Compartments in microfluidic devices: 
From manipulation to analysis of droplets and vesicles

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...’”

- Isaac Asimov
Abstract

Microfluidics is a variety of technologies to handle small liquid volumes. It is especially useful, when small compartments of fluids, such as droplets or vesicles are the target of analysis. These nano- to micrometer sized compartments serve as small containers for substances, where the fluid content is isolated from the surrounding fluid. While the giant unilamellar vesicles (GUVs) with their lipid membrane resemble aqueous compartments found in nature and are used as cell model systems, the fully synthetic droplets gained great attention for the isolation of chemicals or cells in small liquid volumes. Both types of compartments can be used to study cells or cellular processes and properties, like fusion or membrane permeability, to gain insights about cellular processes, which otherwise cannot be monitored individually. However, the preparation and handling of such small volumes for a analysis is challenging. Microfluidic devices provide excellent tools to manipulate these compartments and allow a subsequent analysis. In this thesis, platforms are developed to exploit the benefits of microfluidics for the controlled electrofusion of vesicles, a model system to study the compartmentalization of cells and an introduction system for inductively coupled plasma mass spectrometry (ICPMS).

The first microfluidic device is designed to induce and study controlled electrofusion of GUVs. This enables analysis of mixing of membrane lipids and vesicle content in a reproducible manner. It contains microelectrodes patterned onto a glass surface and an array of traps. In combination these features allow to precisely position two or more GUVs on top of the microelectrodes for electrofusion. Upon applying a voltage pulse to the electrodes individual fusion events can be monitored by fluorescence microscopy. Mixing of the membrane lipids is investigated with a Förster resonance energy transfer (FRET) assay. Additionally, the kinetics of a complexation reaction triggered by the fusion of GUVs is measured.

With the second microfluidic device, a new model system for studying the inner compartmentalization of cells is introduced. The device uses droplet microfluidic technology to generate and prepare multivesicular droplets (MVDs) for analysis. In a MVD, multiple GUVs are encapsulated in a droplet. They mimic the hierarchical structure of cells, with the droplet representing the outer cell membrane and the GUVs substituting the cell organelles. In the device the MVDs can be trapped in an array for detailed analysis by fluorescence microscopy. The effects of compartmentalization, like isolation and enrichment of substances, are investigated with a membrane-staining and a compartmentalized enzyme cascade.
The third microfluidic platform compartmentalizes aqueous sample solutions or cell suspensions into droplets size in the range of 40 – 60 µm for a subsequent elemental analysis by ICPMS. A straightforward, cheap and disposable microfluidic chip forms monodisperse droplets of the sample solution or suspension separated by a perfluorinated carrier phase. In a stream of the carrier phase, the sample droplets are ejected and by a custom transport assembly introduced to the plasma of an inductively coupled plasma mass spectrometry (ICPMS) setup, where the elemental analysis of solutions and cells in native buffers is performed. The flexible platform also enables the integration of advanced on-chip sample pretreatment or multiplexed sample introduction.

In summary, this thesis presents microfluidic platforms specifically designed for their applications in bioanalytics, biomimetics and chemistry. Microcompartments, such as droplets and vesicles can be precisely handled and analyzed.
Zusammenfassung


Mit dem zweiten mikrofluidischen System wurde ein neues Modellsystem für die Untersuchung der inneren Kompartimentierung der Zellen entwickelt. Die Plattform verwendet Droplet-Mikrofluidik, um multivesikuläre Droplets (MVDs) zu erzeugen. Bei der Erzeugung werden mehrere GUVs in einem Droplet eingeschlossen. Dadurch ergibt sich


Zusammenfassend zeigt diese Arbeit mikrofluidische Plattformen, die speziell für ihre Anwendungen in der Bioanalytik, Biomimetik und Chemie entwickelt wurden, um Mikrokompartmente wie Droplets und Vesikel zielsicher zu handhaben und zu analysieren.
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XVI
Abbreviations

**Ca**  Capillary number.
**Re**  Reynolds number.
$\mu$**TAS**  Micro total analysis system.

**A**  Absorption.
**APD**  Avalanche photodiode.
**BSA**  Bovine serum albumin.
**CAD**  Computer-aided design.
**CCD**  Charge-coupled device.
**CMOS**  Complementary metal-oxide-semiconductor.
**DC**  Direct current.
**DiI**  1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.
**DMF**  Digital microfluidics.
**DMSO**  Dimethyl sulfoxide.
**DNA**  Deoxyribonucleic acid.
**DOPC**  1,2-dioleoyl-sn-glycero-3-phosphocholine.
**DSPE**  1,2-distearoyl-sn-glycero-3-phosphoethanolamine.
**EDTA**  Ethylenediaminetetraacetic.
**EMCCD**  Electron-multiplying charge-coupled device.
**ESI**  Electrospray ionization.

**EWOD**  Electrowetting on dielectric.

**F**  Fluorescence.

**FEP**  Fluorinated ethylene propylene.

**FRET**  Förster resonance energy transfer.

**GOx**  Glucose oxidase.

**GUV**  giant unilamellar vesicle.

**HRP**  Horseradish peroxidase.

**HS**  Hosting solution.

**IC**  Integrated circuit.

**IC**  Internal conversion.

**ICP**  Inductively coupled plasma.

**ICP-OES**  Inductively coupled plasma optical emission spectrometry.

**ICPMS**  Inductively coupled plasma mass spectrometry.

**IS**  Intravesicular solution.

**ISC**  Intersystem crossing.

**ITO**  Indium tin oxide.

**LA-ICPMS**  Laser ablation inductively coupled plasma mass spectrometry.

**LADE**  Liquid-assisted droplet ejection.

**Laser**  Light Amplification by Stimulated Emission of Radiation.

**LED**  Light-emitting diode.

**LoC**  Lab-on-a-chip.

**LUV**  Small unilamellar vesicle.

**m/Q**  Mass-to-charge ratio.
**MALDI** Matrix-assisted laser desorption/ionization.

**MLV** Multilamellar vesicle.

**MS** Mass spectrometry.

**MVD** Multivesicular droplet.

**MVV** Multivesicular vesicle.

**P** Phosphorescence.

**PBS** Phosphate-buffered saline.

**PDMS** Poly(dimethylsiloxane).

**PE** 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine.

**PEG** Polyethylene glycol.

**PFH** Perfluorohexane.

**PFPE** Perfluoropolyether.

**PMMA** Poly(methyl methacrylate).

**PMT** Photomultiplier tube.

**POPC** 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine.

**PS** Phospholipid solution.

**PTFE** Polytetrafluoroethylene.

**PVC** Poly(vinyl chloride).

**RF** Radio frequency.

**RSD** Relative standard deviation.

**RT** Room temperature.

**SD** Standard deviation.

**SNARE** Soluble N-ethylmaleimide-sensitive-factor attachment receptor.

**SUV** Small unilamellar vesicle.

**TE** Transport efficiency.
**UV** Ultraviolet.

**VR** Vibrational relaxation.
1 General introduction
1. **General introduction**

1.1 **Artificial cell systems**

The term artificial cell describes a synthetic system that mimics one or more cellular functions. Thomas M. S. Chang performed first experiments that are covered by this definition in 1964. He encapsulated enzymes in micrometer sized semipermeable polymers microcapsules to observe the diffusion across the membrane.[1] Today, artificial cell systems are not only used with the goal to build self replicating synthetic cells [2], but are also employed to investigate specific cellular processes, which cannot be studied on cells due to their overwhelming complexity[3]. Amongst others, the used model systems are based on vesicles and droplets.[4] Since these compartments are in the size range of cells or organelles, their handling for an analysis is challenging. In the following chapter microfluidics as a technology to handle small objects is presented. Furthermore, droplets and vesicles as model systems are introduced in detail.

1.2 **Microfluidics**

Inspired by the rapid success of the miniturization of integrated circuits (IC) since the 1960s and driven by the desire to improve analytical methods by downscaling, in 1979, the first miniaturized analytical device was developed. Terry et al. showed a miniaturized gas chromatograph whose micrometer-sized channels were fabricated by etching of a silicon wafer, which was initially developed for IC industry.[5] Since these early days microfluidics has evolved into a highly interdisciplinary field with a multitude of applications ranging form single cell analysis[6] and DNA amplification[7] to the synthesis of nanoparticles[8]. Especially for analytical purposes microfluidic systems are a great success, which lead to the introduction of the term micro total analysis system (µTAS) by Manz et al. in 1990.[9, 10] It describes the integration of all processing steps to perform a chemical analysis into a single microdevice. In the following years it turned out that this principle is not limited to the chemical analysis of samples, but it can be more generally applied to laboratory processes. Today, the integration of one or more laboratory processes on a microfluidic chip is referred to as lab-on-a-chip (LoC). In 2006, George M. Whitesides proposed the following nowadays widely accepted definition for microfluidics.

“It is the science and technology of systems that process or manipulate small (10⁻⁹ to 10⁻¹⁸ litres) amounts of fluids, using channels with dimensions of tens to hundreds of micrometres.”[11]
1.2 Microfluidics

In this size range, the behavior of fluids differs from the macroscopic behavior. At the micrometer scale volume forces like inertia and gravity become less significant and surface forces such as shear stress and surface tension dominate. This behavior can be described for an object with characteristic length \( l \) by the following scaling law (equation 1.1).[12]

\[
\frac{\text{surface forces}}{\text{volume forces}} \propto \frac{l^2}{l^3} = l^{-1} \xrightarrow{l \to 0} \infty
\]

In microfluidics the consequences of this scaling effects are exploited. Among other benefits they allow the precise handling and processing of small fluids amounts. Additionally, the surface-to-volume ratio is high in microfluidic systems, which enable precise temperature control and a fast heat transfer. This advantage can, for example, be used to control the temperature zones on microfluidic continuous-flow PCR devices.[7, 13] In the closed microchannels, small liquid amounts can be manipulated without fast evaporation, that occurs in conventional systems, like pipetting robots. For the processing of rare samples and high-throughput screening applications the low sample consumption, which is a result form the small channel dimensions, is a benefit. With channels in a similar size range as cells microfluidics is ideal for handling cells or cell size objects like bacteria [14], mammalian cells [15, 16], algae [17], yeast cells [18], GUVs[19] or droplets[20]. The small size of microfluidic systems also allows processes to be parallelized in order to achieved a high throughput. An additional benefit of these miniaturized systems is the footprint reduction of lab equipment. The high level of integration achieved today even enables operation scenarios beyond the lab, with portable point-of-care devices.[21]

1.2.1 Fluid dynamics

In fluidic system the Navier-Stokes equation describes flows of Newtonian fluids like water and air. This equation is essentially the continuum version of Newton’s second law on a per volume basis:

\[
\rho \left( \frac{\partial u}{\partial t} + u \cdot \nabla u \right) = \nabla \cdot \sigma + f = -\nabla p + \eta \nabla^2 u + f
\]

(1.2)

Where \( \rho \) is the fluid density [kg m\(^{-3}\)], \( u \) is the relative fluid flow velocity [m s\(^{-1}\)], \( \sigma \) the fluid stress [N m\(^{-2}\)], \( p \) the pressure [Pa] and \( \eta \) dynamic viscosity of the liquid [Pa s].
1. **General introduction**

Additionally, mass conservation requires:

\[
\frac{\partial \rho}{\partial t} + \nabla (\rho u) = 0
\]  \hspace{1cm} (1.3)

At micrometer range inertial forces are small compared to the viscous forces (see equation 1.1). Therefore, the Navier-Stokes equation can be simplified for the description of microfluidic systems to the Stokes equation for an incompressible fluid with no slip boundary condition:

\[
\rho \frac{\partial u}{\partial t} = \nabla \sigma + f = -\nabla p + \eta \nabla^2 u + f
\]  \hspace{1cm} (1.4)

A further consequence of the small channel sizes and the scaling law (equation 1.1) is that the dimensionless Reynolds number \( Re \) is typically low. It is derived from the Navier-Stokes equation (equation 1.4) and describes the ratio between the inertial forces and the viscous forces:

\[
Re = \frac{\text{inertial forces}}{\text{viscous forces}} = \frac{\rho ul}{\eta}
\]  \hspace{1cm} (1.5)

For a straight, circular channel at \( Re \) above 3000, the inertial forces dominate and the flow is turbulent. At \( Re \) 2000 to 3000 the system is in the transition between turbulent and laminar flow. Below a \( Re \) of 2000 the flow is strictly laminar.[12, 22] Most microfluidic systems are operated at \( Re \) well below 1. Consequently, the flow inside the channels is laminar, streams of fluids merging in a microchannel flow parallel and no turbulences are observed. The mixing is only driven by diffusion. Thus, reagent concentrations can be controlled in space and time.

