of the “DNA prism” device was a self-patterned 3D crystalline nanoarray used for fractionating smaller DNA molecules (2-50 kbp) in continuous flow.\textsuperscript{15} Other separation principles, including Ogston sieving, entropic trapping, and electrostatic sieving, were demonstrated by Fu et al. using a nanofabricated two-dimensional (2D) sieving array operated under pulsed electric fields to fractionate DNA in continuous flow.\textsuperscript{16}
The micro- and nano-fabricated sieving devices briefly reviewed above allow for optimization of the separation process in spatially-controlled sieving matrices, optimally exploiting the basic physical principles of DNA separation. However, defect-free fabrication of the 3D nanostructures, such as the nano-post array\textsuperscript{11} and the crystalline nanoarray,\textsuperscript{15} is extremely challenging. Although the manufacturing of 2D nanostructures (for example, the anisotropic nanofluidic sieving array\textsuperscript{16}) is relatively easier, these nanostructures intrinsically yield low sample throughput. An ideal sieving matrix should thus have simple design and facile fabrication steps, yet provide high-resolution and high-throughput separation.

Remarkably, a relatively larger device (in the order of a few cm) filled with bulk agarose gel has been demonstrated for the purification and concentration of high molecular weight DNA (in the order of ten thousands bp), using the field-dependent mobility of DNA in a method called synchronous coefficient of drag alteration (SCODA).\textsuperscript{17-19} However, this study overlooked the fact that the field-dependent mobility depends on the DNA fragment size, which will be shown in this work to provide an alternative method for continuous flow DNA fractionation that has hitherto gone unnoticed.

We demonstrate a microscale gel electrophoresis (\(\mu\)GEL) device operated under orthogonal pulsed electric fields with strongly differing magnitudes for continuous flow fractionation of sub-10-kbp DNA molecules. Preparative fractionation is shown over a broad frequency range (3 decades). The \(\mu\)GEL device presents two major advances over the aforementioned microchip-based systems. First, agarose gel is used as the sieving matrix: requiring much less effort to fabricate a defect-free 3D network of nanopores for high-resolution separations, and offering considerable flexibility in varying pore size for the DNA fragments of interest. Second, we can combine both separation and purification functions by maximally exploiting the field-dependent mobility: fragments in a range of 0.5-10 kbp are separated from each other and from other ionic species (fluorescein sodium salt was utilized in this work as an example) in continuous flow.

### 5.2 Methods

#### 5.2.1 Microchip fabrication

The microchips were fabricated in the MESA+ cleanroom facility at the University of Twente. Fused silica glass was selected for its high optical clarity and its smooth microchannel surfaces after the deep reactive ion etching (DRIE) process. Microchips consisted of a processed top and an unprocessed bottom parts. The top part contained the separation chamber, microchannels, and buffer reservoirs. A bonded microchip is presented in Figure 5.1a and
5.1b, consisting of a 10 by 10 mm square chamber connected to the buffer reservoirs via microchannels (50 µm x 10 mm, 50 µm periodicity) on each side. The microchannels serve for the generation of a uniform electric field over the separation chamber. The overall dimension of the microchip is 35 by 35 mm.

DRIE was utilized to define the microchannels and separation chamber. The process flow is shown in Figure 5.2. Before DRIE, the wafer was spin-coated with SU-8 (MicroChem), which served as a mask for the underlying part. The SU-8 layer was patterned by photolithography and stripped in a piranha bath after the DRIE process. The height of the structures was measured as 20 µm using a Dektak 150 Surface Profiler (Bruker). Buffer reservoirs were opened on the backside of the top part. After coating and photolithography of Ordyl tape resist (BF410, Tokyo Okha Kogyo), it was developed using a NaHCO$_3$ (0.2%, w/v, Sigma Aldrich) solution. Subsequently, powder blasting process was performed and the tape resist was removed using acetone. Finally, both top and bottom parts were cleaned and thermally bonded at 1080°C. The bonded wafers were then diced into individual microchips.

Agarose hydrogel was prepared by dissolving agarose powder (1.2%, w/v, Invitrogen) in deionized water. The mixture was boiled in a microwave and pipetted into the warmed microchip (70°C on a hot plate). The empty space in the microchip was filled entirely by capillary forces. The microchip was then immersed in a buffer solution for at least one night. The agarose gel increased in volume during the immersion process due to swelling, which ensured an airtight filling of the microchannels and eliminated leakage and air

**Figure 5.1. (a) An image of the microchip. (b) Schematic illustration of the microchip and its components. (c) Schematic illustration of the in-house made chip holder including all main components. The chip holder is placed on an inverted epi-fluorescence microscope for observation of DNA fractionation.**
bubble problems. The buffer solution consisted of tris borate EDTA (TBE, 0.1X, Invitrogen), β-mercaptoethanol (3%, w/v, Sigma Aldrich), and deionized water.

5.2.2 Sample preparation
Individual DNA fragments (0.5 kbp, 1 kbp, 2 kbp, 5 kbp, 10 kbp) were obtained from New England BioLabs. Fragments were then labelled with an intercalating fluorescent dye YOYO-1 (Invitrogen) at a 1:5 dye-to-bp ratio. The final DNA concentration was 12.5 ng µl⁻¹. YOYO-1 solution was added before DNA solution to prevent precipitation. The mixture was incubated at room temperature for 1 hour, and then 0.1% (v/v) β-mercaptoethanol was added to prevent photo bleaching. The final mixture is stable at room temperature.

5.2.3 Experiment setup
An epifluorescence microscope (Leica, DM-IRM) equipped with a thermoelectrically-cooled CCD camera (Hamamatsu ORCA-ER C4742-80-12AG) and GFP filter cube (Chroma) was used for fluorescence imaging. A 100 W mercury lamp (Leica) was set to the highest intensity level for illumination.
Electric fields were applied to the microchip using a LabSmith HVS448LC High Voltage Sequencer. In-house made platinum electrode connectors were mounted in an in-house made chip holder to apply the electric fields. Figure 5.1c illustrates the chip holder and connections.

5.2.4 Simulations

Two-dimensional finite element calculations of the µGEL device were performed to estimate the electric field distribution in the separation chamber, using the AC/DC module in COMSOL Multiphysics 5.2 software in the stationary condition. The agarose gel and TBE buffer-filled separation chamber was modelled with a specific conductivity of 4.20 S m$^{-2}$ and a relative permittivity of 80. The potentials applied to the reservoirs were defined as given in Figure 5.3. The system responses to the applied potentials were governed by Maxwell’s equations under the electric currents interface of the software.

5.2.5 Resolution calculations

We calculated the separation resolution between two adjacent flow streams $R_s = \frac{\Delta X}{2\sigma_1 + 2\sigma_2}$, where $\Delta X$ is the spatial distance between the streams, and $\sigma_1$ and $\sigma_2$ are the standard deviations of the stream widths.

5.2.6 Image processing

Matlab software was used for plotting the fluorescence intensity graphs. To obtain images with comparable intensities, the background of the images was subtracted, the noise was reduced by applying a Gaussian filter to every 5 x 5 pixels, and the contrast was increased by 2%. The fluorescence intensity profile was plotted by drawing a line along the y-axis of the image (in front of the microchannels), where all the samples were collected. The resulting intensity plots were used to calculate the resolution of the separation. Resolution values between peaks are provided in Table 5.1.
5.3 Results and discussion

5.3.1 Design and operation of the µGEL device

Figure 5.2 outlines the microchip fabrication and hydrogel patterning processes, while Figure 5.1 presents the design of the µGEL device and the chip holder used in the experiments (see Methods section). The microchip consists of a 10 mm by 10 mm chamber connected to buffer reservoirs via parallel microchannel arrays (50 µm x 10 mm, 50 µm periodicity) on four sides. The microchannels provide a high resistance area, preventing current leakage from the separation chamber and generating a fairly uniform electric field (Figure 5.4).22,23

In all separation experiments, two electric fields, $E_1$ and $E_2$, were alternately applied across the separation matrix at various frequencies. Figure 5.3 presents the magnitude and angle at the injection point—relative to the x-axis—of all transverse electric fields. Throughout the experiments, the electric field strength ratio $E_1/E_2$ at the injection point varied between 2.4 and 3, while the angle between the fields varied around 90° ($85°-98°$). DNA fragments were continuously injected into the agarose matrix and separated fragments were continuously collected at different side microchannels.

Figure 5.4 shows the simulated electric field distribution in the separation matrix at typical $E_1$ and $E_2$ values. Both the magnitude and direction of the electric fields vary slightly over the µGEL device: the angle between $E_1$ and $E_2$ becomes more acute (up to 60° at some locations in the microchip) and
the ratio $E_1/E_2$ gradually increases (up to 3.1) as calculated using COMSOL Multiphysics software.

5.3.2 DNA fractionation results and discussion of physical separation principles

We investigated the continuous flow separation of 0.5, 1, 2, 5, and 10 kbp fragments, at a wide range of DC electric fields $E_1$ and $E_2$ (from 1 V cm$^{-1}$ to 135 V cm$^{-1}$) and switching frequencies (from 0.016 Hz to 33 Hz). Figure 5.5 shows photomicrographs of the flow streams of individual DNA fragments observed at such electric fields and switching frequencies. The deflection angle ($\phi$) between initial stream and the flow stream exit locations was measured to quantify the separation between the fragments (Figure 5.6f).

Table 5.1. Resolution values between peaks of DNA fragments shown in Figure 5.5.

<table>
<thead>
<tr>
<th>Electric field (V cm$^{-1}$)</th>
<th>Fragments</th>
<th>$R_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1$</td>
<td>$E_2$</td>
<td></td>
</tr>
<tr>
<td>9.7</td>
<td>3.8</td>
<td>0.4</td>
</tr>
<tr>
<td>29.1</td>
<td>9.7</td>
<td>0.5</td>
</tr>
<tr>
<td>89.8</td>
<td>34.4</td>
<td>1.0</td>
</tr>
<tr>
<td>59.5</td>
<td>24.6</td>
<td>1.0</td>
</tr>
<tr>
<td>134.6</td>
<td>55.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The semilog plots of the deflection angle as a function of switching frequency, given in Figures 5a-e, show that DNA fragments can be separated over more than 3 decades of frequency. To explain this observed behavior, we propose a mechanism based on the combination of the field-dependent mobility and the “switchback” mechanisms, with the former mechanism dominating at low frequencies and the latter mechanism dominating at high frequencies.
The switchback mechanism was previously demonstrated in micromachined devices by Huang et al.\textsuperscript{12} and Zheng et al.\textsuperscript{16}, and theoretically analyzed by Chen et al.\textsuperscript{24} When the applied field switches direction, DNA...
fragments need time to reorient, $t_\omega \approx \frac{L}{\mu E}$, where $L$ is the DNA contour length, $\mu$ the DNA mobility, and $E$ the applied field (Figure 5.9). For the small fragments, the reorientation time is shorter. At the corresponding reorientation frequency $f_\omega = \frac{1}{2t_\omega} = \frac{\mu E}{2L_{DNA}}$, small fragments will thus follow an intermediate path in between both applied field vectors ($E_1$ and $E_2$). For large DNA fragments $f_\omega$ is shorter and they will mainly follow the direction of the large
applied field \((E_1)\). The migration angle at frequencies close to the reorientation frequency, thus depends on DNA fragment size. The fractionation at frequencies around or above \(f_w = \frac{1}{2t_w} = \frac{\mu E}{2L_{DNA}}\) can be explained by the switchback mechanism. In Figures 5a-e, the frequencies around or above \(f_w = \frac{1}{2t_w} = \frac{\mu E}{2L_{DNA}}\) are indicated with a purple background.

The physics of DNA reorientation in agarose gels has been studied by Aakerman et al.\(^{25}\) The reorientation frequencies of DNA fragments in the µGEL device lie between 2.4 Hz for 10 kbp fragments and 50 Hz for 0.5 kbp fragments at an applied field of 5  9.5 V cm\(^{-1}\). Optical measurements in the µGEL device confirmed the validity of the above equation for the switching frequency. Calculate d orientation times of 10 kbp fragments were 0.24 sec when \(E_1\) was switched to \(E_2\), and 0.42 sec when \(E_2\) was switched to \(E_1\), upon the alternating application of 59.5 V cm\(^{-1}\) \((E_1)\) and 24.6 V cm\(^{-1}\) \((E_2)\) fields. The observed reorientation times were found in good agreement with theory, calculated as 0.2 sec and 0.4 sec, respectively. At frequencies far below the switching frequency \(f_w = \frac{1}{2t_w} = \frac{\mu E}{2L_{DNA}}\), DNA fragments will quickly reorient along the new electric field direction, and will spend most of the application time moving at steady state in the field.\(^{22}\) The fractionation observed at these frequencies can be explained by the variation in field-dependent mobility of DNA molecules based on their length. The biased reptation with orientation principle states that the mobility \(\mu\) of a DNA fragment in an agarose sieving matrix is a function of the electric field and DNA contour length.\(^{24,26-29}\) This behavior was experimentally confirmed in the µGEL device: Figure 5.7 shows

![Graph showing measured mobility of individual DNA fragments as a function of electric field. The red and dark blue figures illustrate the molecular conformation of large and small fragments at low and high electric fields, respectively.](image)

**Figure 5.7.** Measured mobility of individual DNA fragments as a function of electric field. The red and dark blue figures illustrate the molecular conformation of large and small fragments at low and high electric fields, respectively.
the field-dependent mobilities measured. At low frequencies, the reorientation
time can be neglected and the migration trajectories can simply be calculated
by adding the trajectories at high \((E_1)\) and low \((E_2)\) fields (Figure 5.6f). The
larger the fragment, the larger the mobility increase between the two applied
fields \(\Delta \mu = \frac{d\mu}{dE} (E_1 - E_2)\) (Figure 5.6f and Figure 5.7), leading to differently-
sized fragments following different trajectories.

Adding the contributions of both separation mechanisms, the migration
angle of DNA molecules with respect to the horizontal axis \(\varnothing\) can be
approximated by the following equation:

\[
\varnothing = \text{atan} \left( \frac{\sin \theta_1 + \left( \frac{E_1 \mu_2 - 2fL}{E_1 \mu_2 - 2fL} \right) \sin \theta_1}{\cos \theta_1 - \left( \frac{E_1 \mu_2 - 2fL}{E_1 \mu_2 - 2fL} \right) \cos \theta_1} \right)
\]

Equation 5.1

Here, \(\mu_{1,2}\) is the mobility of the fragments at the two applied fields \(E_{1,2}\); \(\theta_{1,2}\) the
angle between the transverse electric fields \(E_1\) or \(E_2\) and the \(x\)-axis; \(f\) the applied
frequency; and, \(L\) the DNA length.

The calculated (using equation 5.1) and measured deflection angles of
the fragments for 59.5 V cm\(^{-1}\) \((E_1)\) and 22.4 V cm\(^{-1}\) \((E_2)\) are presented in
Figure 5.8. The comparison of Figure 5.8a and 5.8b demonstrates that four flow
streams are obtained at all frequencies below 1 Hz (Figure 5.5a and 5.6c). This
finding corresponds to the separation of the 0.5-5 kbp fragments and it can be
attributed to the biased reptation mechanism. Since 5 and 10 kbp fragments
have approximately equal mobilities \((\mu_1/\mu_2)\) at \(E_1\) and \(E_2\), they cannot be
separated by the biased reptation mechanism. We however obtained five flow
streams in the frequency range of 0.5 to 10 Hz (Figure 5.5a and Figure 5.6c)

![Figure 5.8](image)

**Figure 5.8.** Deflection angle \(\theta\) variation for individual DNA fragments at different
frequencies. (a) Measured deflection angles at \(E_1 = 59.5\) V cm\(^{-1}\) and \(E_2 = 24.6\) V cm\(^{-1}\).
(b) Calculated deflection angles for the same fields.
with > 1 resolution between 2-10 kbp fragments. For the 5 and 10 kbp fragments, we thus expect that the reorientation mechanism is contributing to the separation, since the reorientation frequencies of these DNA lengths are 2 and 4 Hz, respectively (Figure 5.9). It was furthermore found that the calculated angles were lower than the measured angles. This difference can be explained by (1) the bent electric field lines and the variation in field magnitude throughout the microchip which leads to larger than predicted angles (Figure 5.4); and, (2) the fact that the separated flow streams fluctuated along the y-axis at low frequencies, leading to an increased uncertainty in angle determination. A rough correction for the curved field lines can be based on the measured angle of the fluorescein sodium salt trajectory (Figure 5.10), for which $\mu_1/\mu_2 = 1$.

According to the field-dependent mobility mechanism, the best separation performance for all DNA lengths is expected when $\mu_1/\mu_2$ varies maximally for all DNA lengths. According to Figure 5.6, the best separation performance should be achieved around $E_2 = 25$ V cm$^{-1}$. In our observations, the highest resolution separation was indeed obtained within this field region; specifically, when applying 59.5 V cm$^{-1}$ ($E_1$) and 22.4 V cm$^{-1}$ ($E_2$).

Figure 5.5c shows that five flow streams with good resolution were obtained when applying 134.6 V cm$^{-1}$ ($E_i$) at 20 Hz (the reorientation frequency of DNA fragments in this field is 100 Hz for 0.5 kbp DNA and 5 Hz for 10 kbp DNA). At this frequency, both the field-dependent mobility (for small
fragments) and switchback mechanisms (for large fragments) are expected to contribute to the resolution.

At low frequency ranges (0.05–10 Hz) and using 89.8 V cm\(^{-1}\) (\(E_1\)), separation was consistently obtained for four flow streams as seen in Figures 5.4d and 5.5b (6 Hz < \(f_n\) = 120 Hz for DNA fragments). As another example, applying 29.1 V cm\(^{-1}\) (\(E_1\)) at 2 and 1 Hz frequencies yielded a satisfactory separation (\(R_s,\text{mean} \approx 0.7\)) with four independent flow streams (Figure 5.5d). At this field, 2 Hz < \(f_n\) < 40 Hz for DNA fragments, indicating that the field-dependent mobility is the major contributor to separation. As seen in Figure 5.6e, the switchback mechanism does not work well in the \(\mu\)GEL device when operated under low electric field strengths: separation performance decreased when \(f_n < 0.1\) Hz. The poorest separation was obtained when 9.7 V cm\(^{-1}\) (\(E_1\)) was applied at the lowest frequency, 0.016 Hz; here, only two flow streams of fragments were separated.

The field-dependent mobility mechanism theoretically yields an angle \(\emptyset\) independent of frequency. Figures 5.5a-e show this independence only at low frequencies. The observed decrease in angle with increase in frequency is most likely due to the reorientation mechanism. For example, 5 and 10 kbp fragments were separated at 59.5 V cm\(^{-1}\) (\(E_1\)) and 22.4 V cm\(^{-1}\) (\(E_2\)) owing to the decreasing angle with increasing frequency. The agarose gel does not seem to yield satisfactory separations in the switching regime using the applied voltage protocol, despite the resolution decreases for all field strengths at higher frequencies.

Throughout the experiments, the angle between \(E_1\) and \(E_2\) varied around 90° (85°-98°), which narrowed down to an angle of 60° at the exit location of the flow streams. In PFGE (where the reorientation mechanism is used), the best separation performances were always obtained when the angle between two equal transverse electric fields was 120° (and not 90°)\(^{25}\). However, in the case of the field-dependent mobility mechanism, migration velocity differences are important for the separation. We optimally exploited the velocity differences with the applied orthogonal fields.

![Figure 5.10](image.png) DNA purification from fluorescein sodium salt in continuous flow. Fluorescence images present separation of (a) only 0.5–10 kbp DNA fragments and (b) separation of 0.5-10 kbp DNA fragments and fluorescein sodium salt when \(E_1 = 59.5\) V cm\(^{-1}\) and \(E_2 = 24.6\) V cm\(^{-1}\) were applied at 2 Hz frequency. Each image was recorded with 12 sec exposure time.
5.3.3 Separation performance and throughput of the µGEL device

Up to 80 different experiments could be performed with the same microchip, using a large variety of voltage and frequency protocols, and yielding similar (> 95%) results between the replicates of the experiments. Figure 5.6 shows the standard deviation of the measurements performed using the same microchip. Different microchips could also be used for fractionation and purification with reproducible results (> 70%).

Due to the low-height (20 µm) channels and separation chamber, Joule heating is not expected to contribute significantly to the separations in the applied field range. According to Figure 5.11, Joule heating might have affected the separation performance at the highest field strengths ($E_f = 134.6$ V cm$^{-1}$). The ionic strength of the buffer solution was kept constant throughout the experiments; the reservoir volume is 2000 times larger than the inner volume of the microchip and the buffer solution was frequently refreshed.

The µGEL device has a calculated throughput of 0.18 ng of molecules per hour at the DNA input concentration used (12.5 ng µl$^{-1}$), and is comparable to previously reported microfabricated device.\textsuperscript{22,30,31}

5.3.4 Band broadening and peak purity

In our experiments, we found that the width of the band at the injection site (110 µm) did not significantly differ from the width of the bands at flow stream exit locations (120-200 µm). Thus, band broadening was minimal during the separation process, as in accordance with the results suggested by Huang \textit{et al.}\textsuperscript{12} and Lerch \textit{et al.}\textsuperscript{32} At low frequencies, however, the sawtooth movement of the DNA fragments is a major contributor to band broadening. The contribution of the alternating field protocol can be quantified by

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{current_voltage_graph.png}
\caption{Current-voltage graph demonstrating the absence of Joule heating in the microchip at the applied field strengths.}
\end{figure}
where \( L_{fr} \) is the distance along the vertical axis travelled by a DNA fragment in one switching period. According to Equation 5.2, the contribution of the field switching (i.e. fluctuation of fragment flow streams along the separation matrix) to the total band broadening will be less than 30% (~33 µm band width) when \( f < 0.05 \) Hz and \( E_2 = 24.6 \) V cm\(^{-1}\). Since the width of the injection band is the main contributor to the band variance, the resolution of the µGEL device could be improved by decreasing the injection channel width.

The peak width variance between the injection channel and flow stream exit locations at > 10 Hz and > 2 Hz is less than 5% and 15%, respectively. Peak width variations are given in Figure 5.5.

For the DNA recovery calculations, we integrated the fluorescence over the width of the flow stream both at the inlet and the outlet, and compared the results after subtracting the baseline intensity. For example, upon the alternating application of 59.5 V cm\(^{-1}\) \((E_1)\) and 24.6 V cm\(^{-1}\) \((E_2)\) fields at 2 Hz frequency (we observed the highest resolution by applying these fields), the integrated the fluorescence over the width of the flow stream at the inlet is calculated to be 2897 (dimensionless number), while that of the outlet (sum of all fragment streams) is calculated as 2783 (dimensionless number). This corresponds to a recovery of 96.06%, which is as good as previously reported sequencing devices.\(^{30-33}\)

For the peak purity, we calculated the purity of the fragment streams using the resolution values by assuming that the fragment intensity profiles have Gaussian peak shapes. Peak overlap percentage was calculated and peak purity was determined accordingly.\(^{34}\) The purity of the streams were found to be 99.5% (between 0.5 kbp and 1 kbp), 88.5% (between 1 kbp and 2 kbp), and 99.7% (between 2 kbp and 5 kbp, and between 5 kbp and 10 kbp) for the image presented in Figure 5.5b.

### 5.3.5 Purification from other ionic substances

For other ionic species, no reptation occurs and the molecules have the same mobility under both electric fields. Thus, the \( 2fL \) term drops and \( \mu_2 = \mu_1 \), reducing Equation 5.1 to

\[
\varphi = \tan^{-1} \left( \frac{E_1 \sin \theta_1 + E_2 \sin \theta_2}{E_1 \cos \theta_1 - E_2 \cos \theta_2} \right) \quad \text{Equation 5.3}
\]

Periodic application of \( E_1 \) and \( E_2 \) results in the ionic species following a path at angle \( \varphi < \varphi \) (Figure 5.10).\(^{28}\) When 59.5 V cm\(^{-1}\) \((E_1)\) and 22.4 V cm\(^{-1}\) \((E_2)\) fields were applied, we calculated \( \varphi = 45^\circ \) for fluorescein sodium salt,
predicting clear separation from DNA fragments with calculated $\phi < 38.5^\circ$. (Figure 5.8). We experimentally confirmed the purification by separating a mixture of 0.5-10 kbp fragments and 10 mM fluorescein sodium salt by applying $E_1 = 59.5$ V cm$^{-1}$ and $E_2 = 24.6$ V cm$^{-1}$ at 2 Hz (Figure 5.10).

5.4 Conclusions

The advantages of the proposed device include its simple design and fabrication. Here we apply it for separating sub-10-kbp fragments, but the use of agarose gel as the sieving matrix provides flexibility, in principle enabling fractionation of other DNA fragment sizes outside the 0.5-10 kbp range studied here by changing gel concentration. A similar strategy is used in the commercially available mate-pair sequencing device, BluePippin (SageElf), which performs capillary electrophoresis for DNA fractionation in a precast agarose gel cassette with orthogonal sample plug extraction wells. BluePippin can process between a few tens to thousands of base pairs, simply by changing the agarose concentration and voltage protocol. However, fractionation of sub-10-kbp DNA fragments requires 4 hours using BluePippin, while the µGEL device presented here requires only 2 min.

The band resolution for 0.5 to 10 kbp fragments and fluorescein sodium salt varied between 0.9 and 1.4, when 59.5 V cm$^{-1}$ and 24.6 V cm$^{-1}$ fields were switched at a frequency of 2 Hz. Since the main contributor to band variance was the injection band width, we expect that resolution in the µGEL device can be further improved by decreasing the width of the injected band. Improvement in band separation will be attempted by optimizing the voltage protocol for separation of the fragment sizes.

Interestingly, Chen et al. predicted that large and small fragments at low frequencies ($< 2/t_\infty$) will migrate through isotropic matrices such as agarose gels at similar angles and no continuous flow fractionation would be possible. However, $\mu_1/\mu_2$ was assumed to be constant by Chen et al., which we have demonstrated to be an erroneous assumption by achieving fractionation using the field-dependent mobility of DNA.

We have introduced a novel microfluidic platform for the purification and high-resolution fractionation of DNA molecules by basing the separation on the variation in field-dependent mobility of DNA molecules with their length. This device would be of a broad interest for second-generation sequencing and clinical diagnosis applications as it can achieve similar performance with much less effort in terms of fabrication and operation in comparison with currently available devices. In addition, the µGEL device can further be extended to protein gel electrophoresis by replacing agarose with polyacrylamide gel.
5.5 References


Preparative DNA Fractionation


Multicompartmental Hydrogel Arrays for 3D Tissue Culture*  

We demonstrate an in vitro microfluidic cell culture platform that consists of periodic 3D hydrogel structures with controllable shapes. The microchip is composed of 21 microfluidic channels separated by discontinuous collagen gel patches locally patterned in between PDMS pillars. Collagen compartments in the closed fluidic microchip were fabricated by capillary line pinning with 95% patterning success. The compartmentalized design of the microchip and continuous fluid delivery enabled long-term culturing of Caco-2 human intestine cells that started to grow spontaneously into 3D folds on the 3rd day. On 8th day of the culture, Caco-2 cells were co-cultured for 36 hours with an intestinal bacterium (E. coli) which adhered to the cells without affecting the cell viability. Continuous fluidic perfusion also enabled the preliminary screening of drug treatment on the intestinal epithelial cells. The co-culture of Caco-2 and E. coli was treated with 34 µg ml⁻¹ chloramphenicol during 36 hours, which resulted in the death of the bacterial cells. Finally, Caco-2 cells were cultured under static conditions in different compartment geometries with large and small hydrogel interfaces, leading to differences in the proliferation and cell
spread profile of Caco-2 cells. The novelty of the microchip lies in the periodic 3D hydrogel compartments and in the facile fluidic control, that allows dynamic regulation of culture conditions.

*This chapter is in preparation to be submitted as Gumuscu, B.; Albers, H.J.; van den Berg, A.; Eijkel, J.C.T.; van der Meer, A.D. “Multicompartmental Hydrogel Arrays for Controlled Microfluidic 3D Tissue Culture”.*
6.1|Introduction

The integration of cell biology research and microfluidics has led to the emergence of organ-on-chip technology, allowing cells to be cultured in physiologically realistic microenvironments, where mimicking various in vivo tissue functions is enabled in vitro. Organ-on-chip technology is critical not only for improving our understanding of organ physiology, but also for curtailing the high costs and complexities associated with the use of in vivo animal models. In microfluidic platforms, compartmentalized culture models have been shown to provide spatio-temporally controlled microenvironments for monitoring intercellular activity, and high-throughput cell handling.¹² For example, multiple replicates of a tissue construct can be simultaneously tested in microscale compartments, and various environmental physiological conditions can be screened at the same time in organ-on-chip platforms.³⁵

Several techniques have been introduced previously for immobilizing cells on predesignated regions in microchips.⁶⁻¹⁶ Micromolding methods have been used to encapsulate individual cells within microgel structures.⁶ However, micromolding has a low consistency in the patterning success with respect to e.g. photolithography when it comes to the fabrication of periodic micron-sized arrays. Cell encapsulation has also been achieved by applying photolithography on photocrosslinkable synthetic polymers. This technique is widely used to create two-dimensional (2D)⁷⁻⁹ and three-dimensional (3D) cultures,¹⁰⁻¹² including cell-laden hydrogel microdroplets with precisely controlled geometries.¹³ Despite offering high throughput, photolithography and microdroplet techniques require dedicated equipment. As an alternative, microprinting has been used to create free-form patterned arrays of cell-laden materials.¹⁴ For example, sphere-shaped functional tissues and organoids have been fabricated via bioprinters using natural and synthetic hydrogels.¹⁵ However, the extended surface area of sphere-shaped droplets containing the cells makes the droplets vulnerable to drying during the fabrication process. Dielectrophoretic forces have been utilized to concentrate cells into specific locations on microchips, although this process has advanced design and application requirements; therefore, it is not versatile.¹⁶

The aforementioned methods paved the way for high-throughput and scalable cell handling assays. Overall, these methods do not provide the ability to culture cells in a closed fluidic environment, which is critical for mimicking physiologically relevant conditions, such as molecular transport, absorption, and response to drugs. Fluidic integration and fine fluidic control are essential if micropatterned cells are to be used for engineering organs-on-chips.¹⁷,¹⁸ This requirement has been recently addressed by the phaseguide technique, which was used to manufacture a 3D co-culture of two different cell types embedded in adjacent lanes of gels.¹⁹ The phaseguide technique, however, only allowed for limited control over fluid flow in the microchannels due to the high
hydrodynamic resistance of the connecting channels in the design. Further development of the compartmentalization approach is thus warranted for culturing cells in closed fluidic environments without any flow rate constraints.

Previously, our group has introduced the capillary pinning technique for in situ fabrication of periodic hydrogel compartments in a glass microchip. The capillary pinning method would be ideal for micropatterning of cells in 3D compartments since it allows for microfluidic integration. In this study, we show the promise of this approach by applying the capillary pinning technique to fabricate approximately 500 periodic cell culture compartments in a single microchip made of PDMS. As a proof of concept demonstration, we embedded and cultured human intestine epithelial cells (Caco-2) in the microchip inside well-defined hydrogel structures, in which the cells formed 3D structures. Intestinal epithelial cells play a central role in intestinal functions, such as forming 3D villi structures for nutrient absorption and providing attachment surface for beneficial bacterial cells under continuous flow. The device geometry allowed us to screen the glucose consumption rate and 3D fold formation via high-throughput on-line measurements. Long-term co-culturing of intestinal bacteria (E. coli) and Caco-2 cells was also achieved in the microchip operated both in static conditions and under flow. The co-culture was treated with an antibiotic, chloramphenicol, for a preliminary drug screening test under continuous fluidic perfusion. Finally, we demonstrated the versatility of this technique by fabricating cell-laden hydrogel compartments with different geometries.

6.2 Methods

6.2.1 Microchip fabrication

We fabricated the microchip from polydimethylsiloxane (PDMS, Dow Corning) polymer using standard soft lithography techniques. The microchip consisted of one glass and two PDMS layers. The upper PDMS layer consisted of one inlet and one outlet. The lower PDMS layer contained pillars, capillary barriers, microchannels, and buffer reservoirs. The glass layer (microscope slide) did not contain any structures and it was directly bonded to the lower PDMS layer. An assembled microchip is shown in Figure 6.1a and 6.1b, consisting of a 14.5 mm by 9.5 mm rectangular chamber, connected to the buffer reservoirs via microchannels (200 μm x 5 mm with 200 μm periodicity) on upper and lower sides (Figure 6.1b-d). The microchannels serve for obtaining a uniform flow distribution over the culture chamber (Figure 6.1c). An array of rectangle pillars and capillary barriers placed in the culture chamber served as a mechanical support for the fabrication of periodic hydrogel patches. Pillars and hydrogel compartments were of the same dimensions and 200 x 500 μm. In
The second design, the hydrogel compartments were 600 x 500 µm while the pillar dimensions remained the same. The microchannel height was measured as 67.5 µm after the first photolithography, while the capillary barrier height was 7.5 µm after the second lithography. The total inner volume of the microchip was approximately 40 µl.

The lower PDMS layer was prepared by casting a prepolymer (10:1 w/w ratio of PDMS to curing agent) on a SU-8 master that was fabricated by photolithography in the MESA+ cleanroom facility at the University of Twente, The Netherlands. The process flow is shown in Figure 6.2. The SU-8 master contained the negative pattern of the microchip design and consisted of two SU-8 layers (MicroChem). The first layer contained the pillars and microchannels, while the second layer contained pillars, microchannels, and capillary barriers. The height of the first layer was 67.5 µm and that of the second layers was 7.5 µm, which were measured using a Dektak surface profiler (Bruker). After curing the prepolymer at 60°C for 3 h, the PDMS layer was peeled off from the SU-8 master. The rectangular buffer reservoirs were then cut using a blade. The patterned surface of the lower PDMS layer was treated with oxygen plasma at 500 mTorr and highest power for 45 seconds, and immediately bonded with an oxygen plasma treated glass layer. The buffer inlet and outlet were opened using a 1.5 mm hole puncher in the upper PDMS layer, which was assembled with the lower PDMS layer after hydrogel patterning. The microchip was used immediately after preparation. The plasma cleaning was found to be sufficient.
to sterilize the microchip. The tubing and the microfluidic connectors that were used in the experiments were sterilized by rinsing with 70% (v/v) ethanol and 1X PBS (phosphate buffered saline, Sigma Aldrich) solutions.

6.2.2 Hydrogel patterning

A cell-containing collagen mixture was prepared by mixing 1 M NaOH (1.38%, v/v, Sigma Aldrich), DMEM high glucose Glutamax medium (36%, v/v, ThermoFisher Scientific) suspended with Caco-2 cells \((6 \times 10^6 \text{ cells ml}^{-1})\), Collagen (0.3%, v/v, Trevigen Rat Tail Collagen type I), and deionized water.\(^{23}\)

The patterning process occurs via capillary action and it is affected negatively by the hydrophobic recovery of PDMS after the oxygen plasma treatment. For this reason, the hydrogel-cell mixture was injected into the microchip immediately after the assembly. Microchip was completely filled with 40 µl of the mixture and the excess mixture in the main microchannels was removed using a Pasteur pipette connected to a vacuum pump. In this stage, the
collagen mixture only remained between the pillars and capillary barriers due to the capillary pinning process. No air bubbles were trapped during the entire patterning process as described in our early work on the design and working principles of capillary barriers. After the patterning process, the microchip was placed in the incubator for 1.5 h to allow the collagen mixture crosslinking.

6.2.3 Cell culture

Human Caco-2 intestinal epithelial cells (ATCC HTB-37 Caco-2 cell line) were cultured as a monolayer on the tissue culture polystyrene container (Nunc) in DMEM high glucose Glutamax medium (Gibco) supplemented with 20% (v/v) Fetal Bovine Serum (FBS, Gibco), 100 units ml⁻¹ penicillin (Gibco), and 100 units ml⁻¹ streptomycin (Gibco) using an incubator set at 37°C and 5% CO₂ (Binder). The culture medium with this composition was used in all experiments unless stated otherwise. During the culturing process, the culture medium was refreshed every 3 days until the cells reached 80% confluency. Caco-2 cells were then harvested using trypsin/EDTA solution (0.05%, v/v, Gibco) and suspended with DMEM Glutamax medium with a final cell concentration of 6.67·10⁶ cells ml⁻¹ prior to the patterning of collagen mixture. At this cell concentration, aggregation or superposition of cells was not observed in hydrogel patches. After the patterning process, the microchip was incubated for 1 hour and culture media was subsequently pumped into the microchip at a constant flow rate (30 or 300 µl h⁻¹) using a Harvard PhD 2000 syringe pump.

Control studies were performed using both a static microchip culture and static cultures of Caco-2 cells in Transwells (Corning) with 0.4 µm pore polyester membrane inserts, which were pre-coated with the same collagen mixture with the same cell concentration (as described in the cell-laden hydrogel patterning process). The medium in the microchip was refreshed every three days. Similarly, the medium in the Transwell plates was refreshed every three days.

In all experiments, pipetting was done using Eppendorf pipettes and Star Lab pipette tips as well as Integra Pipetboy in combination with Greiner Bio-One pipettes. The brand of the 10 and 50 ml tubes used in the experiments was sterile Greiner Bio-One.

6.2.4 Glucose measurement

We measured the glucose consumption over 14 and 21 days of the cell culture in the microchip and Transwells, respectively. For Transwell measurements, culture medium from the bottom chamber, which did not contain any cells, was collected every two days of the culture. For the microchip measurements, culture medium was collected from the outlet every two days. Collected samples were then transferred to a 96 Transwell (Greiner Bio-One) to quantify the glucose concentration using the Multiskan GO (ThermoFisher...
Scientific) microplate reader at 278 nm. Unconditioned culture medium was used as the blank. The calibration curve of glucose was obtained by measuring different concentrations of the cell culture media containing 25 mM glucose. The resultant glucose concentrations were between the range of 25 mM and 2.5 mM (Figure 6.3). The obtained data points were normalized to the glucose consumption rate on day 7 of the culture in either the microchip or Transwell.

**6.2.5 Bacteria co-culture**

*Escherichia coli* cells were used to study cell-bacteria attachment and interactions. *E. coli* cells were incubated in 10 ml sterile Luria Bertani broth (Sigma Aldrich) overnight at 37°C on a rotary shaker operating at 125 rpm. The culture was subsequently centrifuged at 200 rpm for 2 min and the supernatant was removed in order to transfer the bacterial cells in a sterile DMEM Glutamax medium with FBS and without any antibiotics and without Caco-2 cells. The bacterial cells were incubated in this medium for at least 30 min at room temperature. The bacteria concentration of this medium was found as $3.8 \times 10^8$ CFU ml$^{-1}$ by inoculating the serial dilutions of the medium on LB agar plates overnight.

In order to study cell-bacteria interactions, Caco-2 cells were cultured in the microchips for 8 days under both static and flow conditions. In the last day, the culture medium was switched to antibiotic-free culture medium in both microchips. Two microchips (one was treated under static, the other was treated under fluidic conditions) were then filled with the above mentioned bacteria-culture medium mixture and incubated for 36 h. A control study was performed in a second set of microchips (one was treated under static, the other was treated under flow conditions) that were incubated without bacteria-culture medium mixture and incubated for 36 h. Live/dead staining was applied to screen the
survival of Caco-2 cells using calcein-AM and ethidium homodimer-1, respectively.

For the preliminary drug screening study, GFP expressing *E. coli* [pRSETB] were incubated for 1.5 h in a microchip containing 8-days cultured Caco-2 cells. The microchannels were then carefully washed with antibiotic-free cell culture medium for 1.5 h to remove non-adherent *E. coli* cells. Finally, a chloramphenicol supplemented (34 µg ml\(^{-1}\)) culture medium was pumped into the microchip at 300 µl h\(^{-1}\) flow rate for 36 h in order to remove the adherent *E. coli* cells from Caco-2 cells under 37° C and 5% CO\(_2\) culture conditions.

6.2.6 Morphological analysis

Phase contrast and fluorescence images were recorded through experiments using an EVOS FL imaging system (ThermoFisher Scientific) equipped with EVOS phase contrast objectives, and GFP, RFP, and DAPI filter cubes. For the fluorescence staining, F-actin, nuclei, and tight junctions of the cells were stained in Caco-2 cells after fixation in 4% (v/v) paraformaldehyde and permeabilization in 0.3% (v/v) TritonX-100 (Sigma Aldrich) using FITC-phalloidin (Sigma Aldrich), NucBlue (ThermoFisher Scientific), and/or Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen), respectively. After the staining process, the cells were scanned using the EVOS FL imaging system.

6.3 Results and discussion

6.3.1 Microchip design, fabrication, and culture conditions

The microfluidic cell culture platform was developed for enabling long-term cell culturing in periodic 3D compartments. A degassed PDMS pre-polymer solution was poured on the SU-8 master to create the microchips with 4 or 5 mm thickness. PDMS microchips were then assembled with a glass layer to form the

![Figure 6.4. Pseudo-colored top-view phase contrast microscopy image of patterned collagen (a) without and (b) with Caco-2 cells on day 8 of culturing. Grey squares are glass pillars and blue rectangles are hydrogels. Microchannels and hydrogel compartments were artificially colored based on grey-scale differences.](image-url)
closed microfluidic channels. A collagen pre-gel and Caco-2 cell mixture was patterned in the microchip using capillary line pinning. The patterning success of the capillary barriers was evaluated on a passed or failed basis. Hydrogel compartments covering the entire gap between PDMS pillars were counted as passed. All other configurations were counted as failed. We found the patterning success rate as 95.0±7.6% (mean ± standard deviation, n = 15), based on our analysis on five representative images that were collected from random spots in the microchips. The patterning success rate is in accordance with previous reports. Figure 6.4 illustrates the top-view phase contrast microscopy images of the channel structure and patterned hydrogel compartments without and with Caco-2 cells.