1.3 **Droplet microfluidics**

Droplet microfluidics also referred to as segmented flow microfluidics is one of the most important subcategories of microfluidics. Here the microfluidic channels are used to form and handle nano- to picoliter emulsion droplets or foams. Thorsen *et al.* presented in 2001 for the first time the droplet generation in a T-junction microchannel geometry.[23] In the following years other droplet generation geometries have been introduced and droplet microfluidics became an increasingly important research topic. The applications of droplet microfluidics range from formation of monodisperse foams[24] or
emulsions\cite{25} to complex single cell studies\cite{26}, where the droplets are used to handle individual cells. Often droplets are used as small reactors in high throughput screening to minimize the sample consumption and to enable a precise control of the reaction conditions.\cite{27} A prominent example for this kind of experiment is the droplet-based screening of protein crystallization.\cite{28} Here, multiple parameters such as concentrations of various compounds can be tested and a large number of nucleation experiments can be performed with minimal sample consumption in a short time. Droplet technology is also used to prepare samples for matrix-assisted laser desorption/ionization mass spectrometry\cite{29} by spotting droplets onto a plate and to introduce samples in electrospray mass spectrometers as polydisperse aerosol.\cite{30, 31}. Both techniques offer a high sample throughput and are therefore a good match with droplet microfluidics. By sequential repetition of multiple droplet emulsification steps multicomponent multiple emulsions can be formed.\cite{32} This allows the encapsulation of immiscible or incompatible compounds for chemical reactions or the formation of multicompartment materials. Besides their intended applications for cosmetics, drug delivery or food processing\cite{33} these kinds of emulsions are also proposed as cell model systems\cite{34}. More details about this type of droplet usage can be found in chapter 1.3.3.

Common to all these techniques is the use of at least two immiscible phases. Usually an aqueous phase, that forms the droplet body referred to as droplet or dispersed phase and a hydrophobic carrier phase are used and often a surfactant is added to the carrier phase, for a better stability of the droplets. An example of aqueous droplets interspaced by a perfluorinated carrier phase can be seen in Figure 1.1. The droplet formation in microfluidic channels is a highly reproducible process, but it requires tight control of flow rates and channel geometry (see chapter 2.1). Thousands these finite monodisperse droplets can be generated every second. By using droplet splitting techniques droplet volumes down to femto liter scale are possible.\cite{35}

![Figure 1.1: Bright field image of aqueous droplets loaded with a food dye in a microchannel interspaced by a perfluorinated carrier phase (FC-40). Scale bar 25 µm.](image)

This high production rate and the low sample amount needed for a single droplet make this technique particular useful for high-throughput screening experiments such as cell assays and polymerase chain reactions.\cite{36, 37} Here each droplet can be used as an individual microreactor. Due to the small volume and the recirculating flow pattern
inside the droplets contents are mixed rapidly. Droplets embedded in the carrier phase are not evaporating rapidly like droplets on open surfaces.

For the manipulation of droplets, numerous techniques are available. They include capabilities for droplet fusion\[38\], direct liquid injection into droplets\[39\], droplet fission\[38\], droplet sorting\[40\], ultra fast mixing in droplets\[41\] and droplet trapping (by geometric obstacles (traps)\[42\] or lateral cavities on a channel\[43\]). Droplet analysis is mostly done by optical fluorescent detection but also detection by electronical systems\[44\], capillary electrophoresis\[45\], X-ray diffraction\[46\] and mass spectrometry\[29, 47\] are known. Together, these techniques allow to perform complex studies using droplet microfluidics, such as nanoparticle synthesis\[48\], protein crystallization experiments\[49\] and single cell studies\[26\].

Today, perfluorinated compounds such as FC-40, FC 72 (perfluorohexane (PFH)), perfluorodecalin and HFE-7500 are typically used as hydrophobic carrier phase. These substances are characterized by a low solubility of water (ppm regime) and most other hydrophilic and lipophilic substances.\[50\] The high gas permeability of these compounds make them also particular useful for cell experiments inside droplet.\[51\] Furthermore, they are compatible with materials used to build the microfluidic chips, such as poly(dimethylsiloxane) (PDMS).

The geometry and surface properties of the channel where the droplets are formed influence the droplet formation process. Additionally, the physical properties of the two immiscible fluids impact the droplet generation, e.g. droplet size. These properties are expressed by the dimensionless capillary number \( Ca \). It describes the relative effect of the viscous forces inside the flow versus the interfacial forces between two fluids. The interfacial forces for example have to be in a range, where they are not to strong and prevent droplet brake up, but also high enough to prevent subsequent fission of the droplets. Parameters, like flow velocity or composition of the fluids, have to be adjusted for every droplet generation geometry to obtain a stable droplet formation. For a fixed channel geometry this number can be used to describe the dynamics during droplet formation.

\[
Ca = \frac{\text{viscous forces}}{\text{interfacial forces}} = \frac{\eta u}{\gamma}
\]  

(1.6)

Where \( \eta \) is the dynamic viscosity of the liquid [Pa s], \( u \) is the fluid velocity [m s\(^{-1}\)] and \( \gamma \) is the surface tension [N m\(^{-1}\)].

Besides the channel-based droplet microfluidics which is subject of this thesis, also electrowetting on dielectric (EWOD) based digital microfluidics (DMF)[52] as well as the
1.3 Droplet microfluidics

Piezo-driven or valve-driven droplet-on-demand techniques are available for droplet generation and handling. In the following two sections the two major designs for continuous passive droplet generation are discussed in detail.

1.3.1 T-Junction design

The T-junction design was the first reported geometry for the droplet formation in microfluidic devices. Until today this design is widely used, but was not subject of this thesis.

The T-junction consists of two channels that merge perpendicular (see Figure 1.2). The dispersed phase is pushed into the straight main channel of the carrier phase and blocks this channel as it elongates into it. As a result a pressure is build up in the channel of the carrier phase. At a certain point in the channel the dispersed phase thins and breaks up into a droplet. Droplet sizes can be controlled by the fluid flow rates, the channel width or the relative viscosity of the two phases. Three different regimes of droplet formation depending on the $Ca$ are known for the T-junction geometry. At low $Ca$, the droplet breakup is pressure dominated, which is referred to as squeezing regime. For intermediate $Ca$, shear starts to dominate the droplet formation (dripping regime). High $Ca$ marks the beginning of the jetting regime. The droplet size is decreased and the droplet frequency is increased when the $Ca$ is increased (for a constant channel geometry). Stable formation of monodisperse droplets is achieved at low to medium $Ca$. The T-junction is suitable for application that require low flow rates and larger droplets.

![Figure 1.2: Scheme of the T-junction design for droplet generation. Adapted from [56].](image)

1.3.2 Flow focussing design

Smaller droplets at higher flow rates can be formed by a flow focussing geometry. This symmetric design (planar flow focussing) was first introduced in 2003 by Anna et al. and is today frequently used for droplet generation. It consists of three channels that merge into a single main channel with an optional orifice (see Figure 1.3). Similar
1. **General introduction**

Figure 1.3: *Scheme of the flow focussing design for droplet generation.* Adapted from [56].

to the T-junction, three regimes of droplet formation are known for the flow focussing design and are listed here in the order of increasing $Ca$: squeezing, dripping and jetting regime. In the squeezing regime the dispersed phase enters the junction and blocks the downstream channel. Subsequently a pressure builds up and at a certain point causes the release of a droplet. The dripping regime works similar to the squeezing, but is characterized by a droplet break up caused by viscous drag on the dispersed phase. As a result, the formed droplets are smaller than the channel. In the jetting regime, the dispersed phase is drawn to a thin jet that breaks up into droplets. The reason for this break up is the Rayleigh-Plateau instability [58]. A stable formation of monodisperse droplets by flow focussing can be achieved for medium and high $Ca$.[55] For this geometry droplet size and frequency can be best controlled by the flow rate of the carrier phase.

### 1.3.3 Droplets for artificial cell systems

Even before droplet formation by microfluidic techniques was developed the principle to use aqueous droplets as cell-mimicking systems was known. In 1961 Boris Rotman used polydisperse droplets that were created by a spray method to measured the activity of enzymes.[59] Since the development of the monodisperse droplet formation by microfluidic means the focus of the studies of biochemical reaction in droplets has shifted to drug discovery.[60, 61] Additionally, droplets are also used to directly measure cell response or properties of single cells. Typically, libraries of organisms are screened for their cellular behavior such as gene expression.[62]

Droplets are beyond that also used as templates for the production of monodisperse vesicles, whereas conventional bulk methods usually produce only polydisperse vesicles. Several of the droplet microfluidic approaches are variations of the emulsion
transfer method (see chapter 2.2.2). Typically, the emulsification step is performed by microfluidic techniques. More advanced approaches are able to generate segmented vesicles, which are then used to study enzyme cascades in these model systems. Further assembly line approaches merge all processing steps into a single microfluidic device. On the one hand the post mediated phase transfer method uses a hurdle that promotes the transfer from droplet to vesicle. On the other hand the step junction transfer method facilitates the formation of vesicles by height change of the droplet containing channel. Vesicles can also be generated by solvent extraction of a double emulsion template. Hereby, droplet microfluidics is used to form a water-in-oil-in-water emulsion with a lipid containing oil phase. Subsequently, the solvent is extracted from the oil phase and only a lipid bilayer remains. The lipid coated ice droplet hydration technique employs frozen aqueous droplets as templates, which are formed in a purpose-built microfluidic system. Many of the above mentioned techniques require surfactants for the stabilization of the droplets prior to the vesicle formation or can contain remains of the oil phase in the lipid membrane. This can restrict their usage for cell model studies.

Droplet interface bilayers is a technique to study pore formation and permeation in lipid membranes. Two aqueous droplets in a lipid containing solution are brought into close contact and a lipid bilayer is formed in between the vesicles by self-assembly. More advanced systems are capable of automated assembly, where multiple droplets are positioned in defined geometries to resemble tissue-like structures.

In order to mimic of the hierarchical build up of cells multiple emulsions can be used. Similar to cells this kind of emulsion droplets have a compartmentalized substructure. Moreover they serve as templates for multicompartment polymersomes. Although the generation of these compartmentalized structures is possible, their use as artificial cells is limited. They lack the biological relevance of a lipid membrane and in some cases barely resemble the cellular structure. Also the possibilities for functionalization with receptors, membrane pores or antibodies are restricted. For a review about droplet microfluidics for the study of artificial cells see reference.

1.4 Vesicles

The word “vesicle” originates from Latin, vesicula, -ae, small bubble. In the context here it is used to describe an in vitro formed compartment confined by one or more layers of membranes (see Figure 1.4). These membranes are build from amphiphilic molecules with hydrophilic (head) and hydrophobic (tail) parts. Beside lipids synthetic surfac-
1. *General introduction*

![Diagram of vesicle, lipid bilayer, and lipid](image)

**Figure 1.4: Composition of vesicles membranes.** From left to right: Schemes of a vesicle, lipid bilayer and lipid. Chemical structure of the phospholipid POPC.

Tants or amphiphilic block copolymers can be used as amphiphilic compounds to form vesicles.[79]

1.4.1 *Vesicle classification*

Vesicles can be classified by their membrane or lumen composition as well as their size or lamellarity (see Table 1.1). Vesicles with a single membrane layer are referred to as the unilamellar vesicles. Depending on their size they are categorized as: small unilamellar vesicle (SUV), large unilamellar vesicle (LUV) and giant unilamellar vesicle (GUV). More complex vesicular structures with more than one bilayer can be divided into two different categories: The multilamellar vesicles MLVs have several concentric lipid bilayers. In contrast, the multivesicular vesicles MVVs comprise of smaller vesicles in a larger vesicle with typically one bilayer per vesicle (see Figure 1.5).[80]

<table>
<thead>
<tr>
<th>Table 1.1: Vesicle classification [81, 82].</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbreviation</strong></td>
</tr>
<tr>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>Giant unilamellar vesicle</td>
</tr>
<tr>
<td>Multivesicular vesicle</td>
</tr>
<tr>
<td>Multilamellar vesicle</td>
</tr>
</tbody>
</table>
1.4 Vesicles

1.4.2 Electrofusion

Fusion of lipid membranes is vital in many biological processes, such as cell-to-cell communication, intracellular signaling and transport of hydrophilic molecules. Using artificial cell model system to study these fusion events can contribute to the understanding of the mechanisms, that are for example involved in the mixing of membrane lipids or the distribution compounds in the lumen. It offers important insights for the construction of more complex cellular model systems. The fusion of vesicles is energetically unfavorable as steric, hydration and electrostatic repulsion forces keep the membranes separated and prevent spontaneous fusion.[83] In nature particular membrane proteins like the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) help to overcome these energetic barriers.[84] There are also several in vitro techniques to induce fusion of vesicles, like the addition of fusogenic chemicals like Ca$^{2+}$ ions[85] or peptides[86]. Another method for in vitro fusion of vesicles is based on the findings of U. Zimmermann et al. that cell membranes can be reversibly destabilized by an electric field.[87] This technique is referred to as electrofusion. The principles of this technique are described in detail below.

For electrofusion, vesicles are placed in between two electrodes that form a capacitor (see Figure 1.6). A short voltage pulse is applied to the electrodes and an electric field ($E_c$) is formed. Inside this field, the membranes electrically behave like a capacitor filled with a dielectric medium. The resting potential difference across the membrane is called membrane potential ($V_m$). If an external electric field is applied perpendicular to the membrane, the potential difference increases and the charge separation across the

![Diagram of multivesicular and multilamellar vesicles](image-url)
1. **General introduction**

Figure 1.6: **Scheme of electrofusion.** From left to right: Vesicles are placed in between the plates of a capacitor. Upon applying a voltage to the capacitor, the electric charges cause membrane thinning. If the electric field exceeds a certain threshold ($E_c$) the membranes break down and pores are formed. The membrane lipids rearrange and the vesicles fuse. Adapted from [91].

membrane induces a compression of the membrane. At certain electric field strength ($E_c$) the membrane breaks down, a pore is formed and the potential is discharged. It is possible to deliver DNA or drugs into vesicles or cells through this pore without permanent damage.[88–90] As long as the number and size of pores in relation to the membrane surface is small they can be closed again. The lipids of such a reversibly destabilized membrane can rearrange in a way that two or more vesicles are fused together. For even stronger electric fields, the higher number and larger size of the membrane pores cause an irreversible breakdown. The vesicle is mechanically destructed. For a successful electrofusion the geometry, field strength and duration of the electric field are critical to avoid the destruction of the vesicles. Also it is necessary to position the GUVs in close contact.

The first reported electrofusion of GUVs were performed in bulk, offered only a limited control over the process and had a low fusion efficiency.[92] For more detailed investigations micropipette aspiration techniques were employed to position two GUVs in contact.[93, 94] The aspiration lead to a unwanted deformation of the vesicles that increases the membrane tension. Although this is a very laborious technique with a limited throughput it was anyhow used to investigate the mechanisms involved in electrofusion.[95, 96]

For vesicle fusions microfluidic systems offer a precise control over the environment.[97] Here, triggers such as fusogenic molecules like PEG can be delivered in a precise way.[98] Moreover, the observation can be done directly in the device. The fist im-
implementation of electrofusion on a microfluidic device used optical traps to arrange the vesicles and micromanipulators for positioning of the microelectrodes. This method is only capable of performing a single fusion at a time and requires a large amount of off-chip equipment. Other methods tried to overcome this disadvantages by integrating electrodes in the chip, which are also used to arrange the vesicles for the fusion. This position method allowed only little control over the number of vesicles that were fused. Additionally, the simultaneously initiation of all fusions limits the number of observable fusion events. Wang et al. introduced a microfluidic system with electrodes embedded in the channel walls. He used these electrodes to form the vesicles on-chip by electroformation and fused these by an electric pulse. This system offers no control over the number of vesicles that are fused and delivered images of unsatisfying image quality due to the lipid-coated electrodes. Overall the precise control over the position and number of the vesicles that are involved electrofusion in an easy to use device is lacking. Besides the fusion of vesicles microfluidic devices with electrodes were also used to investigate electroporation and fusion of droplets.

1.4.3 Lipid vesicles as a model system for cells

Vesicles are investigated in fields of biomimetic chemistry and biomembrane physics. Their cell-like structure makes them particular useful for the creation and investigation of artificial cells, in the spirit of the famous quote by Richard P. Feynman:

“What I cannot create, I do not understand.”

Vesicles are used to build artificial cell systems that can help to gain insights in cellular processes, which otherwise can not be monitored without interference by other reaction in the cell. GUVs with a similar size compared to cells have been used to study cellular processes and properties like vesicle fusion, vesicle fission, membrane permeability, effects of the cytoskeleton and for gene expression in vesicles. For a review about the application of GUVs see reference. A large number of preparation techniques can be used to create GUVs (see section 2.2). Other cell-mimicking systems are the MVVs which have a cell-like hierarchical inner structure. Therefore, they are suited to recreate the cell with its organelles and to study the effects of compartmentalization, like accumulation of substances, that the organelles can cause. Apart from their applications as cell model systems, MVV were proposed as drug delivery systems and as multi-compartment micro-reactors.

For the investigation of such model systems by microscopy the positioning is important. A particular challenge is the typically large polydispersity and deformability
of the vesicles. Placement and immobilization of vesicles can for example be done by pipetting, micromanipulation[99] or optical trapping[114]. These systems usually require a skilled operator and offer only a low throughput. Microfluidic systems have the ability to precisely control small amounts of liquid. This can be used to control the surrounding media of the vesicles, for example to initiate reactions.[115] For the placement of vesicles in microfluidic systems optical[99] or electrical[100, 101] techniques are known. Immobilization in such systems can also be achieved by surface modification techniques.[116] Stamou et al. presented a system where the surface is first coated with biotinylated BSA. In a second step a cholesterol-PEG-biotin linker attaches the vesicles to the surface. The hydrophobic cholesterol integrates into the membrane and the biotin is bound to biotinylated BSA by an avidin linker. The resulting surface is covered with bound vesicles. GUVs can also be physically confined for analysis.[19] Here, two small post with a gap in between are located inside the channels and hold the vesicles. For the previously described electrofusion systems these current positioning systems are not sufficient as the either are laborious, lack control over the number of vesicles or are only able to capture single vesicles in place. However, for droplets[16, 42] and cells[117] physical trapping arrays for pairing are known. Typically, specialized post arrays are used to capture two or more vesicles.
1.5 Scope of the thesis

This thesis focuses on the development of microfluidic devices for the manipulation and analysis of droplets and vesicles. Custom made platforms for controllable electrofusion, droplet-based cell models and ICPMS introduction are presented. In the following sections these three specific aims are listed and the main challenges of the overall thesis are given.

Understanding and controlling vesicle fusion is important for many natural processes. Fusion of natural vesicular systems can be found, for example, in cell-to-cell communication, fertilization or virus infections of cells. To study this fusion processes in detail, artificial vesicles are a convenient model system. However, suitable platforms to induce fusion of these artificial vesicles in a controlled environment and at a desired time point are laborious. Microfluidics is a suitable technique which is capable of handling these small compartments in a straightforward way. Additionally, a microfluidic device can be equipped with microelectrodes to induce electrofusion of vesicles.

The aim of the project, presented in chapter 3, is the development of a microfluidic platform for the investigation of vesicle fusion. Its main goal is to achieve spacial and temporal control over the fusion of GUVs in a straightforward setup, which enables the observation of lipid mixing and reaction kinetics in fused vesicles. In order to achieve spacial control over the fusion events multiple GUVs have to be trapped and isolated in a post array. For initiating the fusions at a desired point in time electrofusion is employed. Microelectrodes were integrated to direct the required voltage pulse to the vesicle. Here, a particular challenge was the optimization of the electrode design and the parameters of the voltage pulse. Both parameters have a strong influence on the electric field, which has to be tightly controlled for a successful fusion of the vesicles. Additionally a ring shaped valve was integrated around each trap that isolates the GUVs allowing to create a defined microenvironment in which the fusion takes place. To increase throughput the system is designed in a modular way for multiple parallel and consecutive electrofusions in a single experiment. The device was designed to study dynamics of lipid mixing with a FRET assay. Furthermore, complexation reactions initiated by electrofusion of two GUVs demonstrate the capabilities for reaction monitoring.
1. **General introduction**

Lipid membranes are not only the outer shell of a cell but also their internal structure is compartmentalized into smaller vesicular units. Many of these cell organelles have important tasks, such as storage or degradation of material, as well as energy production. Artificial model systems for this compartmentalized structure can be built with lipid vesicles. Especially the MVVs are suitable to mimic the inner compartmentalization of cells, but the controlled formation of MVVs is challenging.

In chapter 4 the development of multivesicular droplets (MVDs) is described. The aim is a straightforward competitive droplet based cell model systems to investigate compartmentalization. The MVDs combine droplet microfluidics with conventional GUV preparation techniques for a convenient production. They consist of multiple GUVs which are encapsulated in monodisperse aqueous droplets in a perfluorinated carrier phase containing a biocompatible surfactant. In order to generate and analyze the MVDs, a microfluidic platform is developed. A particular challenge is the analysis of MVDs which are generated in a stream of the carrier phase. To overcome the issues associated with the continuous movement of the MVDs high-speed microscopy is employed used to monitor the formation of MVDs. For a detailed bright field or fluorescence inspection of the MVDs a trapping array is adapted. The enrichment compounds in compartmentalized system is investigated with membrane-staining and an enzyme cascade.

Sample introduction systems are a crucial component of every ICPMS setup. However, conventional systems, such as nebulizers, which create a polydisperse aerosol of the sample, suffer from a high sample consumption and an incomplete sample transport. Their small nozzles also bare a high risk of clogging, when concentrated salt solutions or undigested biological fluids have to be analyzed. An alternative approach is the single-droplet introduction by piezo-electric or thermal inkjet droplet dispensers. Although these systems are promising for the efficient introduction of small sample volumes as monodisperse droplets, they are prone to clogging and are difficult to clean especially when nanoparticles or biological samples are processed. Moreover, these rather expensive devices are also limited to a fixed volume in terms of droplet size.

The aim of the project, described in chapter 5, is to build an improved introduction system for ICPMS. A particular focus is the introduction of small sample volumes and undigested biological samples. Also the system should supply the sample inform of monodispers droplets of adjustable size and frequency. In order to meet these requirements a novel droplet microfluidic-based sample introduction system is developed. It consists of a chip which generates monodisperse droplets of an aqueous sample in a stream of PFH by flow focusing. Here droplets of various size and frequency can be generated. A major challenge was the development of a technique to transfer the droplets.
from the chip to the plasma. In a first step the droplets are ejected from the chip in a PFH jet. The intact aqueous droplets are then dried and transported to the ICP by a custom-built desolvation setup, which removes the PFH as vapor before the dried residues enter the plasma. In addition the fabrication of this chip was a challenge as the interface between chip and transport system has to be airtight. Replica moulding with special casting forms enables manufacturing of these round, cheap and disposable chips, which can be mounted in a socket of the transport system. Moreover since the chips are only used a single time it also eliminates the need for cleaning. As demonstration of the capabilities the elemental analysis of solutions and cells is presented. Furthermore, the implementation of additional microfluidic modules for on-chip sample pretreatment or multiplexed sample introduction is investigated. The combination of droplet microfluidics and ICPMS offers a new label-free way of analyzing droplets. Compared to the conventional optical droplet detection methods, no changes in color or fluorescence, which typically require dyes, are necessary.
2 Materials and methods
2. Materials and methods

2.1 Microfabrication

The term microfabrication describes a collection of technologies to fabricate structures in the micro- to nanometer range. One of the most important technologies among them is photolithography. It is a patterning method that uses ultraviolet light (UV) to transfer geometric structures from a photomask to a light-sensitive substance (photore sist). In the following sections, the employed microfabrication processes are explained in detail.

2.1.1 Photolithography

Before the beginning of the photolithography process, the desired design is drawn with a computer-aided design (CAD) program. This design is used to print out a photomask. For resolutions down to 5 µm, a polyester-based film coated with a dense photographic emulsion is printed. Higher resolutions can be achieved on soda lime or quartz substrates coated with an opaque chrome layer. Fabrication of these masks is typically done by commercial providers.

In order to avoid particle contaminations, which could interfere with the photolithography, the actual process is performed in a cleanroom. First the substrate, usually a silicon wafer, is dehydrated by heating. For some photoresists it is recommended to apply a promoter to enhance the adhesion to the substrate. The prepared substrate is placed on the chuck of a spin coater. A vacuum retains the substrate and photoresist is poured on it. The spin coater rotates the substrate at high speeds and the centrifugal force exerted on the photoresist causes it to spread. Determined by the viscosity of the photoresist and the spin speed, a uniform resist layer with a certain height is formed. After a soft bake to reduce the solvent amount in the photoresist film, the substrate is loaded into a mask aligner for UV exposure. A mask aligner consists of a vacuum chuck for the substrate, a photomask holder, an UV light source and a microscope. As source for the UV light commonly mercury-vapor lamps with filter for the I-line (365.4 nm) and H-line (404.7 nm) are used. The relative x-y-position of the chuck with respect to the mask holder is adjustable and can be used to align mask and substrate. The positioning can be checked with the microscope. Before the exposure, mask and substrate are brought into close contact to reduce scattering artifacts. Next, the coated substrate is exposed through the photomask for a certain time. Some photoresist require a post exposure baking step, before unpolymerized resist is removed in bath of development solution.
2.1 Microfabrication

Depending on the type of photoresist used either the unexposed areas (positive photoresist) or the exposed areas (negative photoresist) of the photoresist remain on the substrate. An image reversal photoresist can be processed as either positive or negative resist. The reversal from positive to negative behavior is typically initiated by an image reversal bake and a subsequent flood exposure. This type of resist allows to create sloped photoresist sidewall to improve lift-off (see chapter 2.1.4 for details about this kind of process).