6.3.2 | Culture conditions

Concentration of the collagen mixture and seeding density of the cells were optimized for maximum cell survival in order to facilitate a microenvironment mimicking the human intestine. Four different collagen concentrations ranging from 0.3% to 0.45% (w/v) with 0.05% (w/v) increments were tested in the microchip. Caco-2 cells did not proliferate at concentrations higher than 0.3% (w/v) collagen (Figure 6.5a-d). In the preliminary experiments, four different cell concentrations in a range between 1.67·10⁶ and 1.33·10⁷ cells ml⁻¹ were tested. Cell concentrations lower than 6.67·10⁶ cells ml⁻¹ resulted in no growth in the cell population. Therefore, 6.67·10⁶ cells ml⁻¹ concentration was used in all of the experiments reported in this study.
6.3.3 Glucose diffusion and flow velocity distributions

Flow velocity and glucose diffusion in the microchip were simulated using COMSOL Multiphysics software. Creeping flow, laminar flow and transport of diluted species modules were used in simulations. A volumetric flow of 300 µl h⁻¹ and a glucose concentration of 25 mmol l⁻¹ were chosen for the simulations. Creeping flow module was operated by applying steady flow, incompressibility, no turbulence, negligible inertial forces and a shallow channel approximation conditions. For the laminar flow, we applied steady flow, incompressibility, no turbulence, and a shallow channel approximation. The transport of diluted species was coupled with the calculated convection from either the laminar or the creeping flow module. Diffusion rate of glucose in water is $D_{cw} = 6.80\times10^{-10}$ m² s⁻¹, the diffusion rate of glucose in the hydrogel patch is $D_{ch} = 1.44\times10^{-10}$ m² s⁻¹. For the reaction rate of glucose, the glucose uptake of 500 Caco-2 cells per hydrogel patch was estimated to be $-20\times10^{-2}$ mol m⁻³ s⁻¹ based on Olejnik’s report. The simulation results showed very similar velocity fields for laminar and creeping flow (laminar flow displayed in Figure 6.6a). They also showed that the velocity in the outer microchannels was 30% lower than in the center microchannel, due to path length differences resulting in a different hydrodynamic resistance. In future designs, this aspect could be corrected by adjusting local channel width. The simulations furthermore
showed that the expected glucose concentration distribution over the microchip is almost uniform (Figure 6.6b).

6.3.4 Morphology changes in long-term cultures

The cells were cultured under static and fluidic conditions during 14 days in microchips. Figure 6.7a-j and 6.8a-g provide the morphological changes of the cells.

When no flow was present in the microchannels, Caco-2 cells spread in the hydrogel patch in the first 6 days. On day 8, the cells started migrating towards the microchannels (Figure 6.8f). In Figure 6.8g, cellular density, which was determined by the number of nuclei, was found to be low in the microchannels when compared to the hydrogel compartments. Cellular protrusions seem to be localized more to the microchannels with regards to the hydrogel compartments.

Under 30 µl h⁻¹ flow rate, the cells were often observed to localize next to the capillary barriers. After 21 days of culture, the cells did not occupy the microchannels, however cell proliferation was not observed in all cell-laden compartments. This result indicates that the flow rate was not sufficient to supply nutrients to the cells (Figure 6.7a-e). This result is not consistent with the diffusion simulations (Figure 6.6b) because the simulation model shows the diffusion profile in hydrogels without cells. Here, the presence of the cells might reinforce nutrient availability in the microchip.

When culture medium was perfused through the microchannels at 300 µl h⁻¹ flow velocity, Caco-2 cells could form 3D structures in hydrogel compartments on day 3. We observed that most of the compartments (~90%) were fully occupied by Caco-2 cells on day 8 of the culture. Between days 8 and 10, we observed that Caco-2 cells started spreading the microchannels

Figure 6.7. Pseudo-colored top-view phase contrast images of long-term Caco-2 cultures in the microchip. The results are shown for the microchip perfused under (a-e) 30 µl h⁻¹, and (f-j) 300 µl h⁻¹ flow rates. Images were artificially colored based on grey-scale differences. In the fluorescence images (bottom line, day 15) staining was applied: blue colors present cell nuclei (NucBlue), and green colors present cytoskeleton (F-actin). Microchannels and hydrogel compartments were artificially colored based on grey-scale differences. Scale bar for the images is 125 µm. The scale bar in the close up image is 40 µm.
After day 15, the cells over-proliferated in the microchip and occupied the walls of the microchannels together with the entire space in hydrogel compartments (Figure 6.7g-h). In summary, the phase contrast microscopy images show that application of an increasing fluid flow rate accelerated cell proliferation and migration, as well as the formation of 3D structures. Interestingly, 3D structures appeared under both static and fluid flow conditions after 6 days of culturing. 3D structure formation and cell proliferation rates were higher in the microchip perfused with culture media, as seen in Figure 6.8. The timing of 3D structure formation in this study is consistent with the villi-like formation observed by in vitro study of Kim et al.\textsuperscript{21} and in vivo.\textsuperscript{29,30} Immunofluorescence microscopic studies using antibodies directed against the F-actin filaments confirmed that Caco-2 cells were observed to form confluent polygonal epithelial monolayers throughout the microchip on day 15 day of the culturing period (Figure 6.7j). The immunofluorescence staining should also be performed before 15 days of culture for a complete characterization.

### 6.3.5 Glucose consumption

To compare the viability of Caco-2 cells in the microchip or Transwells, glucose consumption over 21 days was measured using UV-vis spectroscopy. For this study, the microchip was perfused at 300 $\mu$l h$^{-1}$ flow rate. The results shown in Figure 6.9 reveal that the cells continuously consumed glucose with gradually increasing amounts in the first 10 days of the culture in the microchips. Glucose consumption stabilized after day 10, which corresponds to overgrowth of the cell population into the microchannels where they formed...
confluent monolayers (Figure 6.7j). In Figure 6.9, the trends of glucose consumption rate show similarity in both microchip and Transwell, revealing that cell population in microchip and Transwell grew in a similar pattern and they have an active metabolism. These findings suggest that the periodic hydrogel compartments in the microchip provide a well-controlled cell culture platform for long-term cell culturing. Owing to the advantage of using a fluidic platform, sampling is enabled from the outflow for direct tracking of the glucose consumption in the culture over time, allowing for e.g. comparison of the growth dynamics of cell population in the microchips and in Transwells from measurements of the metabolome. The microchip therefore enables high-throughput on-line monitoring, when compared to bio-microreactor systems, because of the multiple microchannels designed in parallel to each other.

6.3.6 Bacteria co-culture

Bacteria and human intestine epithelial cells were co-cultured in the microchip in both long-term and short-term experiments to determine the capability of our microchip system to support physiologically relevant dynamic co-cultures.

In the long-term study, an intestinal bacterium, *E. coli*, together with the culture media were injected into the microchip starting on the 8th day of culture for 36 h. Here, 36 h co-culture can be considered as a long-term study owing to the rapid proliferation rate of bacteria. The experiments were performed under both fluid flow and static conditions to provide a comparative study. The microchannels at each side of the hydrogel compartments were used to introduce bacterial cells into the microchip. Figure 6.10a-d shows the live/dead
staining of the culture with calcein-AM and ethidium homodimer-1, respectively. Caco-2 cells showed no indication of cell death (no red colored cells were observed in Figure 6.10d) when the culture was treated with 300 µl h⁻¹ flow rate with \textit{E. coli} cells for 36 h. The viability of Caco-2 cells was observed to be similar under the same conditions without \textit{E. coli} cells (Figure 6.10b). Under static conditions with bacteria, approximately 30% of Caco-2 cells were dead after 36 h, as observed in live/dead staining images (Figure 6.10c). Under static conditions without \textit{E. coli}, 100% of Caco-2 cells remained alive (Figure 6.10a). Caco-2 cells spread towards the main channels when fluid flow was not present. Contrarily, the cells stayed confined in the hydrogel compartment when fluid

\textbf{Figure 6.10.} Pseudo-colored top-view phase contrast microscopy images of live/dead assay bacteria co-culture operated (a) under 300 µl h⁻¹ flow rate without \textit{E. coli} cells, (b) under 300 µl h⁻¹ flow rate with \textit{E. coli} cells, (c) without fluid flow and without \textit{E. coli} cells (d) without fluid flow with \textit{E. coli} cells. The nuclei of Caco-2 cells were stained with DAPI (blue). Alive Caco-2 cells are shown in green and dead Caco-2 cells are shown in red colors. In image (d), the dark clouds in the microchannels and the compartments are \textit{E. coli} colonies. Microchannels and hydrogel compartments were artificially colored based on grey-scale differences. Scale bar is 250 µm.

\textbf{Figure 6.11.} Top-view phase contrast image of bacteria co-culture with transgenic \textit{E. coli} with GFP. Caco-2 cell culture on day 42 (a) before bacteria inoculation under 300 µl h⁻¹ flow rate, (b) after 1.5 h \textit{E. coli} inoculation, (c) after 36 h treatment of chloramphenicol (34 µg ml⁻¹) applied under 300 µl h⁻¹ flow rate. Green fluorescent color represent the bacterial cells. Live/dead conditions of bacterial cells are independent from their fluorescence signal due to the GFP expression of bacterial cells. Scale bar is 250 µm.
flow was present in the main channels. The spread of Caco-2 cells towards the main channels was about 50% higher when compared to the situation where culture media without bacteria was pumped into the microchip. These results demonstrate that the microfluidic perfusability of the microchip effectively enhanced cell viability when co-cultured with *E. coli* cells. A stable co-culturing of human intestine epithelial cells and bacterial cells was also established. The fluidic environment prevented unrestrained over-proliferation of the bacteria in the microchip while Caco-2 cells remained accessible by the bacterial cells in the fluidic culture. In Figure 6.10d, the cloudy regions in both the hydrogel compartments and microchannels—where no fluid flow was present—indicate unrestrained bacteria proliferation. Contrarily, the cloudy regions were not observed in microchannels while they were present in the hydrogel compartments in the microchip, where 300 µl min⁻¹ flow rate was applied (Figure 6.10b).

**Figure 6.12.** Pseudo-colored top-view phase contrast microscopy images of Caco-2 cells cultured in (a-b) larger and (c-d) smaller compartment designs under static conditions. Microchannels and hydrogel compartments were artificially colored based on grey-scale differences. Scale bars are 125 µm.
In the short-term study, bacterial adhesion to Caco-2 cells was observed by injecting recombinant GFP expressing *E. coli* cells into the microchip for 1.5 h (Figure 6.11). During the antibiotic-free culture media treatment, *E. coli* cells adherent to Caco-2 cells remained in the hydrogel compartments while non-adherent cells were washed out with the fluid flow. Chloramphenicol was chosen as the antibiotic in this study as it is commonly used for treatment of bacterial infections. Bacterial proliferation was not observed in the microchip after the incubation with chloramphenicol after 36 h. However, the adherent bacterial cells remained in the compartments as seen in Figure 6.11. These findings demonstrate that the controllable fluidic perfusion in the microchip enables preliminary screening of drug treatment on the co-culture.

6.3.7 Effect of hydrogel compartment geometry on cell growth

Figure 6.12a-d demonstrates the cell proliferation in differently shaped compartments with a smaller or larger hydrogel-culture media interface. Both designs were leak-free and allowed for performing assays due to fast molecular transport across the hydrogel compartments. Under static conditions, Caco-2 cells in the compartments with a large interface (Figure 6.12a-b) spread more evenly over the compartments probably due to the increased hydrogel interface, and therefore increased surface area in the hydrogel compartment. The colony morphology seem to have a more elongated shape when compared to the round-shaped colonies in Figure 6.12c-d. At the 6th day of the culture, cells started to migrate through the microchannels from the larger compartments while they remained inside the smaller compartments. The reason might be the proximity of the PDMS pillars, which are closer to each other in Figure 6.12c-d when compared to Figure 6.12a-b. In the closer proximity case, cells might be given less hydrogel-filled surface to spread and therefore their proliferation rate remained lower. However, an experiment with fluid delivery performed for a longer period is necessary to confirm this result. The main result is that both large and small hydrogel compartments allowed cells to grow over a longer time. The cell growth pattern follows the contours of the compartments, enabling more systematic follow up studies of how compartment sizes and shapes would affect 3D growth patterns.

6.4 Conclusions

In this work, we have developed a new approach to build *in vitro* cell culture platforms for tissue mimicry using periodic 3D cell-laden hydrogel compartments inside closed microfluidic chips. The design concept is based on selectively trapping mixtures of collagen pre-gel and cells in compartments via capillary line pinning. The architecture of the microchip and the ability for continuous fluid delivery enabled long-term and in-parallel culturing of human intestine cells (Caco-2) that spontaneously grew into 3D folds on the 3rd day of
cell culturing. After 8 days of culturing, the cells started spreading to the microchannels and this growth pattern could not be controlled. For that reason, we kept the culture periods under 8 days. Possible solutions would be to increase the flow rate of the culture medium in the microchannels, and to functionalize the microchannel surface selectively to keep them cell-free. The glucose consumption experiments performed in both microchips and Transwells revealed that the dynamics of glucose consumption was similar to cultures in both platforms. We also co-cultured Caco-2 cells with an intestinal bacterium (*E. coli*) to show bacteria-cell attachment and the viability of Caco-2 cells under fluidic conditions. *E. coli* bacteria adhered to Caco-2 cells after 36 h of incubation without affecting the viability of Caco-2 cells, showing cell-bacteria attachment in the microchip as well as mimicking of intestinal integrity in terms of bacteria-cell interactions.

The fluidic control problem of the compartmentalized organ-on-chip devices was addressed in this study to enable on-demand manipulation of the outer cell microenvironment in well-defined structures. In our future work, several features of the microchip will be improved in order to use this approach for effectively mimicking the complex functions of different tissues, including response of the Caco-2 cells to the bacteria-cell attachment and application of different drugs and in the molecular level. Replacing the buffer reservoirs with single microchannels would make a more physiologically realistic *in vitro* model. In this way, the application of fluids with different compositions in each side of the compartments will be enabled.

Our approach offers great promise both for building next-generation organotypic *in vitro* platforms by enabling high-throughput culturing in a microfluidic environment, where approximately 500 hydrogel compartments can be easily fabricated. In addition, this device has potential to be used for creating separate 3D microenvironments, where a gradient of different metabolites can be applied to study tissue functions, drug screening, and toxicology testing. The microchip can be an alternative to the bio-microreactor systems, as it enables high-throughput on-line measurements by the multiple microchannels designed in parallel to each other.
6.5 References


Desalination by Microelectrodialysis*

We report a novel approach for separation of charged species using anion-(AEH) and cation-exchange hydrogels (CEH) in a microfluidic device. The capillary line pinning technique enabled in situ fabrication of alternating anion- and cation-exchange hydrogels that are placed in confined compartments. Adjacent enriched and depleted streams were obtained in continuous flow when a potential difference was applied over the hydrogel stack. The desalination performance of the microchip was demonstrated at different salt concentrations (0.01-1 mM sodium chloride), potentials (10-100 V), currents (12-28 A m$^{-2}$) and fluid flow rates (0-5 µl min$^{-1}$). We demonstrate that the microchip was able to remove approximately 75% of the salt initially present in the depleted outlet streams for inlets streams of 1 mM sodium chloride. Besides desalination, the microchip allows to study ion transport in the ion-selective hydrogels. It can therefore be used to study in detail the interplay of transport phenomena at the electrolyte-hydrogel interface during the desalination process.

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7.1 Introduction

Membrane stacks are widely used for electrodialysis (ED) and reverse electrodialysis (RED) in macroscale systems. Alternating cation- (CEM) and anion-selective membranes (AEM) are stacked with spacers to allow a flowing electrolyte solution between those membranes. When an electric field is applied to this system, the anions in the electrolyte solution will migrate towards the anode, while the cations migrate towards the cathode. However, anions and cations will eventually be blocked by the CEM and AEM respectively, resulting in the formation of alternating depleted and enriched flow streams. As the applied electric field strength is increased, the ion transport rate and therefore the current in the system will rise—up to a critical point. Beyond this point, the system enters the well-known limiting current regime, where the current no longer increases with electric field strength, and the energy efficiency of the ion separation process is reduced. In this regime, the major resistance for ion transport is located in the boundary depletion layer. When increasing the electric field strength further or in the case of membrane discharge due to the developing pH in the membrane, the over-limiting current regime can be reached, resulting in the transport of additional ions obtained through either water splitting or hydrodynamic effects. The hydrodynamic and ion transport phenomena can result in a loss of performance for macroscale ED systems. Microfluidics has therefore been used for downsizing the electrodialysis process to provide more insight into the ion transport phenomena that occur at the microscale. Thanks to the advances in microfabrication techniques, ion-selective materials integrated in fluidic platforms have increased experimental investigation of the aforementioned phenomena. Recently, in situ fabrication of hybrid membranes by photolithography has been demonstrated for charge-based separations in a glass microchip. Kim et al. fabricated two microchannels separated by a Nafion membrane, which was fabricated using micro-stamping, to observe multiple vortical flows inside the ion concentration polarization layer. Kwak et al. fabricated a PDMS microchip consisting of externally mounted commercial ion exchange membranes for visualization of concentration and fluid flow profiles at ohmic, limiting, and overlimiting regimes. In another work, solvent evaporation has been used for in situ fabrication of ion-selective membranes in a PDMS microchip consisting of dual channels for studying ion concentration polarization phenomena.

The aforementioned methods, which have been used for incorporation of membranes into microfluidic systems, pose challenges in terms of robustness, consistency, and ease of fabrication. For example, photolithography techniques bring alignment problems, while evaporation bears the risk of damaging the membranes. Membranes sandwiched between microchannels often lead to fluid leakage. Hydrogels are promising candidates for studying the hydrodynamic and ion transport phenomena in microfluidic devices, as they provide an ion-
selective and hydrophilic matrix, which is versatile, inexpensive, and tailorable.\textsuperscript{14,18} In the past, we introduced the capillary line pinning technique for controllable patterning of hydrogels in microchips.\textsuperscript{19}

In this work, we present a microfluidic device consisting of a stack of alternately-patterned oppositely-charged hydrogel patches fabricated by capillary line pinning for performing electrodialysis. To the best of our knowledge, the use of such a platform has not been demonstrated and investigated before for microfluidic electrodialysis. As a proof of principle, the periodic hydrogel patches were used to obtain alternatingly diluted and enriched parallel streams under different flow rates and applied potential differences. This platform allowed us to study ion transport through the hydrogels as well as salt concentration fluctuations and ion concentration polarization (ICP) in the microchannels. Being able to visualize charge transport in the hydrogels helps to obtain a better understanding of hydrodynamic and ion transport phenomena that occur in ED and RED systems. Besides that, PDMS microchips with \textit{in situ} patterned hydrogels enable low-cost and versatile platforms for microfluidic ion-separations.

7.2 Methods

7.2.1 Microchip and hydrogel fabrication

The SU-8 masters of the polydimethylsiloxane (PDMS, Dow Corning, USA) microchips were fabricated in the MESA+ cleanroom facility at the University of Twente. The masters consisted of two layers of SU-8. The width of the microchannels and the pillars was 700 µm and 1720 µm, respectively. The first layer contained the pillar layout and its thickness was measured as 67.5 µm (Bruker Dektak). The second layer contained both pillar and capillary barrier layouts with a 7.5 µm thickness (see Figure 7.1b and 7.2). Therefore, while the height of the channel was 75 µm, the height of the capillary barriers were 7.5 µm. Therefore, while the height of the microchannel was 75 µm, the height of the capillary barriers was 7.5 µm.

The microchip was fabricated by assembling a PDMS layer with a microscope slide. To achieve that, PDMS precursor was prepared, degassed, poured onto the master, and cured. The cured PDMS was peeled off from the master, treated with oxygen plasma to ensure irreversible bonding with the microscope slide. All microchannels, pillars, capillary barriers, inlets and outlets were structured in the PDMS layer. Figure 7.1a shows an assembled microchip with PDMS pillars and capillary barriers. In the microchip design, each hydrogel compartment (200×500 µm), is interconnected to another via microchannels.
All hydrogel solutions (each with a volume of 500 µl) were degassed at 7 kPa vacuum for 15 min immediately prior to use. All hydrogel precursors were prepared in a nitrogen environment by blending 20% v/v of acrylamide/bis (19:1) (BioRad), 15% w/v N,N’-bis(2-hydroxyethyl) ethylenediamine (bis, Sigma-Aldrich), 10% w/v of 2,2-dimethoxy-2-phenylacetophenone (DMPA, Invitrogen) and 5% w/v of ammonium persulfate (Invitrogen) solutions in a flow hood to avoid dust particles. For the anion-exchange hydrogels (AEH), 1% v/v [2-(methacryloyloxy)ethyl]trimethylammonium chloride solution (METC, Sigma Aldrich), and for the cation-exchange hydrogels (CEH) 1% w/v 3-sulfopropyl acrylate potassium salt (SPAP, Sigma Aldrich) was added to the acrylamide/bis mixture. As the crosslinking reaction is inhibited by oxygen, patterning and polymerization processes were performed in a nitrogen environment.

Figure 7.3 outlines the microchip fabrication and hydrogel patterning processes. A detailed explanation of the capillary pinning technique has been reported by Gumuscu et al.19 After the degassing process, approximately 0.5 µl of AEH and CEH solutions was alternatingly pipetted into the parallel columns by capillary action. Hydrogel precursors remained contained between the PDMS pillars due to capillary pinning at the barriers. Patterned precursors were

![Figure 7.1](image1) Image of the assembled microchip. (b) Schematic representation of a membrane stack containing alternating AEH's and CEH's and the desalination mechanism.

![Figure 7.2](image2) Phase contrast microscopy images of SPAP (red) METC (green) hydrogels (a) after fabrication, prior to water injection to the microchannels, (b) after 1.5 hours in water, (c) after 16 hours in water. The hydrogels are artificially coloured based on grey scale differences.
then exposed to UV light at 400 mW cm\(^{-2}\) for 3 min. Microchips containing CEH and AEH were immersed in 0.1 mM NaCl solution at room temperature. The microchip has six microchannels, which are connected by alternating AEH and CEH as shown in Figure 7.1b.

### 7.2.2 Characterization of hydrogels

**Scanning electron microscopy (SEM)**

Thin films of polymerized hydrogels were casted on a glass surface and freeze-dried for 2 days. Samples were kept in a vacuum chamber overnight and subsequently coated with a 5 nm layer of chromium using a sputtering device (E5000 sputter coater). SEM images were taken using a FEI Sirion HR-SEM.

**FTIR and XPS**

Fourier transform infrared spectroscopy (FTIR) spectra were measured with a Bruker ALPHA (Bruker Optics) using an attenuated total reflectance attachment. Polymerized hydrogels were freeze-dried for 2 days in Eppendorf tubes and kept in a vacuum chamber overnight. Dried samples were directly analyzed without any further sample preparation. For X-ray photoelectron spectroscopy (XPS), 1 g of both polymerized AEH and CEH were freeze-dried for 2 days in Eppendorf tubes and kept in a vacuum chamber.

![Figure 7.3](image_url)

**Figure 7.3.** Photolithography and soft lithography procedures. The schematics on the top line illustrate the photolithography and the schematics on the bottom line present the soft lithography process flows. Blue dashed line presents the cross-sectional area. In bottom right, an assembled microchip with alternately patterned AEH (green) and CEH (pink) are shown.
overnight. Dried samples were analyzed using a Quantera SXM (Physical Electronics Inc.).

**Ion exchange capacity (IEC)**

The IEC is the amount of charged groups in the hydrogels and is measured by titration. For the titration of AEH, 0.7 g of the hydrogel was immersed in 3 M NaCl (Sigma-Aldrich) for 3 days at room temperature to exchange all counter-ions in the AEH for Cl\(^-\). After rinsing it with deionized water, the chloride ions were replaced by sulfate ions by immersing the hydrogel into a 1.5 M Na\(_2\)SO\(_4\) solution for 3 hours. The Na\(_2\)SO\(_4\) solution was refreshed every hour of the experiment and the collected solutions were titrated with 0.1 M AgNO\(_3\) solution. The required volume of AgNO\(_3\) was recorded. The hydrogel was dried in a vacuum oven at 60°C for 48 hours and its dry weight was recorded for IEC calculations. For the titration of CEH, 0.7 g of the hydrogel was immersed in 1 M HCl solution for 3 days at room temperature to exchange all counter-ions in the CEH for H\(^+\). The hydrogel was then rinsed with deionized water and immersed in a 2 M NaCl solution for 3 hours to replace the hydrogen ions for sodium ions. The NaCl solution was refreshed every hour and the refreshed solutions were collected in a separate container. The collected solutions were then titrated with 0.1 M NaOH solution. The hydrogel was similarly dried and its weight was measured for IEC calculations. All of these experiments were repeated three times.

**Water swelling**

Water swelling of the hydrogels was determined by immersion in deionized water and subsequent drying in a vacuum oven. The degree of water swelling \(WS\) was calculated using the following equation: \(WS = (W_s - W_d)/W_d\). Here, \(W_s\) and \(W_d\) represent the weight of the swollen hydrogels and the dried hydrogels, respectively. Also the swelling degree of the polymerized hydrogels was tested by immersing 2 ml of bulk hydrogel after polymerization in 50 ml deionized water, and by immersing the patterned hydrogels on chip in DI water for 72 and 16 hours, respectively.

**Permselectivity**

Permselectivity determines the capability of the hydrogel to discriminate between anions and cations. An in-house built two-compartment-cell made of plexiglass was used in permselectivity experiments. The charged hydrogel was located in between two compartments. One of the compartments was filled with 0.1 M NaCl solution while the other compartment was filled with 0.5 M NaCl solution using a peristaltic pump. Both solutions were recirculated continuously during the measurement and kept at 25°C using a thermostatic bath. Two reference
calomel electrodes were used to measure the electric potential over the hydrogel by connecting the system to a potentiostat (Metrohm Autolab PGSTAT302N) for monitoring the changes in voltage over the hydrogel. The apparent permselectivity $\alpha = \Delta U_{\text{meas}}/\Delta U_{\text{theor}}$ was calculated for both AEH and CEH separately using the ratio of the measured potential difference $\Delta U_{\text{meas}}$ over the theoretical potential difference $\Delta U_{\text{theor}}$. For an ideal permselective membrane. The theoretical potential difference is obtained from the Nernst equation $\Delta U_{\text{theor}} = (RT)/(zF) \ln(c_{1\gamma_1}/c_{2\gamma_2})$. Here, $R$ is the gas constant (J mol$^{-1}$ K$^{-1}$), $T$ the temperature (K), $z$ the electrochemical valence and $F$ the Faraday constant ($96.5 \cdot 10^4$ J mol$^{-1}$). The salt concentrations and activity coefficients of the two solutions are indicated by $c_i$ and $\gamma_i$.

**Membrane resistivity**

The area resistance of both AEH and CEH was measured using an in-house built six-compartment-cell made of plexiglass. In experiments, 0.1 M NaCl was used and the voltage drop $\Delta U$ over the hydrogel was recorded for an applied current density $I/A$. Here, $I$ is the current (A) through the membrane with area $A$ (m$^2$). The resistivity $R_{\rho}$ of each hydrogel, after baseline correction by performing a blank run, was given by the slope of the potential difference versus current density curve. Thus, $R_{\rho} = (A\Delta U)/(Id)$, $d$ being the thickness of the hydrogel (m).

**Electrodialysis experiments**

The desalination performance of the microchip was investigated using solutions of different NaCl concentrations (0.01 mM, 0.1 mM, and 1 mM) containing 5 µM of Alexa Fluor 488 Cadaverine (ThermoFisher Scientific), a negatively charged fluorescent dye. This dye is utilized to qualitatively determine the local ion concentration in the microchannels. Gold coated copper electrodes were placed in the outer two microchannels adjacent to the hydrogels. An electrical potential or current was applied using a Keithley 2450 Source Meter that is controlled by a home-built LabVIEW program. The fluorescent dye was visualized using a Hamamatsu ORCA-Flash 4.0 LT camera mounted on an inverted Zeiss Axiovert 40 MAT microscope. The fluid flow was supplied to the microchip using Harvard Picoplus programmable syringe pumps and Braun Omnifix-F 1 ml syringes. The samples were collected separately from each microchannel and the conductivity of the samples was measured using a potentiostat (BioLogic SP300) combined with an in-house made interdigitated electrode sensor. A calibration curve was plotted and used for the salt concentration conversions (Figure 7.4).
7.3 Results and discussion

7.3.1 Characterization of hydrogels

In Figure 7.5, the molecular structures of charged monomers (METC and SPAP) photopolymerized with acrylamide and bis are shown. The free radicals yielded by DMPA started the crosslinking process by randomly associating with acrylamide, bis, and METC or SPAP monomers and grew polymer chains, which eventually became polymerized in the illuminated region. The photo polymerization process has been explained in detail in our previous work. The SEM images indicate that the hydrogels obtained after photopolymerization were homogeneous (Figure 7.6).

Table 7.1. Bulk material characteristics of crosslinked METC and SPAP polymers, with the 95% confidence interval of the mean indicated. All measurements have been repeated three times.

<table>
<thead>
<tr>
<th></th>
<th>AEH (METC)</th>
<th>CEH (SPAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swelling in deionized water (%)</td>
<td>460 ± 10</td>
<td>450 ± 10</td>
</tr>
<tr>
<td>Ion exchange capacity (mmol g\text{dry}^{-1})</td>
<td>1.95 ± 0.45</td>
<td>0.64 ± 0.09</td>
</tr>
<tr>
<td>Charge density (mmol g\text{H}_2\text{O}^{-1})</td>
<td>0.43 ± 0.10</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Resistivity (\Omega cm) (single measurement)</td>
<td>310</td>
<td>470</td>
</tr>
<tr>
<td>Permselectivity (%) (single measurement)</td>
<td>29</td>
<td>26</td>
</tr>
</tbody>
</table>

Figure 7.4. Calibration curve of the impedance Z as function of the NaCl concentration c. The fitting is performed on a log-log scale to ensure the experimental data is normally distributed around the calibration curve. To improve the reliability of the calibration curve, the data points for 0.01 and 0.02 mM are omitted, as they clearly fall outside the linear range. The minimum measured concentration was also always larger than 0.1 mM. The dashed lines form the 95% prediction bounds. As the calibration line gives \log_{10}(Z) as function of \log_{10}(c), on an absolute scale the size of the error depends on the concentration, and the errors are not symmetric around the determined concentration.
Desalination by Microelectodialysis

**Figure 7.5.** Molecular structures of monomers, crosslinker, and polymers. (a) METC polymer, (b) SPAP polymer.

METC and SPAP are suitable monomers to obtain charge selective interfaces for electrodialysis because they have permanent positive and negative charges, respectively, and their charges are pH independent in the range of pH 2-12.\(^{23,24}\) In addition, the hydrolytic stability of METC and SPAP polymers enables charge based separations in electrodialysis microchips.\(^{23,24}\) In the previous studies, both charged monomers have shown to be super-absorbents, which result in a strong swelling behavior.\(^{25,26}\) The swelling behavior was suppressed by increasing the crosslinking density and the amount of uncharged monomers in this work. We measured the water swelling of the bulk METC and SPAP polymers as 460 ± 10% for METC and as 450 ± 10% for SPAP after drying them in a vacuum oven (Table 7.1). The high swelling degree indicates hydrogels’ ability to absorb water in high contents, which is related to the hydrophilic nature of the PA backbone. Note that the hydrogels already have a high water content right after the photopolymerization. When immersed in deionized water directly after photopolymerization, the additional swelling of the bulk hydrogels was measured to be 15% and 16% for AEH and CEH, respectively. Both hydrogels showed an identical swelling behavior also in the microchip as shown in Figure 7.2. In addition, hydrogels immersed in salt solutions are expected to swell less than they swell in deionized water.\(^{27}\) The reason is that hydrogel swelling decreases with increasing ion concentration, as
the charged side groups present in the hydrogels are increasingly screened by the ions in the salt solution.\textsuperscript{28,29}

The presence of crosslinker and SPAP or METC was validated using FTIR. The FTIR spectra shown in Figure 7.7 verified the presence of SPAP sulfonate groups from S=O stretching bands at 982 and 1043 cm\(^{-1}\) and a SO\(_2\) symmetric stretching absorption at 1178 cm\(^{-1}\).\textsuperscript{30} A relatively weak absorption at 1170 cm\(^{-1}\) was ascribed to N-CH\(_3\) stretching of METC repeat units.\textsuperscript{30} C-N stretching absorptions in general are known to be weak.\textsuperscript{30,31} We measured the FTIR spectra of the corresponding monomers to confirm the location of the peaks observed in the polymers (Figure 7.8).

**Table 7.2.** XPS results for the polymers with different compositions.

<table>
<thead>
<tr>
<th>Element (%)</th>
<th>C (1s)</th>
<th>N (1s)</th>
<th>O (1s)</th>
<th>S (2p)</th>
<th>Cl (2p)</th>
<th>K (2p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>283 eV</td>
<td>399 eV</td>
<td>529 eV</td>
<td>164 eV</td>
<td>200 eV</td>
<td>293 eV</td>
</tr>
<tr>
<td>PA</td>
<td>64.6</td>
<td>16.3</td>
<td>18.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SPAP + PA</td>
<td>62.1</td>
<td>16.1</td>
<td>20.3</td>
<td>1.2</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>METC + PA</td>
<td>64.4</td>
<td>16.3</td>
<td>20.1</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

We analyzed the chemical composition of the hydrogels using XPS to verify the presence of either SPAP or METC monomers. The atomic ratios of carbon, nitrogen, oxygen, sulphur, chlorine, and potassium are shown in Table 7.2. METC and SPAP polymers were compared with PA polymer for the evaluation of the atomic percentage changes. In METC polymer, the amount of carbon atoms remained the same despite a slight increase was expected.\textsuperscript{31} The nitrogen atom content remained the same and this was consistent with the expected composition. The amount of oxygen was expected to increase which was consistent with the measurements in Table 7.2. The detection of chloride clearly showed the presence of charged N\(^+\) groups.\textsuperscript{32-34} In the SPAP hydrogel, the content of carbon atoms decreased, which could be an experimental artefact. On the other hand, the amount of nitrogen atoms remained the same as expected by considering the molecular formulas. The amount of oxygen atoms increased significantly and this can be attributed to the high oxygen content of the SPAP monomer.\textsuperscript{31} Next, the presence of sulfonate groups was validated by the detection of sulphur atoms and potassium atoms. The latter, however, was
present in a lower amount than expected. Here, the potassium atoms might be out of the scanning range due to the orientation of the charged groups on the polymer film.

Table 7.1 shows the material characteristics of the anion exchange (METC) and cation exchange (SPAP) hydrogels. Compared to commercially available ion exchange membranes,\textsuperscript{35,36} METC exhibited a comparable and SPAP a low ion exchange capacity. When considering the charge density (CD), we found both hydrogels to have a low number of charged groups per gram water. Due to the hydrophilic structure of the hydrogels, they are capable of holding

![Figure 7.7. FTIR spectra of binary component ionic hydrogels. The compositions of METC + polyacrylamide (blue column), SPAP + polyacrylamide (red column), and only polyacrylamide are shown.](image)

Table 7.1 shows the material characteristics of the anion exchange (METC) and cation exchange (SPAP) hydrogels. Compared to commercially available ion exchange membranes,\textsuperscript{35,36} METC exhibited a comparable and SPAP a low ion exchange capacity. When considering the charge density (CD), we found both hydrogels to have a low number of charged groups per gram water. Due to the hydrophilic structure of the hydrogels, they are capable of holding

![Figure 7.8. FTIR spectra of (a) METC and (b) SPAP monomers. The blue column shows the C-N stretching at 1178 cm\(^{-1}\) in METC and the red column shows the R-SO\(_3\)\(^-\) groups at 982 and 1043 cm\(^{-1}\) in SPAP.](image)
large amounts of water in their polymeric meshes. This can explain the moderate permselectivity of both hydrogels, as the permselectivity of an ion-selective membrane is related to its charge density.\textsuperscript{35-37} Both anion and cation exchange hydrogels are therefore not fully selective for the counter-ions, which has an adverse effect on the desalination efficiency. However, we performed the desalination experiments using a salt concentration of maximum 1 mM NaCl, which is two orders of magnitude smaller than the salt concentration of 0.1 M/0.5 M, which is commonly used in permselectivity measurements. This low salt concentration leads to decreased screening of the charged groups in the hydrogels, promoting charge separation.\textsuperscript{37} The resistivity of the hydrogels is comparable to commercial membranes with a typical area resistance of 3 Ω cm\textsuperscript{2} and a membrane thickness of 150 µm, giving a resistivity of 200 Ω cm.\textsuperscript{35,36} This relatively high resistivity may be related to the low charge density of the hydrogels.

7.3.2 | Desalination–proof of principle experiments

Upon filling the microfluidic channels with electrolyte solution and applying a gradually increasing potential ($\Delta U = 0-3$ V), we observed migration of the fluorescent dye (and thus presumably of the ions) into the hydrogels. As expected, AEHs were observed to have a high fluorescence intensity, as they absorb the negatively charged fluorescent dye as a counter-ion to a much greater extent than CEHs, where the dye is the co-ion.

To test whether the stack of charged hydrogels can be used for desalination purposes, we filled all microchannels with a diluted salt solution (0.1 mM NaCl) and did not impose any flow through the microchannels. Upon application of a potential difference, depletion zones of the fluorescent dye formed adjacent to the hydrogels in the expected microchannels (which are channel 2 and channel 5 as shown in Figure 7.1b), while the average fluorescence intensity in the enriched microchannels increased. Snapshots of this observation are shown in Figure 7.9a-c. These observations indicate that the hydrogels are charge-selective and can be used for desalination.

![Figure 7.9](image)

*Figure 7.9. Fluorescence images of ion concentration polarization in the microchip. 1 mM NaCl solution with 5 µM of NaAlexa (the fluorescent dye) was injected into the microchip and an IV-sweep up to 3 V was demonstrated. The experiments were performed (a-c) under increasing electric field at no fluid flow.*
IV-sweeps were measured to determine the characteristic under-limiting, limiting and over-limiting current regimes, which are well known for ion-selective membrane systems.\textsuperscript{2,4,8,10} These measurements were performed for different NaCl concentrations (0.01, 0.1 and 1 mM) and flow rates (2, 3, 5 and 10 µl min\(^{-1}\)). The applied voltage was ramped up with steps of 1 V, with a speed of 10 seconds per voltage. Figure 7.10 shows the IV-curves for different salt concentrations.

**Figure 7.10.** IV characteristics for different salt concentrations at flow rates of 3 µl min\(^{-1}\). In (a), the absolute current \(I\) is plotted as function of the potential difference \(\Delta U\). The currents measured strongly depend on the salt concentration, and scales accordingly. In (b), the currents are normalized by the maximum current of the corresponding experiment. The graphs, in particular those for 0.1 and 1 mM, show that around a potential difference of approximately \(\Delta U = 6\) V and \(\Delta U = 22\) V, the slope changes. This indicates the boundaries between the ohmic, limiting and over-limiting current regimes.
concentrations. We observed higher currents for higher salt concentrations, indicating that the current in the ohmic regime is proportional to the ion concentration in the liquid. The maximum current, which is reached at the highest potential difference of $\Delta U = 50$ V in the over-limiting current regime, does not scale one to one with the salt concentration.

Normalization of the current $I$ by the maximum current $I_{\text{max}}$ of the corresponding experiment indicates that for all concentrations the transport behavior is similar. At a potential difference of approximately $\Delta U = 6$ V, ion transport transitions from the ohmic to the limiting current regime. At a potential difference of roughly $\Delta U = 22$ V, the over-limiting current regime starts. Calculation of the derivative $dI/d\Delta U$ shows that at these points the slope of the $I(\Delta U)$-curve changes, indicating a change in the transport characteristics. The transition points are in particular apparent from the curves for 0.1 and 1 Mm NaCl. The fluctuations in current decreased with increasing salt concentration. Different flow rates did not lead to significant differences in measured currents. This can possibly be attributed to incomplete depletion of ions in the diluted streams, implying that there are still ions available for charge transport through the hydrogels for all flow rates.