2.1.2 Fabrication of SU-8 silicon master wafers

The fabrication of SU-8 silicon master wafers for PDMS replica molding was performed in the FIRST cleanroom facility at ETH Zurich. Figure 2.1 shows an illustration of all fabrication steps. The processing parameters for various feature heights are listed in Table 2.1. As an example, the production of wafers with a height of 20 µm is described.

![Schematic of SU-8 processing](image)

Figure 2.1: Schematic of SU-8 processing. First the wafer is dehydrated and spin coated with SU-8 photoresist. After a soft bake UV light and a photomask are used to transfer the pattern of the microfluidic features. A postexposure bake is performed and the photoresist is developed by soaking the wafer in developer. As a final step the wafer is hard baked.

First a polished 100 mm diameter silicon wafer (Si-Mat, Kaufering, Germany) was dehydrated for 10 min at 200 °C. A layer of SU-8 2015 (micro resist technology, Berlin, Germany) was spin coated for 30 s at 1750 rpm. After a soft bake for 240 s at 95 °C, the wafer was exposed (160 mJ cm⁻² measured at 365 nm) on a mask aligner (MA-6 mask aligner, Süss MicroTec, Garching, Germany). Therefore, the transparency photomask (Micro Lithography Services, South Woodham Ferrers, United Kingdom) was attached to soda lime glass. The exposed parts of the SU-8 start to polymerize. To promote the
2. Materials and methods

Table 2.1: List of SU-8 process parameters.\[118, 119\]

<table>
<thead>
<tr>
<th>Thickness [µm]</th>
<th>Photoresist</th>
<th>Spin speed [rpm]</th>
<th>Soft bake at 65 °C [s]</th>
<th>Exposure at 65 °C [mJ cm(^{-2})]</th>
<th>Post exposure bake at 95 °C [s]</th>
<th>Development [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>SU-8 2002</td>
<td>2000</td>
<td>-</td>
<td>60</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>SU-8 2005</td>
<td>2000</td>
<td>-</td>
<td>120</td>
<td>110</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>SU-8 2010</td>
<td>2500</td>
<td>-</td>
<td>180</td>
<td>130</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>SU-8 2015</td>
<td>2750</td>
<td>-</td>
<td>210</td>
<td>140</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>SU-8 2015</td>
<td>1750</td>
<td>-</td>
<td>240</td>
<td>160</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>SU-8 2025</td>
<td>2100</td>
<td>150</td>
<td>360</td>
<td>158</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>SU-8 2050</td>
<td>3250</td>
<td>180</td>
<td>360</td>
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<tr>
<td>50</td>
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<tr>
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<td>510</td>
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<tr>
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<td>300</td>
<td>600</td>
<td>215</td>
<td>120</td>
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<tr>
<td>80</td>
<td>SU-8 2050</td>
<td>1750</td>
<td>300</td>
<td>960</td>
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</tr>
<tr>
<td>90</td>
<td>SU-8 2050</td>
<td>1600</td>
<td>300</td>
<td>1200</td>
<td>240</td>
<td>300</td>
</tr>
<tr>
<td>100</td>
<td>SU-8 2050</td>
<td>1500</td>
<td>300</td>
<td>1260</td>
<td>245</td>
<td>300</td>
</tr>
</tbody>
</table>

polymerization process, a post-exposure bake of 300 s at 95 °C was conducted. To remove the non-polymerized photoresist from the wafer it was developed for 240 s in mr-Dev 600 developer (micro resist technology, Berlin, Germany). The subsequent hard bake was performed for 2 h to evaporate remaining solvent. Finally, the wafer was 12 h exposed to a 1\(H\),1\(H\),2\(H\),2\(H\)-Perfluorodecyltrichlorosilane (ABCR-Chemicals, Karlsruhe, Germany) atmosphere at 100 mbar, to avoid adhesion of PDMS. An image of a finished SU-8 silicon master wafer can be seen in Figure 2.2.

2.1.3 PDMS replica molding

The fabrication of microfluidic chips out of silicon or glass is associated with high material and processing costs. In the beginning this hinderance limited the widespread use of microfluidics. In 1998 David C. Duffy et al. introduced a replica moulding technique to overcome this limitation.\[120\] It requires only a single master wafer to be fabricated in a cleanroom. All other process steps can be performed in a regular laboratory. The material used for the replica moulding is Poly(dimethylsiloxane) (PDMS), a low cost hydrophobic heat curable elastomer. The surface of PDMS can be modified by various
Figure 2.2: Silicon wafer with patterned SU-8 features. The wafer diameter is 100 mm.

ways. In addition, it is optically transparent, gas permeable as well as biocompatible. All steps of the fabrication of microfluidic devices by replica molding are depicted in Figure 2.3. In the next paragraph the fabrication process is described in detail.

![PDMS replica molding diagram](image)

Figure 2.3: PDMS replica molding. In a first step the mixed and degassed PDMS is poured on a silicon master wafer. After curing by heat the PDMS is peeled from the wafer and connection holes are punched. Finally, the PDMS is bonded to a glass slide or a flat PDMS slab.

First, PDMS prepolymer was mixed with PDMS curing agent in a ratio of 10:1 (Sylgard 184 silicone elastomer kit, Dow Corning, Michigan, U.S.A.). The mixture was degassed in a desiccator for approximately 20 min, until all gas bubbles were removed. Next, an aluminum or a Polytetrafluoroethylene (PTFE) casting form was placed on the previously prepared master wafer (see chapter 2.1.2). The degassed PDMS was poured
2. **Materials and methods**

into the casting mold on top of the wafer and cured for 7 min on a hot plate at 150 °C or for 2 h in an oven at 80 °C. Subsequently, the casting form was disassembled and connection holes were punched in the PDMS slab with a 1 mm or 1.5 mm biopsy puncher (Miltex, Pennsylvania, U.S.A.). The result of this molding process are microfluidic channels which are open on one side. They have to be sealed in the next step. One method of sealing the channels is by plasma bonding the PDMS to a glass slide. For this purpose, the PDMS and glass slide were activated in a plasma cleaner (Harrick Plasma, Ithaca, U.S.A.) for 45 s. The plasma induces the formation of hydroxyl groups on the surface of the glass and PDMS, which can covalently bind the glass to PDMS.[121] Alternatively, adhesive bonding of the PDMS to another flat PDMS slab can be used to seal the channels.[122] Here a thin layer of the PDMS curing agent was spin coated on a flat silicon wafer. Then the thin layer of the curing agent was transferred onto the structured PDMS slab and brought into contact with a flat PDMS slab. The small amount of curing agent between the surfaces irreversibly binds the PDMS parts together (see chapter 7.1.3 for details). For images of finished microfluidic devices see Figures 3.2, 4.1 and 5.6.

### 2.1.4 Metallization of glass slides

Glass slides were patterned with metal to form electrodes which can be used to initiate GUVs electrofusion events. The fabrication process steps are depicted in Figure 2.4. The technique was a so-called lift-off process. First a sacrificial layer of photoresist is applied and patterned by photolithography. This layer serves as a mask during the metal deposition by physical vapour deposition. Lastly the surplus metal is removed by dissolving the sacrificial layer. Only in the desired areas, the metal layer remains.

First, the glass coverslips (24 × 40 mm, no. 3, Menzel Gläser, Braunschweig, Germany) were thoroughly cleaned and dehydrated on a hot plate for 10 min at 200 °C. Next, a layer of AZ5214E image reversal photoresist was spin coated onto the glass slides for 30 s at 4000 rpm. After a soft bake for 1 min at 100 °C the coated slides were exposed on a MA 6 mask aligner (Süss MicroTec, Garching, Germany) with 75 mJ cm\(^{-2}\) measured at 405 nm. For this purpose, a transparency photomask (Micro Lithography Services, South Woodham Ferrers, United Kingdom) fixed to a soda lime glass was used. Subsequently, an image reversal bake (100 s at 120 °C) and a flood exposure (300 mJ cm\(^{-2}\) measured at 405 nm) on a MA 6 mask aligner was conducted. The coverslips were developed in AZ726 MIF developer (AZ electronic materials, Wiesbaden, Germany) for 25 s. On the glass slides, only the sacrificial photoresist layer with an undercut for lift-off remains. In the following, physical vapor deposition (Plassys II, Plassys-Bestek, Marolles-en-Hurepoix,
2.1 Microfabrication

Figure 2.4: Schematic of the microelectrode fabrication on glass slides. Glass slides are dehydrated, spin coated with AZ 5214E image reversal photoresist and soft baked. The design of the microelectrodes is transferred using ultraviolet light and a photomask. After a flood exposure the resist is developed and a layer of chromium and platinum is deposited. In a last step remover is used to lift-off the sacrificial layer exposing the microelectrodes. France) of a 10 nm chromium adhesion layer and a 100 nm platinum layer was carried out. Excess metal was removed by placing the glass slides in an ethanol remover bath (lift-off). The sacrificial photoresist layer was dissolved and only the patterned electrodes remain on the coverslips. A resulting glass slide with electrodes is shown in Figure 2.5.

Figure 2.5: Glass cover slip with platinum microelectrodes. The dimensions of the glass slide are 24 mm × 40 mm.
2. Materials and methods

2.2 GUV preparation

For vesicles with diameters larger than 1 µm (GUVs), a large variety of preparation techniques is available. These techniques include for example, the lipid-film hydration process[123], the ether injection method[124], the reverse phase evaporation[125], the dialysis based formation[126], the freeze-and-thaw procedure[127, 128] and the formation from planar lipid bilayers[72]. In the last years also several microfluidic based techniques have been reported.[65, 129, 130] For a review on GUV preparation techniques see [79]. The methods used in this thesis are electroformation and the emulsion transfer method, which are described in detail in the next sections.

2.2.1 Electroformation of GUVs

The electroformation of vesicles was introduced by Miglena I. Angelova and Dimiter S. Dimitrov.[131, 132]. Here, a dried lipid film is rehydrated in the presence of an oscillating electrical field. The separation and bending of the lipid bilayer is influenced by the electrical field (duration, frequency, amplitude and current). Figure 2.6 shows a scheme of the electroformation process.

![Figure 2.6: Scheme of the vesicle preparation by electroformation.](image)

First, a 1 mM solution of 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, U.S.A.) in a chloroform/methanol mixture (9:1 v/v) was prepared. A 1.5 mm thick silicone rubber spacer with 12 holes was placed on top of an indium tin oxide (ITO) coated glass slide (15 – 25 Ω sq−1, Sigma-Aldrich, St. Louis, U.S.A.). Subsequently, 2.5 µL of the lipid solution were deposited in each of the 12 wells and dried overnight under vacuum. Ultra pure water was added to the each well and the top sealed with
another ITO glass slide. This assembly was placed in a custom made holder (see Figure 2.7). With an arbitrary function generator (HMF2525, Hameg, Mainhausen, Germany) 1 V at 10 Hz for 4 h and 1.5 V at 3 Hz for 30 min were applied to form the GUVs. Finally, the device was disassembled and the vesicle suspensions were removed from the wells with a pipet.

Figure 2.7: Image of the assembled device for the electroformation of GUVs.

2.2.2 GUV formation by the emulsion transfer method

The formation of GUVs by the water in oil emulsion transfer method was introduced by Pautot et al.[133, 134] Intravesicular solution (IS) is dispersed in a squalene or mineral oil based phospholipid solution (PS). This droplet suspension is overlaid on top of a hosting solution (HS). Around the droplets and at the interface to the HS, the amphiphilic lipids form a layer. For the next step, it is necessary that the IS has a lower density than the HS. Centrifugation is used to transfer the droplets from the PS to the HS and thereby creating a lipid bilayer around the droplet. A scheme of the GUV formation by the emulsion transfer method is shown in Figure 2.8. This method is especially suited for the formation of GUVs with asymmetrical membranes and the loading with various compounds. However, the density of the IS and HS have to be sufficiently different and it is possible that parts of the oil remain in the membrane.

As a first step, a solution of 200 mM POPC (Avanti Polar Lipids, Alabaster, U.S.A.) in mineral oil (Sigma-Aldrich, St. Louis, U.S.A.) was prepared (PS). All following preparation steps were performed in 1.5 mL polypropylene micro tubes (Sarstedt, Nümbrecht,
2. Materials and methods

Figure 2.8: Scheme of the vesicle preparation by the water/oil emulsion transfer method. Droplets of an intravesicular solution (IS) in a phospholipid solution (PS) are forced by centrifugal force into a hosting solution (HS). This transfer forms a lipid bilayer around the droplet.