At elevated voltages ($\Delta U > 10$ V) we observed the development of ion depletion zones in the depleted microchannels. On the other hand, a local increase of fluorescence intensity was observed in the enriched microchannels,

![Image](image-url)

**Figure 7.11.** Fluorescence images showing the formation of ion depleted and enriched zones in the depleted (D) and enriched (E) microchannels for two constant potential measurements. 0.1 mM NaCl solution with 5 μM Alexa Fluor 488 Cadaverine (the fluorescent dye) was flushed through the microchip at 3 μl min$^{-1}$. The experiments were performed at a constant potential difference of (a) $\Delta U = 20$ V and (b) $\Delta U = 30$ V. The bright colored hydrogel is the anion-exchange hydrogel (METC) and the dark colored one is the cation-exchange hydrogel (SPAP). E represents enriched stream and D represents diluted stream.
indicating the local existence of a higher salt concentration. As shown in Figure 7.11a, the depletion zones were observed to grow in size with increasing applied voltage and distance from the inlet. Figure 7.11b shows that for $\Delta U > 25$ V vortices were formed in the depletion zones. Because of the discontinuous nature of the hydrogel patches—an alternating pattern of charge-selective hydrogels and PDMS—the vortices did not migrate in the direction of...
the imposed flow as reported by Kwak et al.\textsuperscript{13} At the upstream edge of each hydrogel patch, in the depletion zone, a high fluorescence intensity was observed. This suggests that near those points locally the salt concentration was larger than in the rest of the depletion zones. The mixing of the electrolyte solution next to the PMDS pillars by the vortices could explain this behavior, as this mixing increases the ion concentration near the PDMS pillar and consequently also near the upstream edge of each hydrogel. Charge transport is thus affected by the heterogeneity of the ion concentration at the hydrogel.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure7_13.png}
\caption{The design of the microfluidic electrodialysis platform allows visualization of transport in the hydrogels between the salt enriched (E) and depleted (D) channels. In (a) the current $I$ is plotted as function of time $t$ for a fixed potential difference of $\Delta U = 70$ V and flow rate of 3 $\mu$L min$^{-1}$. Before reaching steady state, the change in current can be related to transport in the hydrogel, as indicated by the (b) fluorescence images of the dye in the hydrogels (1 mM NaCl solution with 5 $\mu$M Alexa Fluor 488 Cadaverine). It took about 600 s before the intensity, and thus presumably dye and ion concentration, in the hydrogels was constant. E represents enriched stream and D represents diluted stream.}
\end{figure}
interface. Thus, the heterogeneity could even enhance charge transport when compared to a homogeneous ion-selective membrane, as previously discussed in the literature.  

For quantification of the desalination performance of the microchip, we performed measurements in which we applied a constant potential in the range of 3-7 µA (corresponding to a current density of 12-28 A m⁻²). The resulting currents and potentials were measured to confirm charge transport through the hydrogel patches. A 1 mM NaCl (supplemented with 5 µM Alexa Fluor 488 Cadaverine) solution was pumped to the microchannels at either 3 or 5 µl min⁻¹ flow rates. The conductivity of the solutions collected from the outlets was measured, and the salt concentration was calculated accordingly. As expected, the effluent streams from the microchannels were salt enriched or depleted in an alternating pattern.

In Figure 7.12a the measured salt concentrations are shown for constant potential experiments for two different flow rates (3 and 5 µl min⁻¹). When the flow rate was increased, the residence time and therefore the charge separation decreased. Only the outlet streams of microchannels 2 to 5 were taken into account. In the outer two microchannels 1 and 6, containing the electrodes and both facing only an anion-exchange hydrogel (see Figure 7.1), the solution is not necessarily either enriched or depleted because of electrode reactions.

In Figure 7.13a, the current $I$ is plotted as function of time $t$ for a fixed potential difference of $\Delta U = 70$ V and a flow rate of 3 µl min⁻¹. This data corresponds to the results shown for 3 µl min⁻¹ in Figure 7.12a. Next, Figure 7.13a demonstrates that the average current during this experiment was 7.7 µA (31.3 A m⁻²) at steady state. Based on this current, the total transport was calculated to be $4.8 \cdot 10^{13}$ ions per second, while $6.0 \cdot 10^{13}$ ions entered each channel per second. Based on this calculation, we expect a desalination degree of 80%. This is twice as high as the measured value of 40% for 3 µl min⁻¹, as Figure 7.12a shows. The deviations between the measured and expected desalination degree could be attributed to the low permselectivity of the hydrogels, which leads to non-selective ion transport.

In Figure 7.12b, the salt concentrations of the outlet streams are shown for three applied currents namely 3, 5, and 7 µA (12.2, 20.3 and 28.4 A m⁻²). The data shows that the outlet concentrations did not change when increasing the current $I$. We would expect to see a difference in the degree of desalination because the transport rate of charge carriers is proportional to the current. For all three currents, however, 75% of the initially present salt was removed in the desalinated streams. This suggests that this is the maximum degree of desalination that is achievable with these devices. A valuable property of our microfluidic electrodialysis platform is that it enables real-time visualization of ion transport through the hydrogels using a fluorescent dye. This helps in obtaining a better understanding of relevant electrokinetic transport phenomena. As the curve in Figure 7.13a shows, approximately 600 s was
required before the system reached steady state. We observe that at the very first instance \((t < 10 \text{ s})\), the current decreased. This could indicate the build-up of a concentration boundary layer. Subsequently the current was increasing, reaching a (local) maximum at 250 s. Inspection of the fluorescence images for this experiment, given in Figure 7.13b, shows that the fluorescence distribution can be related to charge transport through the hydrogels. When applying a potential difference, a high concentration of negatively charged fluorescent dye was observed near the interface of the anion exchange METC-hydrogel with the salt-depleted channel. This cluster of dye migrated towards the enriched channel in approximately 250 seconds. Comparison with the profile in Figure 7.12a shows that at that moment, the current reached a (local) maximum. Subsequently, the fluorescence slowly approached a constant intensity distribution, reaching steady state after approximately 600 s. Note that not only in the positively charged anion-exchange hydrogel (AEH) the fluorescence intensity and thus dye concentration increased, but also in the negatively charged cation exchange SPAP-hydrogel (CEH). This indicates that, in line with the measured permselectivity of the bulk polymers, the ion selectively of the hydrogels was not perfect. The dye concentration was however clearly larger in the AEH than in the CEH.

7.4 | Conclusions

Alternatingly patterned anion- and cation-exchange hydrogels were fabricated in a PDMS microchip using capillary barriers. The chemical composition of the hydrogels was tailored to obtain a suitable balance between the degree of swelling and the charge density. The material properties were characterized, and the ion exchange hydrogels showed ion-selective permeation, based on their ion exchange capacity, permselectivity and resistivity. We demonstrated that the hydrogels can be used for desalination of water with salt concentrations up to 1 mM NaCl at various flow rates (0-5 µl min\(^{-1}\)), applied potentials (0-100 V), and currents (3-7 µA or the current densities 12-28 A m\(^{-2}\)). We obtained alternating dilute and concentrated streams at the outlet of the microchip. For example, we achieved a desalination degree of 40\% for an inlet concentration of 1 mM NaCl, an applied potential difference of \(\Delta U = 70\) V and a flow rate of 3 µl min\(^{-1}\). The maximum desalination degree we obtained was 75\%.

By utilizing a charged fluorescent dye, we visualized the ion concentration in the electrodialysis device. The fluorescence images revealed that when a potential difference is applied, ion-depleted boundary layers are formed near the ion-exchange hydrogels. When \(\Delta U > 25\) V, we observed the formation of vortices, enhancing convective transport towards the hydrogels in the overlimiting current regime. The desalination platform described here also enables visualization of charge transport through the hydrogels.
The separation efficiency can be enhanced by increasing the total length of the hydrogel-liquid interface, by increasing the residence time of the solution in the microchannels, or by optimizing the hydrogel properties like the charge density. This would allow desalination of salt solutions with higher concentrations, without the need to apply large potential differences. Our approach might therefore be promising for the development of processes related to water desalination, sample pre-treatment, and high-focusing of proteins.
7.5 References


Desalination by Microelectodialysis


This chapter evaluates the technology development of the preparative DNA fractionation, *in vitro* compartmentalization of human gut epithelium, and microelectrodialysis projects using societal perspectives.
8.1 | Introduction

The aim of this chapter is to understand the emergence period of technology, which in turn can help manage the societal requirements of a lab-on-a-chip product. Here, the emergence period represents the evolution of a new idea from concept to functional product based on the influence of outside perspectives. Technology development is a complex and unpredictable process; therefore, a comprehensive analysis of the emergence period is beneficial for obtaining an in-depth opinion about the research process. This argument will be explored using the example of the techno-scientific devices (see section 8.2) reported in this thesis.

Technology assessment is a scientific and communicative process, which examines the short- or long-term consequences of the societal embedding of the technology. The process of technology creation is shaped by both societal challenges and cognitive reasoning. However, the cognitive reasoning is performed by the technology developers, who often do not take into account the dependence of the future applications on various external perceptions during product development. With this in mind, we attempt to use insights of technology assessment for discussing the functional requirements of the developed technique, its economic value, and its social embedding. Technology assessment can be used for explicitly bringing societal perspectives into the reflection on the research and emergence period. In this chapter, technology assessment is applied using an adapted and simplified innovation value chain. The outsider perspectives define how research problems are posed, which previous knowledge is used, and the criteria required to evaluate the aim of the research and the final product (Figure 8.1).1-3

The next section identifies the techno-scientific device, a fluidic microchip, which will establish the base for technology assessment. In section 8.3, we will explore the outsider perspectives using an innovation value chain for the analysis of the techno-scientific device. Finally, we will conclude this chapter with a reflection on the technology assessment during the emerging period of the techno-scientific device.

![Figure 8.1](image-url) Evaluation of research and technology development by the outsider perspectives.
8.2 | Techno-scientific device

We introduce a techno-scientific product, which is a fluidic microchip containing hydrogel microarrays. The techno-scientific device was developed during 3 years of investigation in a multidisciplinary research environment of bioengineering and fundamental science. The techno-scientific device reflects the hybrid background and integrative thinking of both science fields. The design of the device was aimed to establish a ground for (1) preparative DNA fractionation, (2) in vitro compartmentalization of human gut epithelium (gut-on-chip), and (3) microelectrodialysis applications. The common point of all of these applications is the hydrogel micropatterning technology, which has been discussed in chapter 2 and chapter 3 of this thesis. The microchip was designed and fabricated initially for the preparative DNA fractionation project. Due to the flexibility of the hydrogel patterning technique with respect to design modifications, it quickly became apparent that this technology could be used in diverse application areas. Thus, the device was extended to gut-on-chip and microelectrodialysis applications. Only minor revisions to the microchip design were required between projects, and the device seemed to have a great potential to improve the current state of technology in all three fields (Figure 8.2). The development during the emerging period was considerably influenced by the brainstorming sessions with colleagues and supervisors. In the following paragraphs, we will discuss the general advantages of miniaturization, and the advantages of the device within the context of the aforementioned three application areas.

In general, the main advantages of microchips are their capability for small sample volumes, fast analysis, high throughput, optimization on the small scale, and compatibility with portable readout systems. These platforms are therefore promising candidates to replace routine laboratory techniques such as DNA fractionation, conventional organ-on-chip platforms, and electrodialysis units, which are recently in demand. To fulfill this, we proposed microchips with periodic hydrogel patches for: high quality biomolecule fractionation with improved downstream analysis; increased opportunity to control microenvironments in cell culturing; and, enhanced electrodialysis performance.

Long analysis duration, coupled with complicated chip designs and equipment, have failed to meet the needs of optimal separation efficiency for continuous flow analyses. One of our goals is, therefore, to develop a new generation 2D electrophoresis tool, which can be the first step in commercializing continuous flow DNA fractionation devices. Towards this aim, we integrated glass pillars and periodic hydrogel patterns over a 1 cm² area within a microchip. Periodic hydrogel patterns were planned to provide an ideal tool for studying the DNA molecular dynamics in confining spaces by
creating structural anisotropy, leading different-sized fragments to follow dissimilar trajectories under orthogonally applied electric fields. However, when initial experiments yielded suboptimal separations, we decided to fill a separation matrix with agarose and apply an asymmetric field profile, which served well for the same purpose. Details on the design and working mechanism of this system can be found in chapter 4. The analysis can be performed in a few minutes using nanograms of sample solutions, and is advantageous when the collected sample is too small for a classic analysis or when quick results are required. This modern two-dimensional continuous flow electrophoresis approach enables the fractionation and purification of DNA molecules and ionic species.

Conventional cell culturing techniques do not provide sufficient control of the culture environment to easily increase fundamental knowledge of cell-cell and cell-bacteria interactions. In this area, it was our goal to develop a gut-on-chip platform that mimics the complex functions of the human intestine in vitro, and study the tissue functions and cell-bacteria interactions. In the microchip, both intestinal cells (Caco-2 cell line) and bacteria (E. coli) are co-cultured long-term. The core of the device is a square chamber containing periodic hydrogel arrays in which intestinal cells are grown. The development of the platform is aided by a theoretical model on mass-transport of nutrients and metabolites. Functionality and viability of both intestinal cells and bacteria are observed in the microchip, in order to establish the relationships between

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**Figure 8.2.** The techno-scientific model and its application areas (a) Schematic demonstration of DNA electrophoresis in a lab-on-a-chip device. (b) Schematic view of gut-on-chip device. (c) Schematic view of electrodialysis in a microelectrodialysis device.
cells and microbes. These relationships are important, as they are responsible for both inflammatory and physiological responses.

Desalination devices perform electrodialysis to study the physics of the electrodialysis process, and to produce drinkable water. There is a large demand to fabricate microscale desalination units for optimization of the electrodialysis performance, because the current state of the technology seems to have reached its limits. The integration of hybrid membranes in microdevices has recently been demonstrated for charge-based separations; however, these microdevices consist of a microchannel sandwiched between ion exchange membranes, which often leads to fluid leakage. The goal of our electrodialysis microchip is to overcome this complication by combining microfluidics and charged hydrogels patterned by capillary line pinning. As the technique enables a highly-parallelized configuration, it can remarkably increase the throughput of the electrodialysis process. The microchip has the same layout as the DNA fractionation platform, consisting of hydrogel microarrays, a separation chamber, and reservoirs. Additionally, the microchip contains positively and negatively charged hydrogels, patterned alternatingly in separate lanes, which serve as conductors of the oppositely charged ions in this microchip. Under a constant flow rate and application of a constant current, positively charged ions are attracted to the negatively charged hydrogels, while the opposite occurs for negatively charged ions. This leads to the removal of salt ions from the solution to be desalinated.

8.3|Outsider perspectives and technology assessment

So far, we have introduced the technical aspects of a techno-scientific device and its three applications. Aside from the technical aspects, developers should also consider how research practices can be used to increase the awareness of the broader socio-technical content, as this awareness can lead to a more rigorous research. In this section, we will therefore link the technological development to the societal challenges for our techno-scientific product and its three application areas. We propose the outsider perspectives from three different angles to elaborate on the product, as summarized in Figure 8.3. After a brainstorming session with technology assessment and philosophy in engineering science experts (Dr. Verena C. Schulze Greiving and Ir. Bart M. Walhout, together with the help of Prof. Kornelia E. Konrad and Prof. Mieke Boon), we propose that these dimensions and corresponding notions would serve as a guide to assess the quality for this particular study. We have chosen these three aspects as a conceptual framework, because they were considered to constitute fundamentals for the aforementioned applications. Additionally, these aspects are important for product creation and innovation processes. Functional requirements and economic value are necessitated before valorization, while societal embedding is important for admissibility,
integration, and acceptability of the product in later steps. However, these should be ideally taken into account at an early stage.\textsuperscript{17}

Innovation value chains are tools that make an attempt to model innovation processes. They can be used to identify important factors shaping the dynamics of the technology development, such as technology transfer and commercialization, risk assessment, public acceptance, admissibility, and integration.\textsuperscript{18} Particularly, the innovation value chain begins with research, is followed by a product design step, and continues through development to marketing steps.\textsuperscript{16} Each innovation notion iterates the process and influences the others. In other words, each notion has inter- and intra-connections with the others. Although the innovation value chain may not assess all possible notions involved in the emergence period, it still seems sufficient to address a number of conclusions.\textsuperscript{16-18} It is important to note that real life situations are mostly complex and require a highly complex innovation value chain for evaluation. Here, we will use a simplified and modified version of the innovation value chain to provide an overview of the emergence period from laboratory to product, and to show the mismatch in perspectives, visions, and connections between the different dimensions. It must be stated that we analyzed these aspects and notions by ourselves. It would be highly valuable to confirm our analysis with relevant individuals along the value chain, because this would give a different perception and more insight to our analysis; however, this is out of the scope of our study.

The first criterion concerns the functional requirements, and it encompasses notions of processing time, throughput, ease of fabrication, follow up analysis, resolution (preparative DNA fractionation), mimicking the organ

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{outsider_perspectives.png}
\caption{The three approaches of outsider perspectives.}
\end{figure}
functions (gut-on-chip), and energy efficiency (microelectrodialysis). Most of these notions are technical, and are related to the overall working mechanism and performance of the techno-scientific product. **Resolution** is a quantitative parameter, which assesses how well two eluted bands are resolved. Resolution should be high enough to process two different samples separately downstream, and is important to distinguish, for example, two DNA fragments encoding certain genes linked to certain diseases. **Mimicking the organ functions** is necessary for organ-on-chip platforms, because their usefulness for drug discovery or studying disease models depends on how close the reactions to mimicked culture conditions are in comparison with the patients themselves. Since the platforms directly use human cells, the technology is superior to animal models and is expected to reduce animal testing for drug development or disease model studies. **Energy efficiency** is of interest because quantification of deionization performance is expressed in energy per ion removal, or energy per hydrogel area efficiency, in electrodialysis applications. Sufficient energy efficiency is required to be labeled as a green technology. **Processing time** is important as a functional requirement in all application areas. For example, one of the main aims of the DNA fractionation device is to shorten the analysis time, as patients waiting for quick diagnosis would demand a technology that provides short processing times. **Throughput** refers to the amount of sample transferred from inlet to outlet of the fractionation matrix in a given time. High sample-throughput requires continuous sample flow, so the sample can be detected as it is produced. This notion presupposes separation of the molecules, and gains importance when limited amounts of samples are available for analysis, such as in forensics and diagnostics. **Ease of fabrication** applies to both research and valorization periods. An ideal product should be easily fabricated with cheap materials, yet also be stable and defect-free for the success of follow up analyses. **Follow up analysis** stands for the requirements of the next possible step in the analysis process. The output of the product needs to fulfill the requirements for this next step.

The second criterion is the economic value and it encompasses notions of price, potential customers, and operation and packaging. These notions are important for the future use of the devices, particularly during the from-benchtop-to-shelf period. Not all research outcomes are necessarily commercialized; however, being aware of these notions would benefit when it comes to valorization of the developed technology. **Price** is determined by the materials used to fabricate the product, as well as the product size and storage conditions for shipping. This notion also contains embedding the device in standard procedures, including the use of certain resources such as machines/microscopes. The economic value of the techno-scientific product may change depending on the appearance of similar devices in the market and functional requirements of the device. **Potential customers** notion defines the audience and scope of the product or application. In this step, prediction of
current needs, future needs, and upgrades gains importance, because this prediction enables the developers to think more systematically about the current state and future use of the technology. *Operation and packaging* notions are used to evaluate the ease of use of the product, including the additional equipment needed to run a complete analysis or process. Packaging includes the industrial design, such as a kit including all necessary components for an analysis.

The third criterion is societal embedding, and encompasses notions of admissibility, integration, and acceptance. The societal embedding of a technoscientific device is important in a societal context because irrelevant devices not only squander valuable resources by wasting time and funds, but also provide poor results that may not be considered further by the academic or public audience. Admissibility is the requirement for new products to meet the rules and standards as set by authorities such as government agencies and the sector, to ensure safety and compliance with existing standards. Integration includes valorization of the product, considerations about health, frequent usage of the product, and the implications of the product on society. New products must be integrated in relevant markets. This occurs according to existing practices and cultural repertories. Acceptance is the last notion of the societal embedding criterion, and concerns the effect of the product on the perception of society, including the reactions of (non-)governmental organizations and lobbies. A product can be accepted when societal concern is not overly large, and when there is sufficient articulation of the pros and cons of the product.

### 8.3.1 Assessment of preparative DNA fractionation device

We present the evaluation criteria in Table 8.1, which will be followed by a paragraph discussing the interconnection between the different criteria and notions. The focus will be on DNA fractionation device and its assessment by the outsider perspectives.

<table>
<thead>
<tr>
<th>(a) Functional requirements</th>
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| Resolution | • The device provides high-resolution fractionation for 0.5 to 10 kilo base pair DNA molecules.  
| | • This function is comparable to alternatives such as the commercially available BluePippin device, and the non-commercially available ANA device. |
| Processing time | • The device completes the separation process within 2 min when applying an electric field of approximately 60 V cm⁻¹. |
Exploring Innovation Journeys of a Techno-Scientific Device

| Throughput | • The device provides a throughput of 0.18 ng molecules per hour.  
| • This function is comparable with non-commercial alternatives such as “ANA”. |

| Ease of fabrication | • The device utilizes an agarose matrix, providing some advantages over the aforementioned devices:  
| (1) easier and cheaper fabrication  
| (2) defect-free fabrication  
| (3) no required design considerations such as obstacle geometries. |

| Follow up analysis | • The device is operated under continuous flow to collect sufficient sample for PCR and compile a library.  
| • The continuous flow application is not unique to the device as it is used by aforementioned non-commercial alternatives. |

(b) Economic value

| Price | • The device is fabricated from fused silica (an expensive material) and mass production is possible.  
| • Fabrication from PDMS is an option and has been shown before. In this case, the mass production would require further research.  
| • The device has a smaller footprint and provides cost-effective shipping as it occupies less space than the commercial alternatives (in the order of tens of centimeters). |

| Potential customers | • The aim of the application is the DNA sample purification and preparation for PCR, sequencing, and gene analysis.  
| • Potential customers include:  
| (1) Other research institutes at universities  
| (2) Hospitals  
| (3) Companies involved in diagnosis product projects. |

| Operation and packaging | • Sample is required to be pipetted into the device.  
| • Power supply and control software are needed.  
| • Packaging is done by a chip holder vs. the compact cassette system of the other industrial alternatives. |
Material needs to be kept DNA-free, which may increase the packaging and shipping costs.

### (c) Societal embedding

| Admissibility       | • FDA regulates medical devices:\[24\]
|                    | (1) Good manufacturing practices include electronic signatures, pharmaceutical ingredients, and quality risk management. These practices may influence the functioning of the device such as yielding unexpected or non-stable results.
|                    | (2) Performance standards include purity of the output. This may have a potential influence on follow up analysis.
|                    | (3) Safety (application of high voltages).
|                    | (4) Validations (evaluated by the bullet points in the quality section).
|                    | (5) Reproducibility is a key element for valorization.

| Integration         | • Valorization. Some of the other alternatives are patented, which may negatively affect the valorization of this specific technology. On the other hand, patented alternatives show that the technology is useful for society and in demand:\[24,25\]
|                    | • Health perspective. It must be asked here whether the diagnosis of genetically identified diseases is a need-to-know or a right-to-know of the public. Diagnoses may lead to new regulations from insurance companies. They may affect people positively (healthier lifestyle) or negatively (fear or guilt about current lifestyle, eventually resulting in several diseases or increased suicide rate) since people will know more about their risk for (future) diseases.
|                    | • Cheaper technology allows doctors to use this tool more often to obtain quick results and enables availability of the technology for more consumers.
|                    | • The device can only be used as a part of a more complex diagnostics system with multiple components, including DNA purification, PCR, etc. units, so it will only have indirect implications on the societal level.

| Acceptance          | • Depends on the perception of the technique in society.
|                    | • Spit party example: A company promotes genetic testing by taking spit samples at high-society parties to give customers risk profiles for their potential genetic diseases:\[26,27\] Predicting the patient’s risk of suffering, i.e.
risk of heart attacks, could cause them unnecessary anxiety if the results are not properly interpreted.

The notions mentioned above are also mutually interconnected. For example, ease of fabrication directly relates to the price of the device, while resolution, throughput, and processing time are important for potential customers. For this application, we achieved the best fractionation results using expensive microchip materials (in this example, fused silica), as the cheap alternatives (such as PDMS) seemed to negatively impact the performance due to reduced optical clarity and absorption of the buffer, which would affect the price, admissibility, potential customers, and integration. The follow up analysis notion requires a detailed plan for operation and packaging, which must be taken into account for the admissibility. Potential customers and price are also connected with acceptance and integration, as the users will affect the perception in society. For example, if the results are not properly interpreted, and users are told about their false chance of having a heart attack, the perceived reliability of the device may decrease within the society. The preparative DNA fractionation device needs to be integrated into a large-scale system, which includes DNA extraction and PCR components, to obtain the final diagnosis results. In this sense, the device does not have direct implications on the societal level. The integration in existing systems will not be possible in the near future as it requires further work, including further separation optimization and simplifications in its design.

The biomolecule separation application aims to help with diagnosis of genetics-based diseases. One advantage of the device would be the utilization of a reduced sample amount to obtain results; on the other hand, the device needs to be integrated into a complex analysis system. In this sense, the device might not be readily relevant to the society.

8.3.2 Assessment of compartmentalized gut-on-chip device

We present the evaluation criteria in Table 8.2, which will be followed by a paragraph discussing the interconnection between the different criteria and notions. The focus will be on multicompartamental hydrogel arrays for 3D tissue culture and its assessment by the outsider perspectives.

Table 8.2. Analysis according to outsider perspectives by simplified innovation value chain for compartmentalized gut-on-chip application.

(a) Functional requirements

| Mimicking organ functions | The device generates 3D fold structures resembling intestinal villi; however, the structures are not morphologically identical to human intestinal villi. |
This device mimics organ functions less successfully than other non-commercial (gut-on-chip device by Wyss Institute)\textsuperscript{7} and commercial devices by Emulate Inc.,\textsuperscript{27} because it does not provide apical and basolateral sides, which are found in a real \textit{in vitro} organ system.

### Culturing time
- Cell maturation takes 11 days owing to the fluidic control.
- This performance is comparable to that of other microchip alternatives\textsuperscript{7} and provides shorter culturing time when compared to well-plate cultures (21 days).

### Throughput
- The compartmentalized nature of the device provides 450 in-parallel culturing environments in a single microchip.
- The compartmentalization enables in-parallel testing of multiple conditions in a single device and is introduced for the first time.

### Ease of fabrication
- The device is made from PDMS, and utilizes a simple and unique patterning technique in which a mixture of cells and pre-gel is pipetted into the chip.
- This function provides an easy patterning procedure compared to other techniques, such as laser patterning\textsuperscript{9} or droplet technology, which require expensive and dedicated equipment.

### Follow up analysis
- Staining can be done easily due to the fluidic nature of the device. The fluidic system also facilitates simple changing of the experimental conditions, such as nutrients, oxygen, and drugs.
- Co-culturing with other microorganisms and cells is demonstrated.
- Drug development tests could be performed in future versions of this device.
- The device geometry does not provide apical and basolateral sides of the organoids, and thus is not identical with the conditions in the human body. This problem is solved in a newer version of the device.

### (b) Economic value

### Price
- The device is fabricated from PDMS (cheap material); mass production is not possible due to unstable material properties of PDMS (hydrophobicity).
- Some companies use polystyrene as an alternative to PDMS, which might be a solution for commercializing...
this device. However, further research is needed on this topic.

| Potential customers | • The aim of the application is to provide a versatile platform for culturing different cell types, to reduce/replace animal testing for the purposes of studying drug development and disease models. Replacement of animal models for cosmetic screening may also be interesting.
• Potential customers include:
  (1) Other research institutes at universities
  (2) Hospitals and clinical centers
  (3) R&D departments of pharmaceutical companies. |

| Operation and packaging | • The patterning technique must be performed by a technician (might be eliminated with further research on the design).
• The technique has critical steps (might be eliminated with a user guide).
• Fluidic pumps and microscope usage are required. The integration of these materials into compact systems and current practices would be possible with a detailed industrial design. On the other hand, the system components (such as tubing) have to be kept sterile prior to operation.
• Material needs to be kept sterile which may increase shipping costs. |

(c) Societal embedding

| Admissibility | • FDA regulates medical devices:
  (1) Good manufacturing practices include electronic signatures, pharmaceutical ingredients, and quality risk management. Misuse of these practices may lead to malfunctioning, such as channel blockage.
  (2) Performance standards include properly controlled cell growth. This has a potential influence on follow up analysis for drug development and disease modeling because wrongly treated cells might easily evolve and change their response to drugs or chemicals.
  (3) Safety (use of pharmaceuticals and chemicals).
  (4) Validations (evaluated by the bullet points in the quality section).
  (5) Reproducibility as it is a key element for valorization. |
### Integration

- Valorization. Some of the other alternatives are patented, which may negatively affect the valorization of this specific technology. On the other hand, patented alternatives show that the technology is useful for the society and in demand.\(^{27}\)

- Effects of material and performance (side effects of the PDMS material still need to be tested on the cells). There is an increasing societal demand to stop/reduce animal testing. The attempts to fulfill this demand might be satisfactory for society, but false positive/negative results may cause a problem regarding the reliability of the device.\(^{29}\)

- Cheaper technology allows more disease models to be studied and more drugs to be tested in a cost-effective way.

- The device has indirect implications on the societal level and it can only be used in clinics.

- The device is not a component of a complex analysis system; it does not need integration. It might be connected to other organ-on-chip devices to form a body-on-chip device.

### Acceptance

- Depends on the perception of the technique in society.

- PETA example: The non-profit organization has volunteer supporters from public and is the largest animal rights group in the world. They negatively bias people on animal testing in drug discovery and disease modeling.\(^{29}\) This also applies to cosmetic testing, which is now banned in the European Union.

Interconnections exist between the notions in the above criteria. For example, ease of fabrication directly relates to the price of the device, while cell culturing time, throughput, and recapitulating organ functions are important for potential customers. We considered using a low cost material—PDMS—which affects the price, admissibility, potential customers, and integration. The use of low cost materials could trigger more research on large scale production techniques. However, more research is required for enabling the mass production of PDMS, or finding another alternative low-cost material. The follow up analysis notion requires a detailed plan for operation and packaging, which is taken into account for the admissibility. Potential customers, price, and recapitulating organ functions also connect to acceptance and integration as the users will influence the perception in society. Trainings and workshops might be needed to introduce the function and working principles of the device, and
these will influence admissibility and acceptance or initiate research to improve the ease of use. Trainings and workshops can also be eliminated by further research on the design to increase the ease of use of the product.

The cell culturing application is expected to provide an alternative for animal testing in drug development research. On the other hand, the device needs to be validated by health foundations and organizations. This means that the device may undergo a more detailed analysis with respect to both design and functionality to be validated by the authorities.

### 8.3.3 Assessment of microelectrodialysis device

We present the evaluation criteria in Table 8.3, which will be followed by a paragraph introducing the interconnection of the criteria and notions. The focus will be on microelectrodialysis device and its assessment by the outsider perspectives.

Table 8.3. Analysis according to outsider perspectives by simplified innovation value chain for microelectrodialysis application.

(a) Functional requirements

| Energy efficiency | • Hydrogels separate ions less successfully than membranes because their swelling behavior limits the increase in charge density. However, hydrogels are cheaper than membranes. |
| Processing time | • Sample collection rate is 18 µL per minute and is comparable to other alternatives, such as the non-commercial desalination microchip by MIT\(^{10,11}\). • The desalination limit of the device is 1 mM, which is 100 times lower than the non-commercial desalination microchip. |
| Throughput | • Parallelized nature of the device provides up to 6 times more liquid processing per unit time. • Parallelization of the electrodialysis process on the micron-scale is introduced to the literature for the first time. |
| Ease of fabrication | • The device is made from PDMS. The device utilizes a simple and unique patterning technique, in which the pre-hydrogel mixture is simply pipetted into the chip. • This function provides an easy patterning procedure compared to other techniques such as nanochannel fabrication, which require expensive and dedicated equipment. |
Although the membrane-clamped devices suffer from leakage, this device does not owing to the hydrogel patterning technology.

The device is operated under continuous flow to collect desalinated samples or for purification of mass spectroscopy samples.

This function is not unique to the device and is being used by the aforementioned non-commercial alternative.

The device is expected to be scaled-up in commercial desalination plants, as it provides an optimal platform to study concentration polarization physics.

The device can be coupled to a microbe or heavy metal purification system.

The device is fabricated from PDMS (cheap material). However, mass production is not possible due to the unstable material properties of PDMS (hydrophobicity, shrinking).

The device utilizes cheap hydrogels instead of membranes. However, process efficiency is lower than the aforementioned alternatives.

The aim of the application is to provide an optimization tool for large-scale desalination systems. The device may also be integrated as a sample preparation tool for small-scale samples in mass spectroscopy.

Potential customers include:
(1) Other research institutes at universities
(2) Water purification plants
(3) R&D departments of desalination membrane companies.

The patterning technique must be performed by a technician (might be eliminated with further research on the design).

The technique has critical steps (might be eliminated with a user guide).

Fluidic pumps and power supply usage are required. The integration of these elements into compact systems would be possible with a detailed industrial design.

Material needs to be kept dust-free, which may increase shipping costs.
Integration of the system in a large-scale plant will not be possible due to size constraints. The device is expected to only serve for research purposes. The overall throughput of the electrodialysis device is quite low, namely $3 \text{ µl min}^{-1}$ water.

### (c) Societal embedding

<table>
<thead>
<tr>
<th>Admissibility</th>
<th>Integration</th>
</tr>
</thead>
</table>
| - The Drinking Water Directive regulates electrodialysis and desalination devices\(^{30}\)  
  (1) Good manufacturing practices include electronic signatures, standard guides, practices, and quality risk management. Misuse of these practices may lead to malfunctioning, such as channel blockage and salt contamination in other microchannels.  
  (2) Performance standards include predefined protocols for certain concentration ranges (potential influence on follow up analysis such as sample desalination and optimization for water plants).  
  (3) Safety (use of hazardous chemicals such as hydrogel precursors).  
  (4) Validations (evaluated by the bullet points in the quality section).  
  (5) Reproducibility as it is a key element for valorization. |
| - Valorization (the technology is quite new and there are no commercialized products yet).  
  - Health (side effects of the PDMS material need to be addressed if the output is used as drinking water).  
  - Cheaper technology allows more R&D to be performed on electrodialysis.  
  - The device has indirect implications on the societal level as it has no direct interaction with society.  
  - It is a standalone device, requiring no integration with other complex systems. |

<table>
<thead>
<tr>
<th>Acceptance</th>
<th></th>
</tr>
</thead>
</table>
| - Depends on the perception of the technique in society.  
  - Public might reject water recycling activities due to a "not as clean as spring water" perception, and as a result, clients for this recycled water may not be easily found.\(^{31}\) |

Looking at the interconnections between each of the above mentioned notions, one can see that ease of fabrication directly relates to the price of the device, while the energy efficiency and throughput notions are important for potential customers. We considered using a low cost material, affecting the
price, admissibility, potential customers, and integration. However, here further research is required to enable mass production of PDMS, or to find an alternative material for the replacement of PDMS, e.g. another polymer. The follow up analysis notion requires a detailed plan for operation and packaging, which is taken into account for the admissibility. Potential customers, price, and energy efficiency also are related to acceptance and integration, as the users will influence the perception in the society. Training and workshops might be needed to introduce the function and working principles of the device, and these will influence acceptance and admissibility; however, it would not be critical for R&D companies or institutions. Training and workshops can also be eliminated by further research to increase the ease of use of the product.

The electrodialysis device is designed to help optimization of large-scale desalination units. However, the durability of hydrogels does not allow us to realize this aim, and further research on charged hydrogels is required. The device can still be used for the pretreatment of mass spectroscopy samples. The device might not be directly relevant for society, since the small-scale system would be used to optimize conditions and parameters for a large-scale system, and would be automated with the help of computer scientists and information technology managers.

### 8.4 Reflective opinion on the outsider perspectives

The preliminary evidence gathered in this chapter suggests that the techno-scientific product can potentially be used for various applications. During the research process, we focused on the design, functionality, and the originality of the concept and the techno-scientific device. Functional requirements have become the most prominent aspect for us, because economic value and societal embedding are mostly considered when commercialization of the techno-scientific product is the issue. In that sense, we limited our vision in the emergence period by giving weight to the functional requirements aspect.

As Garud and Rappa\(^\text{32}\) have already argued, researchers have “accepted criteria”, which is meaningful, logical, and theory-compatible, for applying to their research. Accepted criteria can be defined as frames of reference\(^\text{33,34}\) including previous literature, common sense, requirements of the research project, requests of the funder, point-of-view of the technology developer, and the norms of technology developer’s supervisors. Any data produced may be supposed as consistent with the accepted criteria, therefore researchers may tend to ignore, deny, or even manipulate “unexpected” data. In return, this self-filtering process may lead the technology development processes to become tautological. Latour and Woolgar\(^\text{36}\) suggested, “assessment approaches adopted to evaluate a technology eventually become the basis for the construction reality.”\(^\text{19}\) After a while, researchers may trap themselves in their accepted criteria and render themselves blind to possible phenomena happening
“otherwise”, turning the technology creation process in a “haze-maze”. Society may be skeptical about research results, because technology developers are pushed to highlight their polished results in order to sustain their existence in academia. Inappropriate research results are often hidden—especially in “lab-on-a-chip” devices, where the hopes of society are pinned to this infant technology to unveil faster, better, cheaper, and real solutions, which also shapes the selling point of most articles. Society may be aware of this fact and start asking more skeptical questions about the relevance of new techno-scientific devices. In this sense, understanding and considering outsider perspectives is already a big step to solve “malfunctioning” parts of the technology development process and influence the technological road mapping. This would enhance the possibility to move towards the most appropriate technology and enhance societal embedding.\textsuperscript{17} Also, the help of social scientists and marketing professionals might be useful to satisfy the criteria of economic value and societal embedding. The economic value and societal embedding of the product in light of several specific challenges were identified in sections 8.3.1, 8.3.2, and 8.3.3.

The outsider perspectives are helpful to have a detailed look at the emerging period of our technology, and to identify the important aspects and pitfalls of the aforementioned applications. The comparison of our devices with already existing non-commercial and commercial devices helped to identify valuable technological aspects and to reveal the weak points of developed technology, in efforts to turn it into a commercial product. This study could be supplemented with different technology assessment tools including i.e. vision maps, actor maps, fictive scripts, interviews with experts in the field, and workshops to obtain a more in-depth understanding.

Considering the societal aspects has revealed the potential effect of these devices on societal perception. Even though the devices mostly seem to have an indirect effect on the society, outsider perspectives helped us to analyze their impact on the societal level. We believe that outsider perspectives present a valuable approach to improve the functionality, economical value, and societal embedding of the research during the emergence period, assisting researchers to find potential problems and solutions of techno-scientific devices.

8.5|Conclusions

This chapter has viewed the work in this thesis from a different perspective. We have analyzed the technology development of a microfluidic chip, here named a techno-scientific device. To take a closer look at the emergence period and the current state of the technology, we used a perspective where research decisions are modulated by outsider perspectives. Having analyzed the emergence period and current state, we argued that the future issues and dynamics of the technology can be interpreted.
Outsider perspectives consisted of the functional requirements of the developed product, its economic value, and its societal embedding. It is inspired by the innovation value chain, which allowed gathering information on a broad range of specifications from commercialization to societal embedding. This analysis does not include all technological and societal aspects, as this requires a more in-depth study. We believe that outsider perspectives will still be useful in creating a more complete picture of the developed technology, such as obtaining an increased awareness on the user practices, marketing, and public integration.
8.6 References


People for the Ethical Treatment of Animals (PETA).


Guidelines for the Regulation of Desalination, Promotion of Renewable Energies for Water Production through Desalination, 2011.


Conclusions and Future Perspectives

A summary and conclusions of each thesis chapter are given, and several future directions are presented as an outlook for this work.
9.1 Summary and conclusions

A class of polymeric soft materials that absorb large quantities of water are called hydrogels. Many different types of hydrogels have been developed and widely used as smart materials in the past decades. Their material properties can be changed significantly in a controlled fashion by e.g. tuning temperature, pH, and electric or magnetic fields. In this thesis, three biomedical and practical engineering applications of hydrogels are addressed in separate projects: preparative DNA fractionation, in vitro compartmentalization of human gut epithelium, and desalination by microelectrodialysis. The research surrounding and including these applications has been grouped in eight chapters.

In chapter one, a brief historical background with milestones in each field was given. Our motivation to design and demonstrate all these applications on the microscale was explained.

General background knowledge necessary to understand the concepts presented in this thesis was provided in chapter two. Synthesis of hydrogels and characterization of hydrogel structures were explained. The physical and chemical properties of DNA molecules, the basics of electrophoresis, and the physics of DNA fractionation were subsequently discussed. This was followed by basic information on gastrointestinal track microbiota, bacterial metabolism, and intestinal epithelial cells for the in vitro compartmentalization of human gut epithelium project. Finally, the electrodialysis process, including ion transport in charged hydrogels and ion concentration polarization, were explained for the desalination by microelectrodialysis project.

Although the microfabrication of patterned hydrogel arrays on rigid substrates has been extensively investigated, the ability to do the same in closed microchips has not been sufficiently examined. We closed this gap in chapter three and four, by presenting two novel fabrication methods, which enabled simple and reproducible patterning of micron-sized hydrogel arrays inside closed microchips. Notably, in the work reported in chapter three, the glass cover of the microchip was thinned by mechanical grinding and polishing. This procedure reduced the spacing between the photomask and hydrogel precursor, thereby reducing the effects of UV diffraction. Together with this approach, we developed an intermittent illumination technique consisting of short illumination periods followed by relatively longer dark periods, which decreased the diffusion of monomers to enable high resolution patterning. The effects of glass cover thickness and roughness, together with different optical lithography recipes on patterning success, were investigated. The patterning method was tested with two different types of photopolymerizing hydrogels: polyacrylamide and polyethylene glycol diacrylate. The conclusion of chapter three was that the presented method can enable in situ fabrication of well-defined hydrogel patterns for microfluidic platforms. In chapter four, another
Conclusions and Future Perspectives

A method to fabricate micrometer-sized massively-parallel hydrogel patterns inside closed microfluidic chips was demonstrated. The design concept was based on selectively trapping UV-curable hydrogel solution using non-fluorescent capillary barriers. With this technique, we demonstrated the ability to pattern high-resolution hydrogel structures in closed microfluidic devices over large areas, with high reliability and without any defects or distortions. As a conclusion, this approach allowed complete control over selectively filling and emptying monolith glass chips, and was further used in the in vitro compartmentalization of human gut epithelium and desalination by microelectrodialysis projects (chapter six and seven).