Germany). In one micro tube, 500 µL of an aqueous 1 Osmol L\(^{-1}\) glucose (Acros organics, Geel, Belgium) solution was overlaid with 200 µL PS and incubated for 10 min. In another micro tube 500 µL PS and 50 µL of an 1 Osmol L\(^{-1}\) sucrose (Acros organics, Geel, Belgium) solution were mechanically dispersed. Subsequently, 500 µL of the suspension were transferred to the other micro tube and centrifuged for 3 min at 1500 \(\times g\). Excess solution was removed with an aspirator, leaving only the pellet of the resulting GUVs behind. The vesicles were redispersed with 100 µL of the aqueous 1 Osmol L\(^{-1}\) glucose solution and centrifuged again with the previous settings. Again the excess solution was removed and the resulting vesicles were redispersed in 50 µL of the aqueous 1 Osmol L\(^{-1}\) glucose solution.

2.3 Fluorescence microscopy

Fluorescence microscopy is an imaging technique which is widely used to investigate biological and biomimetic samples, such as vesicles.[135] The technique can equally well be used for the detection and analysis of droplets.[38, 136] It is based on the abil-
ity of certain molecules or nanostructures to absorb light of a specific wavelength range and emit light of longer wavelengths. This effect is called Stokes shift named after Sir George G. Stokes. Fluorescent molecules typically contain large $\pi$-electron systems, such as heterocycles or polyaromatic hydrocarbons. They are referred to as fluorophores or fluorescent dyes. When the fluorophores are equipped with the ability to bind to a specific target molecule or to react on a stimulus they are called fluorescent probes. Such a probe can be used to monitor for example the membrane of a GUV or a reaction in a droplet. The fundamental principles of fluorescence are discussed in the next chapter.

2.3.1 Fluorescence fundamentals

All steps of the fluorescence process can be illustrated in the so called Jablonski diagram (see Figure 2.9).[137] Absorption (A) of a photon of sufficient energy by a fluorophore takes femtoseconds. The fluorophore changes its quantum state from a singlet ground state ($S_0$) to an electronically and vibrationally excited singlet state ($S_1$). In picoseconds the vibrational energy is relaxed to the lowest $S_1$ by non-radiative vibrational relaxation (VR). Further relaxation back to $S_0$ ground state can happen by several competing pathways. One pathway is the spin-allowed emission of a photon called fluorescence (F). Typical fluorescence life times are in the order of nanoseconds. Also the radiation-less internal conversion (IC) and subsequent vibrational relaxation can result in the $S_0$ state. Another relaxation pathway is the phosphorescence (P). By intersystem crossing (ISC) the system can be transferred to a vibrational excited triplet state ($T_1$). After vibrational relaxation, the relaxation back to the $S_0$ ground state by emission of a photon is a spin-forbidden process. Thus lifetimes are typically in the millisecond range.

2.3.2 Förster resonance energy transfer

Förster resonance energy transfer (FRET) is a specific kind of non radiative energy transfer named after Theodor Förster.[138] It is an important research tool in biology and chemistry and can be used to gain information about the distance of two different fluorophores. FRET is sensitive in the range of $0.5 – 10 \text{ nm}$. [139]

A Jablonski-diagram of the FRET process is depicted in Figure 2.10. First the donor fluorophore is excited to a vibrationally excited $S_1$ and undergoes vibronic relaxation to the $S_1$ ground state. Apart from relaxing to the $S_0$ ground state by emitting fluorescence, the energy can be transferred in a non-radiative way by dipole-dipole interactions to an acceptor fluorophore in close proximity. Thereby, the acceptor is excited to a vibrationally
2. Materials and methods

Figure 2.9: **Jablonski-diagram**: (A) Absorption, (F) fluorescence, (IC) internal conversion, (ISC) intersystem crossing, (P) phosphorescence and (VR) vibrational relaxation.

excited $S_1$ state. After non radiative vibronic relaxation, it can emit fluorescence with a longer wavelength.

The efficiency of the energy transfer is determined by the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum. Apart from the relative orientation of the donor and acceptor, the distance is the substantial parameter for the FRET efficiency. It can be calculated by the following formula:

$$k_{ET} = \frac{R_0}{r_D \cdot \tau_D}$$  \hspace{1cm} (2.1)

Where $k_{ET}$ is the rate of energy transfer, $R_0$ the Förster distance (distance at which the energy transfer efficiency is 50 %), $\tau_D$ fluorescence lifetime of the donor molecule and $r$ distance between the donor and acceptor.

In a typical FRET experiment, the sample is exited with wavelengths that excite the donor and not or only weakly excite acceptor. The intensity of the fluorescence light emitted by the donor is detected and used gain to information about the distance between
the two probes. Alternatively, the dequenching of the acceptor probe can be measured as increase in acceptor fluorescence intensity.

![Jablonski diagram for the FRET process](image)

**Figure 2.10**: Jablonski diagram for the FRET process: (A) Absorption, (F) fluorescence and (VR) vibrational relaxation.

### 2.3.3 Instrumentation

The common instrument for fluorescence microscopy is the wide-field epifluorescence microscope. For this microscope setup the excitation light which illuminates the sample and the emission light pass through the same objective. A scheme of the beam path can be seen in Figure 2.11.

Mercury-vapor lamps, Xenon arc lamps, light-emitting diodes (LEDs) or Laser can be used as excitation light sources. Optical filters are used to filter out unwanted wavelengths that the light source might generates, before the light is directed to the sample by a dichroic mirror. The fluorescence light emitted from the sample passes the dichroic mirror and is subsequently filtered again to remove all wavelengths except those of the fluorescence of the sample. Detection of the emitted light can be done by imaging sensors (cameras) like the complementary metal-oxide-semiconductor (CMOS), the charge-coupled device (CCD) or the more sensitive electron multiplying charge-coupled device (EMCCD) sensors. Moreover, photomultiplier tubes (PMTs) or avalanche photodiodes (APDs) can be used as detectors.
2. Materials and methods

Figure 2.11: Schematic of an epifluorescence microscope. Not to scale. Adapted from [56].

A better spacial resolution (especially in z-direction) can be achieved by confocal microscopes. Point illumination and a pinhole (for the detector) in the focal plane are used to eliminate out of focus light, thereby only the fluorescent very close to the focal plane is detected. In this thesis a confocal Laser scanning microscope was used for the inspection of GUVs. Here, multiple electronically controlled mirrors are used to scan the sample with a Laser beam and descan the resulting fluorescence. The Laser and detector pinhole are fixed in position.

2.4 Mass spectrometry

Mass spectrometry (MS) is a method to determine the mass of an atom or molecule by measuring the mass to charge ratio m/z of their gas-phase ions.[140] The principle of this technique is known since the 19th century. Based on the findings of his mentor Sir Joseph J. Thomson, it was Francis W. Aston who introduced in 1919 the first functioning mass spectrometer.[141] His device was able to identify some of the isotopes of chlorine, bromine and krypton. Today, MS can be performed for numerous solid, liquid or gas samples. For this purpose, a variety of ionization and detection methods can be used.

In the recent years, MS has gained attention as an analytical method for droplet microfluidics. Both techniques are optimized for high sample throughput. Droplet microfluidic is able to handle ultra small sample volumes while MS has the sensitivity to
2.4 Mass spectrometry

analyze such small samples. Furthermore, MS offers a label free detection of molecules and atoms which can not be performed by fluorescence measurements. The coupling of continuous droplet microfluidics to MS typically requires an interface. Such interfaces are known for the soft ionization methods electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which are commonly employed for the analysis of biomacromolecules and organic molecules. They either operate on the principle of emulsion separation[142], by spotting of droplets onto plates[143] or by directly injecting the droplets[30]. These methods must pay particular attention to the carrier phase between the droplets, as it can suppress the signal or interfere with the signal of the target analyte.

2.4.1 Introduction to inductively coupled plasma mass spectrometry

Inductively coupled plasma mass spectrometry (ICPMS) is a quantitative elemental analysis technique with a detection limit down to the part per quadrillion level and up to nine orders of magnitude dynamic range.[144] It was introduced in 1980 by Robert S. Houk et al.[145]. He was the first who used an inductively coupled plasma as ion source for a mass spectrometer. Details of the working principle will be discussed in the following sections. Nowadays ICPMS is an important routine method in analytical laboratories working in the fields of forensic[146], geological[147], environmental[148] or life sciences[148, 149]. It can be used for many gaseous, liquid or solid samples.

Two main methods are distinguished according to their type of sample introduction systems. Sample introduction techniques are necessary to transport sample (or parts of it) to the plasma. Laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) uses a Laser to ablate small portions of the sample that are carried to the plasma via a gas stream. While this introduction method is well established for the analysis of archaeological and geological samples[150] it also gained interest for biological samples[151]. Another way to introduce samples is as aerosol or small droplets formed from a liquid sample. As this technique is the introduction method used in this thesis, it will be discussed in more detail in the following section.
2. Materials and methods

2.4.2 Setup and working principle of an ICPMS for liquid samples

Figure 2.12 shows a scheme of a typical ICPMS setup for liquid sample analysis. In the subsequent section, a step-by-step description of the sample introduction and analysis is discussed.

![Scheme of an ICPMS setup for liquid samples](image)

Figure 2.12: **Scheme of an ICPMS setup for liquid samples.** The green and blue arrows indicate inlets for argon and liquid sample, respectively. The yellow labelled interface region is operated at a pressure of few 10 mPa. Ion optics, mass analyzer and detector (orange part) are typically kept a pressure of few 100 µPa.

**Sample introduction**

The sample introduction system has the task to generate fine droplets of the sample and transport these droplets into the ICP torch. Usually, pneumatic or ultrasonic nebulizer are used to generate a polydisperse aerosol. The aerosol subsequently passes a so-called spray chamber, where larger droplets are filtered out before the sample droplets are transported to the torch. Sample introduction is one of the most critical parts of the setup. Sample losses in this step have a significant impact on the performance of the whole system, as stated by Robert Thomas:

“The sample-introduction area has been called the Achilles heel of ICP-MS, because it is considered the weakest component of the instrument. Only about 2 % of the sample finds its way into the plasma, depending on the matrix and method of introducing the sample.” [144]

Therefore, many efforts to improve ICPMS are focussed on the sample introduction. Different types of nebulizers and other introduction concepts have been developed to achieve a higher sample transport efficiency (TE), such as the high-efficiency nebulizer[152], the direct injection high-efficiency nebulizer[153] or the single droplet injection techniques[154, 155].
ICP torch

The ICP torch consists of three concentric quartz tubes each equipped with a gas inlet. The outermost tube supplies the plasma gas which is typically argon to cool the torch. A stream of auxiliary gas (typically argon) fed between the middle and outer tube allows plasma formation and to change the position of the plasma. Around the torch, a radio frequency-coil (RF-coil) is located and usually operated at 27 or 40 MHz. It generates a magnetic field that sustains the plasma (initially ignited by a high voltage spark). Here, the argon is ionized by collisions with accelerated electrons and ions. In the analytical zone, the plasma has a temperature of 6000 – 7000 K. Through the central tube the sample droplets from the nebulizer enter the torch and are subjected to the high temperatures of the plasma. First, the droplets dry to a small particle. Afterwards, they are vaporized to a gaseous form, atomized and subsequently ionized by collisions with energetic argon electrons (or lesser argon ion). The positively charged ions are transported into the mass spectrometer. As a side-product, polyatomic argon adducts and other polyatomic species can be formed in the plasma.

ICP interface and ion optics

The ions formed in the plasma enter the mass spectrometer through the first vacuum interface. A sampler and skimmer cone help to transfer the ions from atmospheric to the vacuum part (few 10 mPa) containing the ion optics, mass analyzer and detector.

The following ion optics counteracts the diversion of the ion beam (Coulomb repulsion) and focuses the ion beam into the mass analyzer. Additionally, it prevents neutral species, photons and small particles from entering the mass analyzer and the detector.

Mass analyser

A mass analyzer is a device that separates ions according to their m/z. For ICPMS different kinds of mass analyzers are available, such as quadrupole, magnetic sector and time of flight systems. In this chapter only the principle of a quadrupole mass filter which was used in this thesis is explained.

A quadrupole mass analyzer consists of four cylindrical or hyperbolic metal rods, with the same lengths (typically 15 – 20 cm). The opposing rod pairs are electrically connected and a direct current (DC) of opposite polarity is applied to the pairs (see Figure...
2. Materials and methods

2.13). In addition, a RF voltage is applied between the pairs of rods. For a particular pair of RF-DC voltage, ions of a specific m/z can travel through the center of the rods in the direction of the detector (resonant ion). All other ions are deflected and can not reach the detector (non-resonant ion). By scanning one m/z after the other a full mass spectrum can be recorded.

![Scheme of a quadrupole mass analyzer.](image)

**Figure 2.13: Scheme of a quadrupole mass analyzer.**

**Ion detection**

The last step for the filtered ions is the conversion into an electronic signal corresponding to the amount of ions hitting the detector. This signal is recorded by a computer and corresponds to the relative abundance of the ions that can pass the mass filter. In the resulting spectra, the relative abundance (ordinate) is plotted against the m/z value (given by the mass filter settings).