In chapter five we report a new and simple approach for preparative purification and fractionation of sub-10-kbp DNA molecules in a microfluidic device. For the first time, the variation of the field-dependent mobility of DNA molecules with DNA length was exploited for this purpose, a DNA fractionation principle that hitherto went unnoticed. Agarose gel with 1.2% concentration was used as the separation matrix. A 0.5-10 kbp DNA ladder was fractionated and separated from other ionic species in continuous flow within 2 minutes, simply by periodically switching between two orthogonal electrical fields of strongly differing magnitude. As mentioned above, the separation resolution was demonstrated to be based on the variation in field-dependent mobility for differently-sized DNA fragments.

Compartmentalization in microfluidic chips allows cells to be cultured in a highly parallelized fashion. Previously demonstrated microdevices that use compartmentalization typically lacked complete fluidic control, which is necessary to enable on-demand manipulation of the outer cell microenvironment in well-defined structures. In the work reported in chapter six, we have therefore manufactured a microfluidic cell culture platform that consists of periodic 3D hydrogel structures with controllable configuration and shapes. The design concept was based on selectively trapping mixtures of collagen pre-gel and cells in compartments via capillary line pinning. The architecture of the microchip and the use of continuous fluid delivery enabled long-term and in-parallel culturing of Caco-2 human intestine epithelial cells. The cells underwent differentiation and spontaneously grew into 3D folds on the 3rd day of cell culturing. Caco-2 cells were also co-cultured with an intestinal bacterium (E.coli) which adhered to the cells after 36 h of incubation. The viability was unaffected, showing cell-bacteria attachment while still maintaining controllable fluidic perfusion in the microchip. As a conclusion, this approach may offer great promise, both for building next generation in vitro platforms, and for addressing challenges in drug screening and toxicology testing by enabling compartmentalized 3D cell culturing in a microfluidic environment.

A stack of oppositely charged hydrogels in a microfluidic chip were demonstrated for the first time in chapter seven, and utilized for the
desalination by microelectrodialysis project. For charge-based separations, microdevices containing a single microchannel sandwiched between two ion-exchange membranes have been used, but this configuration often leads to fluid leakage. In our approach, we addressed this problem by \textit{in situ} manufacturing a stack of periodic hydrogel structures in a microfluidic platform by capillary pinning. This technique resulted in alternating anion- (AEH) and cation-exchange hydrogels (CEH) in confined compartments separated by water channels. When an electrolyte solution was flowed through the microchip channels and a potential difference was applied across the membranes, parallel streams of concentrated and ion-depleted water were obtained in continuous flow. Different electric fields (10-100 V cm$^{-1}$), currents (3-7 µA), and fluid flow rates (0-5 µl min$^{-1}$) were investigated to obtain an optimum electrodialysis performance for 0.01-1 mM NaCl concentrations. It was concluded that the approach may be used for the development of a low-cost and hybrid hydrogel system, and applied to areas such as the pretreatment of mass spectroscopy samples.

In \textbf{Chapter eight}, the work reported in this thesis was reviewed from a different perspective. The technological development of a microfluidic microchip was analyzed using a perspective modulated by \textit{outsider perspectives}, which were presented using a technology assessment study. Technology assessment is a scientific and communicative process that examines the short- or long-term consequences of the technology in society. We concluded that \textit{outsider perspectives} allowed gathering information on a broad range of specifications, from commercialization to societal embedding. This approach allowed us to think about societal problems in the emergence period of technology, and then produce ideas and knowledge required to solve those problems.

\section*{9.2 Future perspectives}

Future research following the work in this thesis can be aimed at several key points for each application, as well as further analysis of the technology emergence period, also described within each chapter.

\subsection*{9.2.1 Preparative DNA fractionation}

The development of hydrogel-based separation matrices for DNA fractionation represents a valuable step towards integration of biomolecule separation systems within large-scale diagnosis systems in an economical and efficient way. Modifying separation matrix to separate different size ranges of proteins would be a promising starting point for further research. The separation process, including the hydrogel concentration and electric field application protocol, can further be optimized to serve for separation of a wider range of DNA fragments. Changing the hydrogel type could also enable protein
Conclusions and Future Perspectives

Aside from this, inspiration can be taken from artificial sieving structures as shown in the literature, to enable rapid fractionation of biomolecules. Towards this, we have already designed a hybrid separation matrix consisting of hydrogel patterns and geometrical obstacles. In pilot experiments, we observed that it is possible to separate a few tens of kbp fragments within that platform, and further study would certainly be interesting (Figure 9.1).

9.2.2 | In vitro compartmentalization of human gut epithelium

Compartmentalization of hydrogel patches in a closed microchip with fluid delivery provided an important step for studying recapitulation of complex body functions in controlled microenvironments. Replacing the buffer reservoirs with single microchannels would make a more physiologically realistic in vitro model. In this way, the application of different fluids in each side of the compartments would be enabled. Each hydrogel compartment would also have both apical and basolateral sides in the microchip, opening up possibilities for testing the Caco-2 barrier functionality. We have performed preliminary experiments with a microchip design that fits the description above (Figure 9.2). Together with this improved design, a more detailed study on physiological reactions of the cells to environmental changes (such as aminopeptidase activity as a measure for cell differentiation) would be fruitful for a better understanding of physiological changes. The microchip design can also be used to co-culture different types of human cells. For example, Human Umbilical Vein Endothelial Cells (HUVECs) could be cultured in the microchip.
The HUVECs may be able to form small veins through the hydrogel patches, connecting the two neighboring channels. Another example would be astrocytes and endothelial cells for studying the biology of related diseases.

9.2.3 | Desalination by microelectrodialysis

The first steps towards the implementation of an electrodialysis system in a microchip using charged hydrogels instead of membranes was demonstrated. The results obtained from the microfluidic device highlight the need for detailed transport studies of charge and momentum near ion-selective

Figure 9.2. (a) Schematic illustration of the new generation gut-on-chip device. Two or more different culture media are perfused through the main channels for providing apical and basolateral environments to the Caco-2 cells. (b) Phase contrast microscopy image of the patterned hydrogel and Caco-2 cells inside the compartments at day 0.

Figure 9.3. Time lapse fluorescence images of ion concentration polarization in the microchip. 1 mM NaCl solution with 5 μM of AlexaFluor Cadaverin (fluorescent dye) was injected into the microchip at a 3 μl min⁻¹ flow rate. The voltage-time graph shows the response when the current was set to 5 μA. Ion concentration polarization and vortex formation in the microchannels were observed. The voltage-time graph shows that at a constant current (5 μA), the voltage fluctuates over time depending on the depletion and concentration of the ions in the adjacent microchannels. Letter D represents the desalinated stream and letter E presents the enriched stream.
hydrogels at different applied currents. Figure 9.3 shows the preliminary results of chronoamperometry measurements. We believe that chronoamperometry and microPIV studies supported by finite element simulations could provide substantial understanding of the physics of dynamic electrokinetic flows in the overlimiting current regime. Further, different hydrogels with higher charge densities could be examined to desalinate > 10 mM NaCl solutions. However, finding ion-selective materials with a low swelling degree and high ion density may be challenging. Another alternative research direction would be to add more hydrogel stacks to the microchip to increase throughput.
Appendix A

Fabrication of Fused Silica Microchips for Preparative DNA Fractionation
A.1 | Explanation of typical process steps

The fabrication process consists of three fundamental steps:

(1) Top wafer patterning. Care must be taken that both sides of the wafer are protected with a foil during powder blasting.

(2) Micro channels and capillary barriers are etched into the top fused silica wafer by one-step deep reactive ion etching process using the Adixen DE etcher.

(3) Alignment and thermal bonding of the top wafer with a plain fused silica wafer.

A.2 | Masks

Three masks are needed for the fabrication:

![Image of masks]

Figure A.1. Cross section view of the fused silica microchips and three different masks used for fabrication.

A.3 | Mask layout

![Image of chip design]

Figure A.2. Chip design and overlay of three different masks used for fabrication of microchips. Images on the bottom right show two different pillar shapes, and capillary barriers in between.
The design of DNA separation microchip is shown in Figure A.2. The turquoise area presents microchannels (20 µm deep), the light blue area presents capillary barriers (5 µm high), and the black presents powder blasted holes.

### A.4 | Process parameters

#### A.4.1 | Wafer selection

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Process parameters</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Select wafers</td>
<td>Top and bottom wafers: fused silica, PlanOptik V015.04-1107 (Germany)</td>
<td>4”</td>
</tr>
</tbody>
</table>

#### A.4.2 | Top wafer processing

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Process parameters</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Standard Glass</td>
<td>• 2 min fuming HNO₃ beaker I           • Quick Dump Rinse, QDR</td>
<td>Removal of light organic particles and metals</td>
</tr>
<tr>
<td></td>
<td>Cleaning</td>
<td>• Wet bench 17: 10 sec 25% KOH at 75°C dip</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 10 min ultrasonic in the first rinse water</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• QDR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Fuming: 1 min HNO₃ beaker I + 2 min HNO₃ beaker II</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Spin drying</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Drying</td>
<td>• Dry wafers at 120°C for 20 min</td>
<td>Removal of excess water</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Litho SU-8 2005</td>
<td>• Spin coating SU-8-2005 program for 8 µm thickness</td>
<td>PREBAKE program</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Spin coating</td>
<td>5°C-95°C-2 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Prebaking</td>
<td>2°C-20°C-0 min (cool down)</td>
</tr>
<tr>
<td>4.</td>
<td>UV Exposure</td>
<td>Hard contact mode, 13 sec, manual top side, direct exposure</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EVG 620</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Postbaking

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C-250°C</td>
<td>20 min</td>
</tr>
<tr>
<td>50°C-50°C</td>
<td>2 min</td>
</tr>
<tr>
<td>50°C-65°C</td>
<td>2 min</td>
</tr>
<tr>
<td>50°C-75°C</td>
<td>5 min</td>
</tr>
<tr>
<td>2°C-20°C</td>
<td>0 min  (cool down)</td>
</tr>
</tbody>
</table>

### Development

**RER 600**

* Wet bench 24

- Wash front side with RER 600 once
- Leave it in RER 600 for 10 sec
- Program SU-8 spinner: 3 cycles 20 sec each wafer
- Flush with RER 600
- Flush with IPA
- Spin drying
- Visual inspection at light microscope

### Annealing

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C-130°C</td>
<td>10 min</td>
</tr>
<tr>
<td>2°C-20°C</td>
<td>0 min  (cool down)</td>
</tr>
</tbody>
</table>

### DRIE

**Adixen DE**

- 5 min UV-ozone cleaning
- **Tepla Plasma**
- Dry etch of Cr
- Prog. BFloatExp9/10
- 15 µm etching
- Prog. “short clean” for plasma cleaning of wafers
- After each 20 min etching clean chamber: Clean O₂ (30 min)

**Dektak**

- Sketch the wafer at both center and edge
- Measurement comparison

**Tepla Plasma**

- 5 min UV-ozone cleaning

**Adixen DE**

- Dry etch of Cr
- Prog. BFloatExp9/10
- 15 µm etching
- Prog. “short clean” for plasma cleaning of wafers
- After each 20 min etching clean chamber: Clean O₂ (30 min)

**Dektak**

- Sketch the wafer at both center and edge
- Measurement comparison

**Operation**

- Start the process when SH heating is approx. 10/9.5°C
- (Modify: step time: 9 min, SH options: 120.00 mm)
- Bias voltage: 278 V
- Check center He regulation: < 5 sccm
- Pressure: 9·E⁻³ mbar

**DRIE in Adixen DE**

- up to ~20 µm = 36 min per wafer

### SEM

- Coat Cr (10 nm) in Film sputtering SEM Emitech Chromium
- **Cr etching at wet bench 10**
- Cr etching (Cr layer for SEM)
- Cr etchant for 5-7 sec
- QDR
10. **Repeat steps 3-9**

- Dry etching of capillary barriers.
- The etch depth is 5 µm

| Capillary barrier fabrication |

11. **SU-8 Stripping**

Wet bench 9

- Subsequent Piranha (H2SO4:H2O2, 3:1)
- Cleaning 20 min at 120°C
- QDR
- Spin drying

| Essential to make the process above 100°C |

---

### A.4.3 Powder blasting

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Lamination Ordyl BF 410 foil</td>
<td>Backside of the processed fused silica wafer Laminate BF 410 foil on substrate; • Use the laminator at 105°C, speed 2 • Exposure • Remove thin PET film with thin knife • Use capillary barriers mask • Expose man. bottom side, 15 sec, hard contact, constant time, cross-hair <strong>Wafer to dicing room</strong> • Laminate blue dicing foil on the front side • Hot plate for 120 sec • Remove the transparent film with a thin knife • Development at Chemistry Lab CR 2.516 • 0.2% Na2CO3, spray development (7 min per wafer) • 45°C water spray, 2 min development • Spin drying • Visual inspection</td>
<td>During lamination step, hotplate temperature must not exceed 115°C due to the heat sensitivity of the lamination foil Use silicon support to eliminate reflectance of the chuck</td>
</tr>
<tr>
<td>2.</td>
<td>Powder blasting</td>
<td><strong>Parameters:</strong> Pressure: 5 bar Grain size: 29 µm</td>
<td></td>
</tr>
</tbody>
</table>
Flow: minimal
Y-speed: 10 mm min\(^{-1}\)
X-step: 0.5 mm
Nozzle: 1.2 mm
Nozzle height: 22 mm
Scans: 2x

3. Foil stripping and Delamination Chemistry Lab

- Wash the wafer with warm tap water and subsequently with DI water at the sink
- Remove the dicing foil from the front side by carefully peeling off straight backwards
- Spin drying
- Put the wafer in a new wafer box, to prevent contamination with powder particles

4. Cleaning Wet bench 16

- Remove powder particles by ultrasonic agitation in DI water for 10 min

A.4.4 Thermal bonding

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| 1.   | Standard Glass Cleaning        | 2 min fuming HNO\(_3\) I
Quick Dump Rinse, QDR
Wet bench 17: 10 sec 25% KOH at 75°C dip
10 min ultrasonic in the first rinse water
QDR
Fuming: 1 min HNO\(_3\) I + 2 min HNO\(_3\) II
QDR
Spin drying | Removal of organic particles for fusion bonding |
| 2.   | Aligning and Prebonding       | Manually:
- Put bottom wafer on a black background
- Put 3 pieces of Aluminum foil on the edge
- Put top wafer on top of it
- Align by hand with stereomicroscope |
• Contact wafers
• Apply slight pressure in center of the wafer with tweezers
• Pull out Aluminum foil pieces
• Use tweezers to remove air bubbles if necessary

3. **Bond Annealing**
   - Oven: potter furnace
   - Program P15:
     • 5 hours to 625°C
     • 1 hour at 625°C
     • 10 hours to 20°C

A.4.5 | Dicing process

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dicing</td>
<td>Laminate the wafer stack with dicing foil (holes towards the foil)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Load point</strong></td>
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<tr>
<td></td>
<td></td>
<td>• Blade type TC300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Index 1: distance between cuts // to flat</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Index 2: distance between cuts ⊥ to flat</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Height: distance between blade and chuck</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>During dicing</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Thickness: maneuver distance between blade and chuck</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Speed: 4 mm s⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Wafer diameter:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 4”=110 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Stop count 1: number of cuts direction 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Stop count 2: number of cuts direction 2</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Fabrication of PDMS Microchips for Gut-on-Chip and Microelectrodialysis
B.1 | Explanation of typical process steps

The fabrication process consists of three fundamental steps:

1. SU-8 mold patterning on silica wafer. Care must be taken during aligning of the second SU-8 layer, which has capillary barrier patterns on it.
2. PDMS is prepared and poured on top of the patterned SU-8 structures and peeled off once the material hardens.
3. PDMS microchips are punched to create inlets and outlets, and bonded to a microscope slide to form the final microchips.

B.2 | Masks

Two masks are needed for the fabrication:

![Figure B.1. Cross section view of the SU-8 molds and two different masks used for fabrication.](image1)

![Figure B.2. Chip design and overlay of two different masks used for fabrication of microchips. The Blue mask presents the pillar layout, and the purple mask presents the capillary barriers.](image2)
B.3 | Mask layout

The designs of gut-on-chip and microelectrodialysis microchips are shown in Figure B.2 and B.3. The blue and black areas present microchannels (75 µm deep), the purple and light blue areas present capillary barriers (15 µm high).

B.4 | Process parameters

B.4.1 | Wafer selection

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Select wafers</td>
<td>Silicon wafer, standard</td>
<td>4”</td>
</tr>
</tbody>
</table>

B.4.2 | Wafer processing

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| 1.   | Standard Glass Cleaning | • 2 min fuming HNO₃ beaker I  
• Quick Dump Rinse, QDR  
• Wet bench 17: 10 sec 25% KOH at 75°C dip  
• 10 min ultrasonic in the first rinse water | Removal of light organic particles and metals |
<p>| | | |</p>
<table>
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<tbody>
<tr>
<td><strong>2.</strong></td>
<td><strong>Drying</strong></td>
<td></td>
</tr>
</tbody>
</table>
|   | Wet bench 22 | QDR  
Fuming: 1 min HNO₃ beaker I  
+ 2 min HNO₃ beaker II  
QDR  
Spin drying |
|   |   |   |
| **3.** | **Litho SU-8 2005** |   |
|   | Wet bench 24 | Spin coating SU-8-2005  
program for 67.5 µm thickness  
Spin coating  
10 sec 1000 rpm @ acc.500 rpm sec⁻¹  
30 sec 3000 rpm @ acc.1000 rpm sec⁻¹  
Prebaking |
|   |   | **PREBAKE program**  
5°C-95°C-2 min  
2°C-20°C-0 min (cool down) |
| **4.** | **UV Exposure** |   |
|   | EVG 620 | Hard contact mode, 22 sec, manual top side, direct exposure |
|   |   | Ionic contamination is not important in this case, so the faster glass cleaning process is preferable to the standard cleaning; only an oxygen plasma treatment is also enough |
| **5.** | **Postbaking** |   |
|   | Wet bench 24 | 5°C-25°C-20min  
5°C-50°C-2 min  
5°C-65°C-2 min  
5°C-75°C-5 min  
2°C-20°C-0 min (cool down) |
|   |   |   |
| **6.** | **Development RER 600** |   |
|   | Wet bench 24 | Wash the front side with RER 600  
Leave the wafer in RER 600 for 10 sec  
Program SU-8 spinner: 5 cycles  
20 sec each cycle  
Flush with RER 600  
Flush with IPA  
Spin drying  
Visual inspection at light microscope |
|   |   |   |
| **7.** | **Repeat steps 3-7** | Patterning of capillary barriers on SU-8 |
|   |   |   |
### Fabrication of PDMS Microchips for Gut-on-Chip and Microelectrodialysis

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| 8.   | Annealing | • The final channel height is 75 µm  
      |         | • Increase the number of cycles to 8 during the RER 600 development step |         |
| 9.   | Strengthening the mold | 5°C-130°C-10 min  
      |         | 2°C-20°C-0 min (cool down) | Clean dummy wafer  
      |         | • Standard cleaning  
      |         | Preheat the SU-8 mold  
      |         | • Put the SU-8 mold on a hotplate at 95°C  
      |         | Spin coat SU-8 5 on the dummy wafer:  
      |         | • Dry: 2 min, ≥ 120°C  
      |         | • Spin parameters: 10 sec 500 rpm @ 500 rpm sec⁻¹ spread  
      |         | • 30 sec 4000 rpm @ 1000 rpm sec⁻¹  
      |         | • Dry: 15 sec on 95°C hotplate (until interference color does not change anymore)  
      |         | • Stick both wafers together (by hand); the dummy wafer 45° rotated  
      |         | • Put the wafer stack on 95°C hotplate and carefully push with a tweezer in the center, to remove any air in between the stack; app. 30 sec  
      |         | • Repeat this on 120°C hotplate; app. 30 sec  
      |         | • Finally center both wafers by hand (be careful, quite hot) | Due to the crystal structure, the silicon wafers are easy to break along the crystal planes. Gluing a dummy wafer on the backside, which is 45° rotated, makes them less breakable |

### B.4.3 PDMS patterning

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
<th>Parameters</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>1.</td>
<td>PDMS preparation</td>
<td>• Mix PDMS precursor with Sylgard curing agent with 10:1 ratio</td>
<td></td>
</tr>
</tbody>
</table>
| Chemistry Lab | • Mix two components well using a stirrer  
|              | • Degas the mixture for an hour in a vacuum chamber  
|              | • Pour the degassed mixture on top of the SU-8 mold and bake it for at least 4 hours  
|              | • Gently peel off the hardened PDMS layer  
|              | • Punch the holes  
| 2. Microchip assembly Assembly Lab | • Apply plasma cleaning for 45 sec to activate both PDMS and glass slide surfaces  
| | • Immediately after the plasma cleaning step, assemble these two parts together  
| | Removal of light organic particles and metals |
Appendix C

Experiment Setups
C.1 | Basic requirements
The experiment setup for each project is described below.