Two detection principles can be used to achieve this conversion from ion to electronic signal. The discrete dynode electron multiplier detector and the channel electron multiplier detector use the effect that an impacting ion generates secondary electrons. These electrons are then accelerated to generate even more secondary electrons. Finally the electrons are electronically amplified and recorded. Another less sensitive method of ion detection is a Faraday cup, which is usually only used for high ion currents. It catches the charged ions and the resulting current, which is necessary to compensate their charge is measured.
3 Controllable electrofusion of lipid vesicles: Initiation and analysis of reactions within biomimetic containers

Adapted from T. Robinson#, P. E. Verboket#, K. Eyer, and P. S. Dittrich: Controllable electrofusion of lipid vesicles: Initiation and analysis of reactions within biomimetic containers, Lab Chip 14 (2014) 2852-2859. with permission from the Royal Society of Chemistry. #: These authors contributed equally to this work. Link to the article: http://pubs.rsc.org/en/content/articlelanding/2014/lc/c4lc00460d P.E.V., T.R. and P.S.D. designed the research. P.E.V. and T.R. performed the research and analyzed the data. K.E. conducted bulk fluorescence spectroscopy experiments and provided cells. All authors discussed the results and approved the manuscript.
3. **Controllable electrofusion of lipid vesicles**

3.1 **Abstract**

We present a microfluidic device that is able to trap multiple giant unilamellar vesicles (GUVs) and initiate electrofusion via integrated microelectrodes. PDMS posts were designed to trap and isolate two or more vesicles. Electrodes patterned onto the glass surface of the microchannels are able to apply a short, high voltage pulse across the traps for controllable electrofusion of the GUVs. The entire array of traps and electrodes are designed such that an average of 60 individual fusion experiments can be performed on-chip. An assay based on Förster resonance energy transfer (FRET) is performed to show successful lipid mixing. Not only can the device be used to record the dynamics of lipid membrane fusion, but it can be used for reaction monitoring by fusing GUVs containing reactants. We demonstrate this by fusing vesicles encapsulating femtolitre volumes of cobalt chloride or EDTA and monitoring the amount of the complexation product over time.

3.2 **Introduction**

The process of biological membrane fusion is vital for life. It allows transport of hydrophilic molecules across cell or organelle membranes and is therefore important for cell-to-cell communication as well as intracellular signalling pathways.[156] Other biological processes such as fertilisation,[157] tumourigenesis,[158] and viral infection of cells[159, 160] also depend on membrane fusion. Despite its importance in the development of many different organisms, very little is known about its underlying mechanism.[161] Studying the mechanism of how two membranes fuse together is therefore essential to understanding cellular function and will lead to the development of therapeutic approaches which could interfere with the fusion process.[162] In particular, cell-to-cell fusion has proven useful for membrane research,[163] and has been instrumental in genetic engineering.[164] Spontaneous fusion is prevented in biological membranes because of the large energetic barriers (i.e. hydration, electrostatic, and steric repulsions).[83] In order to overcome these hurdles, cells utilise membrane proteins such as the SNARE protein family which bridge the gap between the membranes.[165] *In vitro*, this can be achieved by the addition of chemicals or by the addition of fusogenic peptides derived from viruses.[86]

Besides the importance of membrane fusion in natural systems, the fusion of lipid membranes is vital for the construction of artificial biomimetic systems. Vesicles prepared from synthetic phospholipids serve as simple microcompartments that mimic the cellular
dimensions. Many biochemical reactions such as cell-free protein expression have been performed in the lumen of lipid vesicles under conditions that are present in a living cell.[79] In this respect, fusion of vesicles is one way to initiate reactions and study the distribution of reactants and products within the lumen. Therefore, understanding and controlling the fusion of model membranes provides the intriguing prospect to build up complex artificial cells. It enables the introduction of new compounds into the lumen or the membrane of an existing, simpler model cell at a defined time and thereby mimics an essential process of the cell.

In the past, lipid vesicles have been extensively used to investigate factors affecting fusion, including mechanical stress,[166] cations,[167] membrane proteins,[168] peptides,[169] and polymers.[170] Another possibility to fuse lipid membranes is the application of an electric field. This so-called electrofusion was first demonstrated in the early 1980s by Zimmerman and co-workers where they showed that an electric field could be used to initiate cell-cell fusion.[88, 163, 171] When subjected to a high voltage pulse, the membranes experience electropermeabilisation which is followed by fusion. Not only can an electric field be used as a means to study membrane fusion, but it can also be used to introduce drugs or DNA into cells without altering the cell’s integrity.[88, 89] The first electrofusion studies involving model membranes were performed using giant unilamellar vesicles (GUVs) consisting of polymerisable lipids.[92] The advantage of using GUVs, which are similar in size and curvature to cells, is that they can be observed by light microscopy to monitor both lipid and content mixing. To increase the efficiency of the electrofusion, two GUVs can be brought into contact using micropipette aspiration.[93, 94] This technique has been used to study the mechanics of membrane fusion in great detail.[95, 96] While this is effective for observing the dynamics of membrane fusion, using micropipettes has limited throughput and due to the bulk environment, the precise electric field strengths are unknown. Moreover, the increased membrane tension that micropipette aspiration requires, may not be appropriate.

Microfluidic systems are well-suited to membrane fusion studies due to the unique ability to control the environment.[97] Electrofusion within a micro-environment has been implemented using a combination of optical traps to manipulate vesicles and electrodes attached to micromanipulators.[99] The drawbacks of this method are the need for expensive external equipment (micromanipulators, optical traps) and only single experiments can be performed at a time. Takeuchi et al. demonstrated that integrated electrodes can be used to trigger GUV fusion.[100, 101] With this approach, a continuous electric field is used to spatially confine liposomes before fusion. The disadvantage of this method is that all fusion events are initiated simultaneously and there is little control over the number of confined vesicles. More recently, electrofusion has been demonstrated in a micro-device...
where electrodes make up the channel walls.[102] Here, GUVs are produced by on-chip electroformation and are therefore attached to the electrodes before and during the fusion events. This approach, however, does not offer precise control over the number of GUVs involved in the fusion event and severely limits the quality of the images due to the presence of the lipid coated electrodes. Estes and co-workers have shown that a device fabricated from transparent indium tin oxide (ITO) electrodes can be used to create a 2-D network of GUVs inside microchannels.[98] There is no need to spatially manipulate the vesicles as they are already densely packed together, but the fusion probability after addition of fusogenic agents was low (∼10%). Other applications of electric fields in microfluidic devices include electroporation studies[90, 103, 104] and water-in-oil droplet fusion.[42, 105]

Giant vesicles are routinely produced by electroformation yielding unilamellar vesicles with diameters between ∼1 and 50 µm. This large polydispersity together with a large deformability make it more difficult to capture them compared to cells or monodisperse water-in-oil droplets. Recently we have shown that microfluidic devices with PDMS posts can be used to trap and isolate single vesicles in order to perform experiments with a controlled chemical condition.[19, 115] Here, we designed posts to trap two GUVs with a specific orientation to each other to facilitate contact of the membranes. The entire chip consists of an array of chambers to allow multiple repeat measurements using the same experimental conditions. Microelectrodes patterned onto the glass surface adjacent to the traps are then used to apply a short electric pulse and hence induce electrofusion. While the electrodes are in close proximity, they are not in contact with the GUVs, which allows us to perform high-resolution confocal microscopy during the experiments.

Successful electrofusion is demonstrated at the membrane by means of a lipid mixing assay based on Förster resonance energy transfer (FRET). Additionally, we use a complexation reaction, initiated by fusion of two GUVs filled the respective reactants, to show the capability of the device for reaction monitoring without content loss.

3.3 Experimental

3.3.1 Design and function of the chip

Fig. 3.1 illustrates the loading of the GUVs. The sizes of the GUVs are not homogeneous after electroformation and typically vary between 1 – 50 µm. Vesicles much larger than 20 µm (height of the channel) do not enter the microchannels, and small vesicles be-
3.3 Experimental

Figure 3.1: **Scheme of trapping sequence and electrofusion.** (a) A vesicle fusion trap with integrated electrodes in a sealable microchamber. (b) Without vesicles, fluid flow is allowed through the gaps of the PDMS posts. (c) The first vesicle trapped occupies the rear of the trap and blocks the central passage from flow. (d) Shows the situation when a second vesicle of equal size enters and the flow is diverted in front of the trap. When a second smaller vesicle is trapped as shown in (e) other vesicles are allowed to enter. (f) Electrofusion is then performed. Blue arrows indicate the fluid flow lines.

Low 8 µm can escape between the posts and are therefore not trapped. We consider it ideal when two GUVs of equal size are sequentially trapped and occupy the entire trap such that no further vesicles are allowed to enter due to diversion of the fluid flow (Fig. 3.1d). In case the second GUV is smaller, additional vesicles are allowed to enter the trap. It should be noted that either situation can be used to study fusion events as the interface is position perpendicular to the electric-field. Once all traps are loaded, circular ring valves in each chamber can be hydraulically actuated to isolate each trap and prevent shear stress on the GUVs.

Fig. 3.2 shows the final electrofusion device consisting of a glass coverslip with patterned electrodes, a lower PDMS layer for fluidic delivery of the GUVs, and an upper PDMS layer for the control of valves. The whole device is held together by a custom-built clamp, which also serves to connect the electrodes to an external pulse generator (See Fig. 3.3). Because we do not bond the PDMS layer to the glass, we can reuse the patterned coverslip lowering the cost of the experiment.

The entire channel network is made of 108 chambers separated by 12 rows all connected to the same inlet and outlet (Fig. 3.4). The inlet contains a filter design to prevent unwanted particles blocking the traps or channels. Each chamber contains a PDMS trap with a cathode and anode positioned either side, separated by 50 µm. In order to suc-
3. **Controllable electrofusion of lipid vesicles**

![Diagram of electrofusion device](image)

**Figure 3.2: Electrofusion device.** (a) A schematic of the different components. (b) A photograph of the final device with red and blue food dyes in the control and fluid layers respectively. A one Swiss Franc coin is shown for scale.

![Diagram of custom build clamping holder](image)

**Figure 3.3: (a) Photograph of the custom build clamping holder.** (b) Exploded view drawing of the holder (screws and nuts not shown). The microfluidic device is placed in a groove in the bottom part, which also serves as microscope insert. A flexible printed circuit board is clamped on the exposed part of patterned microelectrodes on the glass slide. It serves as interface between the microelectrodes and the programmable pulse generator. To seal the microfluidic channels a transparent top part clamps the PDMS slab to the glass slide.
cessful trap two GUVs for electrofusion, the PDMS traps were designed such that at the interface the membranes were perpendicular to the electric-field. This ensures destabilisation of the membranes at the interface and more efficient fusion. Therefore, the traps contain four posts to hydrodynamically capture multiple GUVs sequentially (Fig. 3.5 shows a scanning electron microscope image of the trap).

Figure 3.4: Scheme showing the designs of the electrodes (black), of the fluid layer (blue), and of the pressure layer (red).

Figure 3.5: Scanning electron microscope image of a PDMS trap. Scale bar 20 µm.
3. **Controllable electrofusion of lipid vesicles**

### 3.3.2 Chemical reagents

1,2-dioleoyl-

\[ \text{sn-glycer-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycer-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxa-diazol-4-yl) (NBD-DSPE) and 1,2-dimyristoyl-sn-glycer-3-phosphoethanolamine-N-}(lissamine rhodamine B sulfonyl) (Liss-Rhod-PE) were all purchased from Avanti Polar Lipids (AL, USA). Bovine serum albumin (BSA) was purchased from Sigma Aldrich. SU-8 2015 photoresist was obtained from Microchem Corp (MA, USA). AZ5214E image reversal photoresist and AZ726 MIF developer were purchased from AZ electronic materials (Germany). mr-Dev 600 developer was obtained from micro resist technology (Germany). Ethanol was obtained from Scharla (Spain) and 1H,1H,2H,2H-perfluorodecyltrichlorosilane was purchased from ABCR-Chemicals (Germany). PDMS and curing agent (Sylgard® 184) were both purchased from Dow Corning (MI, USA). Ethylenediaminetetraacetic acid (EDTA), cobalt(ii) chloride, methanol and chloroform were all purchased from Acros Organics. Calcein was obtained from Fisher Scientific AG (Wohlen, Switzerland).

### 3.3.3 Electrode fabrication

The platinum microelectrodes for the electro fusion of vesicles were fabricated on glass cover slips (24 × 40 mm, no. 3 Menzel Gläser, Germany) using conventional photolithography and subsequent physical vapour deposition. The design of the electrodes is shown in Fig. 3.4. In a first step, clean glass coverslips were dehydrated on a hotplate for 5 min at 200 °C. Subsequently, the glass coverslips were spin coated with a layer of AZ5214E image reversal photoresist at 4000 rpm for 30 s. After baking the glass coverslips for 1 min at 100 °C they were exposed with 75 mJ cm\(^{-2}\) at 405 nm on a MA 6 mask aligner (Süss MicroTec, Germany) in vacuum contact mode and with a transparency photomask (Micro Lithography Services, UK). Following a baking step for 100 s at 120 °C the glass coverslips were flood exposed with 300 mJ cm\(^{-2}\) at 405 nm on a MA 6 mask aligner. After development of the glass coverslips in AZ726 MIF developer for 25 s, a 10 nm chromium adhesion layer and a 100 nm platinum layer were deposited by physical vapour deposition (Plassys II, Plassys-Bestek, France). In a last step the surplus metal was removed from the glass coverslips by ethanol leaving behind only the patterned electrodes.

The glass slips were cleaned after usage for a fusion experiment in the following manner. The glass slip were brushed with a detergent solution and sonicated for 5 min in ethanol (USC300T, VWR, PA, USA). Afterwards they were rinsed with DI-water and dried with a nitrogen stream.
3.3 Experimental

3.3.4 Microfluidic chip fabrication

The master moulds for the preparation of the fluidic layer and pressure layer were fabricated on top of standard 100 nm diameter silicon wafers (Si-Mat, Germany). The wafers were dehydrated for 10 min at 200 °C and after, spin coated with SU-8 2015 for 30 s at 1750 rpm. After a soft bake for 240 s at 95 °C the wafers were exposed with 150 mJ cm\(^{-2}\) at 365 nm on a MA 6 mask aligner (Süss MicroTec, Germany) and with a transparency photomask (Micro Lithography Services, UK). Next, a post exposure bake was performed for 300 s at 95 °C. The wafers were then developed in mr-Dev 600 developer for 4 min exposing the 20 µm high features. Finally, the wafers were treated in a 1H,1H,2H,2H-perfluorodecyltrichlorosilane atmosphere at 100 mbar for 12 h to prevent adhesion of PDMS.

The PDMS layers were prepared by mixing oligomer and curing agent at a ratio of 10:1. For the pressure layer, the wafer was placed in a square petri dish, PDMS mixture was poured to a height of 5 mm, and cured at 80 °C for 3 h. For the fluidic layer, the mixture was spin-coated at 2000 rpm onto the wafer to a height of 40 µm and cured at 80 °C for 1 h. Holes were punched in the pressure layer PDMS using a 1 mm outer diameter biopsy puncher (Miltex, PA, USA). The layers were then bonded together, by exposure to air plasma using a custom-built chamber, alignment under a microscope, and placed for 2 h at 80 °C. Afterwards, the fluidic access holes were punched with a 1.5 mm outer diameter biopsy puncher (Miltex, PA, USA).

The assembled PDMS section was manually aligned on top of the glass coverslip with the patterned electrodes exposed to the fluidic channels. To seal the fluidic channels and to establish the electrical connection the assembled chip was placed in a custom made clamping device (see Fig. 3.3).

3.3.5 GUV preparation

GUVs are prepared using the electroformation technique[172] in a custom chamber. The lipid DOPC was dissolved in chloroform/methanol (9:1 v/v) at a concentration of 1 mM with 20 µM NBD-DSPE or Liss-Rhod-PE. Then 2.5 µL of the mixture was deposited in 12 locations on a conductive ITO slide (15 – 25 Ω sq\(^{-1}\), Sigma-Aldrich) surrounded by a 1.5 mm thick silicone rubber spacer and dried in a vacuum overnight. This was hydrated with Millipore™ filtered water, both with and without reagents, and the chambers were sealed by a second ITO slide. GUVs were formed by applying 1 V at a frequency of 10 Hz for 4 h using a function generator (HMF2525, Hameg) and subsequently detached from
the surface with 1.5 V at 3 Hz for 30 min. Collection was achieved by careful pipetting and GUVs were used on the same day.

3.3.6 Microscopy

Optically sectioned microscopy of the GUVs was performed using a confocal Laser scanning microscope (Axiovert 200M, Zeiss) with a 63×/1.4 NA oil immersion objective lens. Calcein and NBD fluorescence were recorded using a 488 nm Argon ion laser line, an HFT 488 dichroic, and a 500 – 530 nm band-pass filter. Liss-Rhod fluorescence was recorded using a 561 nm diode laser, an HFT 405/561 dichroic, and a 575 nm long-pass filter. High-speed wide-field microscopy was achieved using an inverted microscope (IX70, Olympus) equipped with a mercury lamp and a 60×/1.4 NA water objective lens. Fluorescence signals were monitored using the appropriate optical filter sets for calcein and images were recorded with an EMCCD camera (iXon Ultra 897, Andor) at a frame rate of 65.8 Hz.

3.3.7 Chip operation

The assembled chip in the camping device was connected to a syringe (Agilent, CA, USA). All fluids were flushed in at a flow rate of 15 µL min⁻¹ using a neMESYS syringe pump (cetoni, Germany). The fluid exchange in the syringe was performed with the valve of the syringe pump. First, the chip was filled with a 2 % (w/v) BSA solution in Millipore filtered water. After an incubation time of 30 min the fluid was exchanged for Millipore filtered water. Subsequently, the GUV suspension was loaded until all traps were occupied. For the GUV content mixing experiments the chip was flushed again with Millipore filtered water to remove any excess calcein in the surrounding solution.

A custom made LabView (National Instruments, TX, USA) controlled programmable pulse generator was used to supply single pulses of 1 ms in the range from 10 to 50 V. All chambers share a common ground and 12 separate channels to supply 9 chambers each. The setup allows applying voltage pulses to 9 individual chambers simultaneously across a 50 µm gap.
3.4 Results and discussions

3.4.1 Trap design and occupancy

Previously, microfluidic systems with arrays of traps have been used to trap two cells or two water-in-oil droplets.[16, 42, 117, 173] This approach, however, was not suitable for GUVs with their large size heterogeneity and deformability. First, we tested an array design and although GUVs were captured initially, they escaped easily again and could not be stably trapped over a longer time period (data not shown). Therefore, we used traps surrounded by ring-shaped valves to allow isolating the vesicles after capture thereby preventing any flow-induced movement.

We also evaluated two different trap designs, where GUVs were trapped adjacently to each other (Fig. 3.6) or one after the other in direction of the flow (Fig. 3.7). However, in the first design, the contact between two GUVs was only occasionally realized due to the large polydispersity of GUV sizes (Fig. 3.6a). Moreover, when contact was achieved, the interface was often not perpendicular to the electric field (Fig. 3.6b). In the case when contact was made with the correct orientation (Fig. 3.6c), the PDMS posts would insulate the GUVs from the electric field, as shown in a simulation performed using COMSOL 4.3b (COMSOL) (Fig. 3.8). This would require higher voltages, which would cause unwanted water hydrolysis.

![Figure 3.6](image)

**Figure 3.6: Alternative design to trap multiple GUVs adjacently.** Confocal fluorescence images (overlaid with transmitted light images) of trapping (a) two small GUVs, b) two large GUVs, and c) the ideal. The membrane of the GUVs contained Liss-Rhod-PE (orange) and lumen contained calcein (green). Scale bar: 10 µm.

Therefore, we used the traps where the GUVs are orientated towards the direction of flow and the electric field. This not only allows contact between all trapped vesicles,
Figure 3.7: **Trapping of multiple GUVs.** (a) Bright-field image of the electrodes patterned on the glass surface aligned either side of a fusion trap. Scale bar: 100 µm. Confocal fluorescence images (overlaid with transmitted light images) of b) the ideal case when two different GUVs are trapped and c) the case when the second GUV is smaller and will allow more GUVs to enter. Scale bar: 20 µm. The GUV membranes contained either Liss-Rhod-PE (orange) or NBD-DSPE (green).

Figure 3.8: **Simulation of the electrical field in alternative design performed using COMSOL.** The potential difference between the electrodes is 10 V and the chamber is filled with water. (a) View from above. (b) View from the side, including a plot of the electric field strength along the white dashed line. The black dashed lines on the plot indicate the region where the GUVs are typically located. Scale bar 20 µm.
but also ensures that the electric field can reach the membrane-membrane interface via a gap between the posts. Additionally, we designed our device to incorporate patterned electrodes directly at each trap (but not in contact) to minimise the strength of the applied electric field and avoid hydrolysis. A micrograph of the final chamber design with the trap and the electrodes is shown in Fig. 3.7, together with fluorescence images of two loading situations. Here, we introduced a suspension of two GUV populations (1:1 ratio), where the membranes are stained with dyes that are fluorescent at different wavelengths. After loading, an average of 258 GUVs are captured per device, i.e. 2 to 3 are occupying one trap. On average, 55% of the traps contain two or more different GUVs (Fig. 3.7c) and 17% contain two different GUVs, which is ideal for our fusion experiments (Fig. 3.7b). The rest of the traps contain either one single GUV, two or more of the same GUV, or are unoccupied. Based on these findings, the electrodes were designed so that the traps can be pulsed in 12 sets of 9. In this way, the chances are high to obtain 1 out of this set of 9 traps filled with two different GUVs, allowing us to study the fusion process in detail by high resolution microscopy. Furthermore, on average 5 out of 9 traps can be used for fusion because they contain vesicles of different populations. Therefore, we are able to perform 60 fusion experiments per chip, 12 of which are ideal.

3.4.2 Electrofusion

For electrofusion, only transient pore formation is required to destabilise the membranes for fusion. In order to find the upper threshold of the electric-field strength that would induce pore formation without lysis, GUVs containing 100 mM calcein were loaded into the device. We found that applying square pulses of 20 V or more caused leakage of the calcein (Fig. 3.9). The length of the applied pulse was also investigated and it was found that longer pulses of 250 ms or more would cause GUV lysis with complete loss of membrane integrity as well as hydrolysis of the water (data not shown). Therefore in all fusion experiments, we used a DC 1 ms square pulse of 10 V. A COMSOL simulation shows how the electric field strength successfully propagates across the PDMS trap (Fig. 3.10). In the region where GUVs are confined, the field strength is between 110 and 220 kV m\(^{-1}\). Similar to the value required for fusion reported by other groups.[96, 174]

To demonstrate the technology is able to successfully fuse the lipid membranes, we employed a membrane mixing assay exploiting FRET. DOPC lipid GUVs with and without 2% NBD-DSPE and Liss-Rhod-PE were produced and loaded into the traps. Here, NBD is the donor fluorophore and the acceptor fluorophore is Liss-Rhod. At this con-
3. **Controllable electrofusion of lipid vesicles**

Figure 3.9: **Calcein leakage with high voltages.** First a 10 V pulse was applied without loss of calcein. Then a second pulse of 20 V was applied causing leakage of calcein and subsequent loss of fluorescence.

Figure 3.10: **Simulation of the electrical field in final design performed using COMSOL.** The potential difference between the electrodes is 10 V and the chamber is filled with water. (a) View from above. (b) View from the side, including a plot of the electric field strength along the white dashed line. The black dashed lines on the plot indicate the region where the GUVs are typically located. Scale bar 20 µm.

centration, FRET takes place due to the close proximity of the fluorescently labelled head groups and as a result the green fluorescence intensity of NBD is reduced (see Fig. 3.11). After fusion with a GUV containing only DOPC, the labelled lipids are diluted and less FRET occurs.[175] This is monitored by an increase in the fluorescence intensity of the NBD.
Upon experiencing the electric field generated by the electrodes, the two GUVs fuse and the lipids begin to mix (Fig. 3.12).

![Graph showing fluorescence intensity](image)

**Figure 3.11:** The fluorescence intensity of NBD and Liss-Rhod when in separate membranes and in the same membrane. In the same membrane the fluorescent labels are in the close proximity causing FRET and therefore a reduction in the fluorescence intensity of NBD.

The average fluorescence intensity of NBD was plotted over time for different fusion events in 3 separate traps. It should be noted that to overlay the change in fluorescence signal, traps with similar sized vesicles were chosen. Out of the 12 ideal traps per chip, approximately 4 traps met this requirement.

Due to having multiple experiments on a single device, we are able to overlay data from the GUVs of the same diameter to show reproducibility of the fusion events. Having integrated electrodes at a defined position either side of the GUVs allows repeated experiments under identical conditions, which would otherwise be difficult to achieve.

### 3.4.3 Content mixing: initiating and monitoring reactions

The combination of traps and electrodes also permits reactions to be performed using GUVs as micro-reactors. The advantages over bulk reactions are the small volumes of 4 – 4000 fL and fast mixing times of 45 ms (see 3.13 for high frame rate data of content mixing).