C.1.1 | DNA fractionation experiment setup

**Chip holder:** A three-piece chip holder was designed for fixing the fused silica microchips on the microscope stage. The chip holder was made of Delrin® and consisted of three pieces. Liquid reservoirs are interfaced to the power supply via five platinum wires attached to the top plate. Middle plate has rectangular-shaped large liquid reservoirs, which are surrounded by in-house made o-rings at the bottom part. The bottom part is in direct contact with the fused silica microchips. Bottom plate is supported by an aluminum plate to avoid bending, and has a square window for microscope visualization. Figure C.1 shows the design of the chip holder. Care must be taken when assembling the bottom and the middle parts because the material stress caused by relatively flexible Delrin® material may break the microchip.

**High voltage power source:** LabSmith HVS448LC High Voltage Sequencer was used in all biomolecule separation experiments.

**Microscope, UV lamp, and camera:** Leica DM IRM microscope was used in all experiments. A mercury halogen lamp (12 V/100 W) was connected to the microscope and a Leica I3 filter cube was used to enable epifluorescence microscopy in this setup. The microscope was connected to a Hamamatsu camera (Model C4742-80-12AG) to record movies and take snapshots. Figure C.2 shows the setup and its components.

![Diagram of chip holder design](image.png)

*Figure C.1.* Chip holder design. The chip holder has three plates. The middle plate has in-house made o-rings as shown in the image.
Figure C.2. (a-d) Experiment setup for biomolecule separation. Power source, camera, electrodes, chip holder, and microchip are shown.

Figure C.3. (a-e) Experiment setup for organ-on-chip studies. Syringe pump, incubator, chip holder, and fluidic access points are shown.
C.1.2 | Gut-on-chip experiment setup

**Incubator:** A Binder brand incubator facilitated the cell culturing experiments. The inner CO₂% and temperature was constantly kept 5% and 37°C, respectively.

**Syringe pump:** Culture media was supplied to the PDMS microchips via a Harvard PHD 4400 Hpsi programmable syringe pump and Hamilton Gastight® syringes. A Teflon tubing provided the connection between the pump and the PDMS microchips. Figure C.3 shows syringe pump, incubator, chipholder and fluidic access points.

C.1.3 | Microelectrodialysis experiment setup

**Power source:** A Keithley power source was used to apply electric field on the PDMS microchip. Gold-coated electrode pins were used to connect the power supply to the microchip.

**Syringe pump:** Salt solution was supplied to the PDMS microchips via four PHD 4400 Hpsi programmable syringe pumps and Braun Omnifix-F 1 ml syringes. A plastic tubing provided the connection between the pump and the PDMS microchips.

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Figure C.4. (a-d) Microelectrodialysis setup consisting of power source, microchip, fluidic access points, sample connection tubes, electrodes, and tubing.
**Microscope, UV lamp, and camera:** Leica DM IRM microscope was used in all experiments. A mercury halogen lamp (12 V/100 W) was connected to the microscope and a BGR filter cube was used to enable epifluorescence microscopy in this setup. The microscope was connected to a Hamamatsu camera (Model C4742-80-12AG) to record movies and take snapshots for fluorescence experiments (Figure C.4).
Publication List

Peer-reviewed journal articles
Publication list

**Book chapter**

**Patent application**
1. B. Gumuscu, T. Tekinay, “Isolation of novel bacteria strains providing degradation of 2,4,6-trinitrotoluene”, Bilkent University, Turkish patent pending, patent no: 2012-O-111830.

**Conference proceedings and presentations**


Samenvatting

Hydrogels vallen in de categorie van slimme materialen (‘smart materials’), die toepassing vinden in medische diagnostiek en therapie en ook als gereedschap worden gebruikt in de fundamentele wetenschap, bijvoorbeeld voor geminiaturiseerde chemische analysesystemen (‘lab-on-a-chip’). In dit proefschrift ligt de focus op het ontwikkelen van technieken om hydrogel microarrays te patterneren voor miniatuur chemische analysesystemen, en vervolgens op drie hoofdtoepassingen van hydrogels in Lab on a Chipsystemen: voor het fractioneren van DNA (als een alternatief voor dure systemen met lage doorvoer), voor een in vitro model voor de menselijke darm met opgedeelde compartimenten (‘gut-on-a-chip’), en voor het ontzouten door middel van micro-elektrodialyse.

Het gebruik van gepatinerende hydrogels in afgesloten microfluidische chips voor de verschillende onderzoeksvelden als hierboven genoemd is erg afhankelijk van het fabricagegemak van de gebruikte technologie. In dit werk zijn twee simpele fabricagemethoden ontwikkeld voor het patineren van hydrogel in microarrays. In de eerste methode is een onderbroken belichting door een fotomasker toegepast op mechanisch gepolijste microchips. In de tweede methode werden capillaire drukbarrières gebruikt om de positie van de vloeistof-lucht meniscus van de hydrogel precursorsoplossing te controleren, waarna de hydrogel werd uitgehard door middel van fotopolymerisatie of thermische uitharding. Beide fabricagemethoden onderscheiden zich van voorafgaande studies door hun veelzijdigheid en hoge reproduceerbaarheid.

De eerste hoofdtoepassing van de hydrogels, de fractonering en zuivering van korte DNA fragmenten speelt een belangrijke rol bij de tweede generatie basenvolgordebepaling van DNA (‘second generation sequencing’) en voor gepersonaliseerde geneeskunde (‘personalized medicine’). We beschrijven een nieuwe methode voor het gelijktijdig fractioneren en zuiveren van DNA in een continue stroom in een microfluïïdische chip gevuld met agarose gel. Dit werk is om twee redenen van belang. Ten eerste demonstreren we een nieuw principe voor het continue fractioneren van DNA, namelijk door de variatie in de veldafhandelijkheid.
van de mobiliteit van het DNA te gebruiken. Dit scheidingsprincipe bleef tot nu toe onopgemerkt en ongebruikt in de scheidingsliteratuur. Ten tweede voorziet deze methode in een goedkope, robuuste en veelzijdige scheidingsmatrix, omdat hij wordt toegepast op agarose gel. Deze theoretische vooruitgang in combinatie met deze praktische vooruitgang kan leiden tot nieuwe ontwikkelingen in de wereld van biologische monstervoorbereiding. Een basislijn-scheiding van een 0.5-10 kbp DNA-ladder werd bereikt in 2 minuten, wat ~15 keer sneller is als in veelgebruikte commerciële apparatuur. Bovendien is de agarose geltechnologie makkelijk aan te passen voor verschillende DNA groottes, en kan een verandering van het geltype het mogelijk maken om bijvoorbeeld eiwitten te fractioneren. Deze combinatie van eigenschappen maakt deze techniek interessant voor tweede generatie sequencing en medische diagnostiek.

De tweede hoofdtoepassing van hydrogels als beschreven in dit proefschrift is het gebruik van multi-compartment hydrogel arrays voor een 3D celkweek van menselijke darmepitheelcellen. Een juiste vormgeving van de *in vitro* micro-omgeving van het weefsel om *in vivo* weefelsystemen na te bootsen is cruciaal voor het verbeteren van onze kennis over weefselfysiologie, alsmede als voor het verminderen van de hoge kosten en problemen die verbonden zijn met de bestaande technieken. We demonstreren een *in vitro* microfluidisch celkweekplatform, dat bestaat uit periodieke 3D hydrogelstructuren. Wij laten zien dat de gecompartimenteerde structuur van de microchip en de eenvoudige techniek voor vloeistofdoorstroming het mogelijk maken om menselijke darmcellen (Caco-2) te kweken, welke spontaan in 3D structuren groeien op de derde dag van de celkweek. Op de achtste dag van de kweek werden de Caco-2 cellen geweekt in een cocultuur met de darmbacterie *E.coli* voor een periode van 36 uur. De *E.coli* cellen hechtten zich aan de Caco-2 cellen zonder de levensvatbaarheid van de cellen te beïnvloeden. Continue vloeistofverversing maakte het mogelijk de effecten van een chlooramfenicolbehandeling op de bacterieën en darmepitheelcellen te bestuderen. Tenslotte vonden we dat verschillende afmetingen van de compartimenten, met grote en kleine interfaces van de hydrogel met de doorstroomvloeistof, leidden tot een verschil in de proliferatie en het celverspreidingsprofiel van de Caco-2 cellen. Deze microchip maakt het mogelijk om de vloeistofdoorstroming te controleren wat een dynamische regulatie van kweekcondities toestaat.

Microelektrodialyse is de laatste hoofdtoepassing van de gepaternerde hydrogels die onderzocht is in dit proefschrift. De gebruikelijk methode om een microelektrodialyse-apparaat te construeren berust op de verwerking van membranen in microchips, wat grote uitdagingen met zich meebrengt op gebied van robuustheid, consistentie en fabricagegemak. In plaats van deze membranen zijn geladen hydrogels veelbelovende kandidaten voor het ontzouten met behulp van elektrodialyse, omdat ze een ion-selectieve en hydrofiele matrix hebben, die veelzijdig en goedkoop is en ook gemakkelijk aan te passen. We paterneerden daarom ion selectieve hydrogels in een
microfluidische chip voor elektrodialyse. Het resultaat was zowel een experimenteel aangetoonde miniatuurontzouting als een toegenomen theoretisch inzicht in de ion transport fenomenen dit plaats vinden bij ontzouting op de microschaal. In dit werk laten we eerst zien dat parallelle vloeistofstromen van geconcentreerd en onzout water worden gevormd in een constante vloeistofstroom, wanneer een potentiaalverschil wordt aangebracht over de afwisselende rijen van kation- en anion-selectieve hydrogels. Ongeveer 75% van het zout dat initieel aanwezig in een inlaatstroom van 1mM natriumchloride werd op deze wijze verwijderd in de onzoute uitlaatstroom. We toonden ontz outing aan voor verschillende zoutconcentraties en vloeistofstroomsnelheden in de microchip. Ten tweede maakte de microchip het mogelijk om het transport van ionen in ion-selectieve hydrogels en microkanalen te bestuderen, door gebruik te maken van fluorescerende ionen om de ion concentraties te visualiseren. Bij voldoende hoge spanningsverschillen werd een draaikolkpatroon waargenomen dicht bij het grensvlak tussen de hydrogel en de vloeistof, welk effect bijdraagt aan een verhoogd convectief transport naar de hydrogels bij spanningen waar dit transport normaal gelimiteerd is.
Contributions

Some of the results described in this thesis were achieved through collaboration with many people. The collaborators and their contributions are listed below. Prof. dr. Jan Eijkel and Prof. dr. ir. Albert van den Berg from BIOS Lab on a chip group in University of Twente provided input to all of the projects in the form of fruitful discussions.

Chapter 3

I would like to thank Roy Kooijman for helping in microchip polishing, Johan G. Bomer for microchip fabrication. I also thank Dr. Allison C.E. Bidulock and Dr. Mark A. Hempenius for thorough discussions.

Chapter 4

I would like to thank Dr. Wim J. Hendrikson for supplying PEG DA, Johan G. Bomer for microchip fabrication. I also thank Dr. Allison C.E. Bidulock, Jan van Nieuwkastele, and Dr. Paul Vulto for thorough discussions.

Chapter 5

I would like to thank Jan W. van Nieuwkastele and Ad Sprenkels for technical support, Dr. Loes I. Segerink and Hai Le The for their kind help in image processing, Johan G. Bomer for microchip fabrication, Hans L. de Boer for helping in chip holder design. I also thank Dr. Allison C.E. Bidulock and Joshua Loessberg-Zahl for thorough discussions.

Chapter 6

This chapter is the result of collaboration with Hugo Albers from BIOS Lab on a chip group. I would like to thank Johan G. Bomer for helping in microchip design, Hans L. de Boer for providing PDMS cutting blades, Dr. Rik. R. Rurup for providing the GFP expressing E. coli cells, Dr. Anne M. Leferink
and for Marinke W. van der Helm their advices on cell culturing, Kirsten A. Leijenhorst-Groener for her help in bacteria culturing, and Dr. Andries van der Meer for thorough discussions.

Chapter 7
This chapter is the result of collaboration with Sander A. Haase and Anne M. Benneker from SFI group. I would like to thank Mark Smithers for SEM imaging, Mark Ankoné for freeze-drying, Timon Rijnaarts for permselectivity and resistivity measurements, Harmen J. Zwijnenberg for titration measurements, Jan van Nieuwkastelee for technical support. I also thank Dr. Mark A. Hempenius, Dr. Jeffrey A. Wood, and Prof. dr. ir. Rob G.H. Lammertink for thorough discussions.

Chapter 8
This chapter is a result of collaboration with Dr. Verena Schulze-Greiving, Bart Wallhout, Dr. Kornelia Konrad and Prof. dr. Mieke Boon. I also thank Daan Shuurbiers for his comments on the chapter.
Acknowledgements

I have been waiting for this moment to come in the last 3 years and 11.5 months (and, yes, plus 26 years). Looking back to the last bit of the waiting period, I feel quite “satisfied” and lucky with the scientific knowledge I gained, people I have met, and all the fun I had. I am very glad to find a chance to thank everyone in the next lines. So, welcome to the most read chapter of this thesis, and for that reason, I did my best to write it carefully to remember everyone. I promise a compensation drink for the ones that I forgot, come and find me!

I would like to start with my promotor Prof. Dr. Ir. Albert van den Berg. **Albert**, you made my very first day at BIOS memorable, as my very first assignment was to prove my academic skills (risk assessment!?) by riding your Segway fast and not to bump onto the walls. You have never told me if I passed your test, but gave me a good lesson: academia is full of challenges—and fun. Thank you for welcoming me in BIOS group, your critical questions which indeed address the broader picture of the projects that we have done together, giving me a hand to join the Lab on a Chip family, your tremendous support, and of course the BBQs at your place.

I would like to thank my promotor and daily supervisor Prof. Dr. Jan Eijkel. **Jan**, I feel the luckiest person in this universe to have a supervisor like you. I can never forget how you made me laugh, shout, cry, and jump at the same time in front of the Oude Kerk when you called me to say that I was accepted for the PhD position. Thank you for teaching me how to walk in my shoes, sharing my enthusiasm when having success, supporting me when getting failure, gearing me down when I try to fly without wings, encouraging me when I was close to give up, sharing my happiness and grief, and being such a father figure in science for me. You added a lot to me in not only scientific but also personal level. I wish all the best for you and **Jeanette** in the future.

I would also like to acknowledge my project partners, Prof. Dr. Ir. Rob Lammertink, Assoc. Prof. Mark Hempenius, Sander Haase, Anne Benneker, Hugo Albers, and Dr. Andries van der Meer. **Rob**, thank you for taking part in
Acknowledgements

my graduation committee and being always positive, helpful, and open to collaborate. It was a big pleasure to work with you as your input to the project lead us to a great piece of work. **Mark**, I really appreciate your collaboration in the desalination project. It was very comfortable to work with a person like you, being extremely patient when answering some fundamental chemistry questions with a great enthusiasm. I also would like to thank **Sander** and **Anne** for teaming up to turn the desalination by hydrogels idea to reality. We not only collaborated but also had loads of fun together. I always came to our meetings very happy and almost laughing because of the positive environment sourced by you both. With this, I would also like to thank all lovely members of **SFI** and **MST** groups for the round table conversations whenever I visited Meander. Among my collaborators, **Hugo** has a special place, being my officially first master student. We’ve been through lots of struggles in the gut-on-chip project, but always came up with sound solutions (of course, not talking about the IV tube gadget made for pumping the culture media using gravity). I look forward to see you defending your PhD thesis in a few years. **Andries**, thank you for always being there to have chat about science and “future plans”. I am glad to work with you in gut-on-chip project, which will hopefully be continued under your lead in the future.

Furthermore, I would like to thank my other promotion committee members, **Prof. Dr. Marcel Karperien**, **Prof. Dr. Alexander Kros**, **Prof. Dr. Patrick Doyle** for evaluating my thesis and giving the approval to defend it publicly. I also thank **Prof. Dr. Hans Wallinga**, for chairing my defense.

I’d like to extend my great thanks to Johan Bomer, without whom this thesis would not have been that thick. **Johan**, thank you for teaching me how to work with silicon and glass wafers—one of the greatest experiences I gained in the last four years—, your endless patience to my questions regarding with the process flows, our discussions about life and Turkey’s last political situation when waiting for the wafers to bake, and being always there whenever technical support was needed. I feel privileged to work with you. Talking about technical support, it’s just time to thank Jan van Nieuwkastelee. **Jan**, I was very lucky to have you in both BIOS and SFI groups. For me, you are the secret hero having special powers on everything about technology. I would also like to thank **Hans de Boer** for helping me to design chipholders and handling the platinum wires; **Paul ter Braak** for his support in the lab (ja, met grote blijdshap) and being one of the most cheerful people in BIOS; and **Hermine Knol-de Vries**, for handling all the arrangements and organizations flawlessly for such a big research group. Thank you **Ad Sprenkels** for helping me to find the correct relays for the voltage switch box within your very limited time at the university. **Eddy de Weerd**, thank you for keeping your door always open for me, especially during my adoption period to the group. Finally, I would like to thank **Edward Borggreve** for welcoming me in his office with a big smile to answer my finance related questions.
Next, my PhD would be quite painful without the technical support of the following people. I would like to express my gratitude to **Kirsten van Leijenhorst-Groener**, who opened the lab doors of NBP group for me to culture bacteria cells; **Roy Kooijman** for helping me with the grinding and polishing of 0.5 mm thin glass wafers; **Huib van Vossen** for his cool suggestions to rarely faced cleanroom related problems; **Ton Jenneboer** and **Peter Linders** for the NICE training; **Rene Wolf** and **Hans Mertens** for having funny chats while waiting for Adixen machine to warm up; **Mark Smithers** for being unique by singing electro tech music when recording awesome scanning electron microscope images; **Samantha Ooijman** for all the fun we had in +10 academic events; **Gerard Kip** for the XPS measurements and SEM training; **Gerard Roelofs** for always being kind to me and making MESA+ cleanroom facility to work flawlessly.

Great thanks goes to the professional “volunteers”, who helped me to complete the most difficult chapter to write in this thesis: Innovation Journey of a Techno-scientific device, or in other words “the philosophy chapter”. Despite the fact that we intended to write a philosophy chapter at the beginning, it turned out to be a technology assessment chapter due to some insider and outsider views issues (I’m sure that everybody will remember that long shot story). Chapter eight wouldn’t have existed in my thesis without my stubbornness and the huge help of a few patient people: The first one is Dr. Verena Stimberg (Schulze-Greiving). **Verena**, thank you very much for bridging the “fundamental science world” and the “social science world” for me, and keeping me on track every time I got lost in “thinking”. I also would like to express my gratitude to **Ir. Bart Wallhout** and **Assist. Prof. Kornelia Konrad** for their valuable inputs to the chapter. I can’t finish this paragraph without mentioning Prof. Dr. Mieke Boon. **Mieke**, thank you for making time for me in your superbusy schedule and teaching me about philosophy of science. You have definitely opened a new and interesting window in my scientific approach.

Apart from the big family of supporters mentioned above, I have spent most of my working hours in a few m² place together with a few people: **Wesley van den Beld**, **Sourav Bhattacharjee**, **Liza Rassaei**, **Jean Philippe Frimat**, **Adithya Sridhar**, **Floris van den Brink**, **Stefan Dekker**, and **Jeroen Vollenbroek**. Thank you guys for putting up with me and making the office such a lovely and funny place all the time. Stefan and Jeroen, thanks for helping me with the translation of the samenvatting part.

It was a privilege to organize one of the stunning BIOS workweeks in 2015 to England together with Floris, Wesley and **Yawar Abbas**. I had so much fun during our meetings and during the journey (especially when our bus was very closeby that innocent jeep, the ferry journey, and of course the flat in Edinburgh). I was also a part of the amazing BIOS Nano-team together with Jan E., Jan N., Verena, **Lennart de Vreede**, **Laura Hendriks-Folkertsma**, and **Marinke van der Helm**. It was exciting to see the expression on the kids’ faces
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