GUVs were created in solutions of either 10 µM calcein and 10 µM cobalt chloride or 200 µM EDTA in Millipore water. Afterwards, they were mixed together and introduced into the device. Once loaded, the external solution was exchanged for Millipore water.
3. *Controllable electrofusion of lipid vesicles*

![Image of GUV fusion](image)

Figure 3.12: **Membrane mixing assay.** a) Time series illustrating the fusion of two GUVs using a 1 ms pulse of 10 V at time 0 seconds. Scale bar: 20 µm. b) The average increase in NBD fluorescence over the entire membrane reports loss of FRET via lipid mixing. Data are taken from 3 experiments on the same chip using vesicles of 20.3 ± 2.1 µm diameter.

(an advantage of the microfluidic format). At first, the fluorescence intensity of calcein is quenched in the presence of cobalt. When fused with the second GUV, the EDTA chelates the cobalt to form a complex and the fluorescence intensity of the calcein increases (Fig. 3.14a & b). The reaction is given as follows:

\[
Co^{2+} + EDTA^{4-} \rightleftharpoons [Co(EDTA)]^{2-}
\]  

(3.1)

Fig. 3.14c shows reaction kinetics from four separate fusion events taken from traps containing GUVs with different sizes. Due to the relative differences in volume, the concentrations of reactants will not be equal after fusion. These concentrations are calculated using the original reagent concentrations and the sizes of the GUVs. The reactions were monitored for the first 4 minutes after fusion and accordingly, the reactions with less cobalt resulted in less product formation. The initial rates in first 20 s were obtained using
3.4 Results & discussions

Figure 3.13: **High frame-rate analysis of content mixing.** GUVs with and without 100 µM calcein were loaded into the traps. Electrofusion was performed and fluorescence images were recorded at 65.8 fps. Data was averaged from 4 experiments and error bars are taken from the standard deviations.

A linear fit and the rate constant ($k$) for reaction was found to be $(2.5 \pm 1.1) \times 10^2 \text{M}^{-1} \text{s}^{-1}$. An experiment was also performed in bulk solution (without GUVs) in a fluorescence spectrometer (LS 50B, Perkin Elmer) using the same conditions but with mixing by stirring and $k$ was measured to be $17.9 \pm 6.6 \text{M}^{-1} \text{s}^{-1}$. We believe that the difference in the measured rates is primarily a consequence of the different mixing times. Although based solely on the diffusion of the reactants into the newly formed GUV, the mixing in the GUV reactor is fast. We can estimate that Co$^{2+}$ diffuses across the diameter of 10 µm in only about 39 ms (with the diffusion coefficient of Co$^{2+}$ as $1.29 \times 10^{-9} \text{m}^{-2} \text{s}^{-1}$ taken from ref. [176]), which fits well with the above mentioned experimentally observed mixing time of 45 ms.

These findings do not only show that the device can trigger the fusion of lipid vesicles for use as micro-reactors, but it also demonstrates efficient content mixing. This is important because lipid mixing can in some cases occur without content mixing.[177]

We believe that the microfluidic device can be employed for various applications in the field of membrane biophysics, e.g. membrane fusion studies and the realisation of artificial cells. While cells and viruses can make use of biochemical methods to provide contact between two membranes for fusion, e.g. by proteins and fusogenic peptides,[86, 165] our device enables this by the flow that pushes the two vesicles into the trap. In this context of forming cell-like systems, we also fused GUVs containing smaller vesicles or “artificial organelles” to build larger systems (Fig. 3.15). Finally, it is worth mentioning
3. **Controllable electrofusion of lipid vesicles**

![Image](image.png)

**Figure 3.14: Content mixing assay.** After fusion, calcein fluorescence increases by complexing EDTA with cobalt for the ideal case a) and for three vesicles b). Scale bars: 5 µm. c) Increase in product concentration upon mixing for [EDTA]/[cobalt] ratios of 3.3, 7.3, 15 and 20 corresponding to concentrations of 28.5, 53.3, 86.8 and 100 µM of EDTA and 8.6, 7.3, 5.7 and 5 µM of cobalt.

that the device holds the capability of fine-controlling the electrical fields around vesicles or cells to induce lysis or electroporation (Fig. 3.9 & 3.16).
Figure 3.15: **Fusion of the outer membrane of multivesicular vesicles (MVVs).** a) Fusion of three GUVs (left), two with and one without calcein inside (indicated with a large dashed circle). The central vesicle contains a smaller vesicle (small dashed circle), which remains inside the final fused vesicle (right). b) Fusion of two GUVs with a third vesicle inside (left). After fusion, the smaller vesicle is engulfed by the large GUVs, which have also fused together (right). Scale bar: 20 µm.
3. Controllable electrofusion of lipid vesicles

Figure 3.16: **Cell lysis and electroporation.** a) Micrograph from the final assembled device using the traps designed for hosting one cell or vesicle. The traps in this example were optimized to trap single mammalian cells. Here, U937 suspension cells in PBS buffer were introduced and trapped using a flow rate of 5 $\mu$L min$^{-1}$. The image is an overlay of a bright field image of the device, and a fluorescence image of the cell stained with calcein AM. The cell is confined between the electrodes so that lysis or electroporation can be achieved efficiently. Scale bar: 50 $\mu$m. b) Example curves from cells subjected to pulses from the electrodes. Upon switching on the electrical field, the fluorescence signal is reduced, i.e. the cellular integrity is compromised. Depending on the strength, length and repeats of the pulses, complete loss of the calcein AM signal (red, 500 V, 10 pulses, 2 $\mu$s, 100 $\mu$s delay) or electroporation (blue, 500 V, 5 pulses, 2 $\mu$s, 100 $\mu$s delay) can be achieved.
3.5 Conclusions

We have presented a microfluidic device that can hydrodynamically trap multiple GUVs. Once captured in the PDMS posts, membrane fusion can be initiated using integrated microelectrodes. The posts are designed to trap two GUVs parallel to the direction of flow. The electrodes are positioned such that the pulsed electric field is perpendicular to the interface of the membranes, therefore allowing efficient electrofusion. Unlike bulk measurements, multiple fusion events can be triggered on demand, providing both temporal and spatial control of the fusion events. Moreover, bulk approaches, such as micropipette aspiration, rely on applying tension to the GUVs. The advantage our device has, is that once the circular ring valves are lowered, there are no external forces acting on the membranes other than the electric field from the microelectrodes.

In general the platform can be used to introduce chemical compounds into vesicles. As shown, this could be a way to initiate reactions and measuring the reaction kinetics. If required, the vesicles carrying the product could be collected by reversing the flow. With some design modifications to improve the yield and size homogeneity of trapped vesicles, the device is also useful for more mechanistic studies on the fusion process, e.g. observing the effect of different buffers.
4 Multi vesicular droplets: A cell model system to study vesicle compartmentalised biochemical reactions

Manuscript in preparation P. E. Verboket, P. S. Dittrich: Multi vesicular droplets: A cell model system to study vesicle compartmentalised biochemical reactions. P.E.V. and P.S.D. designed the research. P.E.V. performed the research and evaluated the data. Both authors discussed the results and approved the manuscript.
4.1 Abstract

Artificial lipid vesicles are widely used as model system for cells. In particular multi vesicular vesicles (MVVs) have been proposed as a platform to study compartmentalisation of cells. However, the preparation techniques are challenging and the control of the size, lamellarity, and loading is demanding. Here we introduce a droplet-based microfluidic device for the formation and examination of multi vesicular droplets (MVDs). A perfluorinated carrier phase with a biocompatible surfactant is used to form monodisperse droplets of an aqueous giant unilamellar vesicle (GUV) suspension. The successful on-chip formation of MVDs was verified by high-speed microscopy. For bright field or fluorescence inspection, the MVDs are trapped in an array. The integrity of the vesicles and droplets is preserved for up to 15 min. On-chip mixing of reagents in the MVDs enabled us to perform membrane-staining assays and a compartmentalised enzyme cascade assay. This straightforward platform is modular and can be used for a wide scope of applications in studying biochemical reactions.

4.2 Introduction

Lipid membranes play an important role for many cellular functions. Cells are not only encapsulated by a lipid membrane but also inside the cell smaller vesicular structures such as the nucleus, vacuoles or mitochondria take over important tasks. These compartments are crucial for material storage, energy production or material degradation.

A sophisticated model system for studying cellular processes, which involve lipid membranes, is based on artificial lipid vesicles. These vesicles are employed to investigate the compartmentalization in eukaryotic organisms and their membrane functions.[79, 178] To replicate cellular compartmentalisation in controllable synthetic environments, giant unilamellar vesicles (GUVs) are of particular interest. With a diameter between 1 to 100 µm they resemble the size range of cell organelles and cells.[79] Today, GUVs have been used to investigate for example mitochondrial cristae formation[179], virus-membrane interactions[180], vesicle fusion[181], or gene expression in GUVs[109]. To mimic the hierarchical build up of cells GUVs are compartmentalized into multi vesicular vesicles (MVVs).[110] It describes the encapsulation of small vesicles in a larger vesicle.[182] In particular MVVs were used to study multi-agent drug delivery systems which offer beneficial release characteristics[111, 112] and multi-compartment micro-reactors[64, 113, 183, 184]. Current synthetic preparation techniques of MVVs include endo-
4.2 Introduction

budding of GUVs,[185–187] methods for the encapsulation of smaller vesicles,[112, 188, 189] vesicle-in-water-in-oil emulsion transfer[190] and double liposomes formed from the spreading of lipid films on a glass substrate[191, 192] as well as reverse phase evaporation. [193] However, these techniques are limited regarding control of the size, lamellarity, and loading, and the preparation processes are laborious.

To establish a straightforward approach that complies for control of size, lamellarity, and loading, we present an easy to use droplet-based microfluidic device. It enables the investigation of inner compartmentalisation of cells with a novel model system. Droplet-based microfluidics offers control over size and loading for the generation of nano- to picolitre sized droplets[27] in micrometer range channels of two immiscible fluids. Commonly, an aqueous phase and a carrier phase are used together with a surfactant for stabilization. Co-encapsulation of multiple aqueous solutions can be implemented which allows for using droplets as individual microreactors.[27] Although the preparation of multicompartent emulsion by means of droplet-based microfluidic is possible[75, 76] the resulting entirely artificial compartmentalised droplets typically lack biological resemblance; e.g. a lipid membrane or a cell-like structure. Based on the application of droplets to encapsulate prokaryotic and eukaryotic cells[194–199] we have developed a system that combines the ease of use and control of droplet microfluidic with the biological relevance of MVVs.

Our approach provides a platform for studying the inner compartmentalisation of cells with a novel model system. Droplet-based microfluidic is used to encapsulate GUVs inside aqueous droplets in a perfluorinated carrier phase to resemble the inner structure of cells and their organelles. Due to their similar structure as MVVs, we refer to them as multi vesicular droplets (MVDs). We show a microfluidic platform to generate MVDs and investigate biochemical reactions within them. MVDs can be trapped and thus analysed by bright field and fluorescence microscopy in a high-throughput manner. In this study we examine the integrity of GUVs inside the MVDs during the encapsulation and while trapped in the array. Additionally we demonstrate the capabilities of the system to perform biochemical reactions with an enzyme cascade assay inside MVDs.
4. **Multi vesicular droplets: A cell model system**

4.3 **Experimentall**

4.3.1 **Chip design and function**

We have developed a microfluidic device to generate MVDs and trap for further inspection. Key component of this microfluidic chip design is the trapping array based on a design by Tan *et al.*[200] and Shi *et al.*[43], see Figure 4.1 and 4.2. The trapping channel features 89 individual droplet traps with a diameter of 50 µm. They are able to trap droplets with diameters between 40 – 50 µm. Each trap features a diversion channel that has a slightly higher hydrodynamic resistance than the trap. The droplets steam flows through the trap until a droplet enters a trap. At the downstream side the trap has a narrow opening that does not allow the droplet to pass. If the passage with the lowest resistance is blocked all following droplet are redirected around the trap. This repeats until all traps are occupied. Droplets can be removed from the trap by an increase of the flow speed. All channels are fabricated with a height of 40 µm. On-chip up to three aqueous solutions can be used to generated droplets by flow focussing. To give a more precise control of stopping the droplet flow a by-pass with an external solenoid vale was connected to the chip. The connection port for the valve is located on a side channel in between the junction for droplet generation and the trapping array. As long as the valve is closed the stream of droplets is directed to the trapping array. Opening the valve directs the droplet flow in the by-pass and the flow of droplets through the traps stops immediately (See video 1 in the supplementary information).

4.3.2 **Chemical reagents**

SU-8 2050 photoresist and mr-Dev 600 developer were obtained from Microchem Corp. (Massachusetts, U.S.A.) and micro resist technology (Berlin, Germany), respectively. 1H,1H,2H,2H-Perfluorodecytrichlorosilane, and FC-40 were purchased from ABCR-Chemicals (Karlsruhe, Germany). The PDMS kit (Sylgard 184) was sourced from Dow Corning (Michigan, U.S.A.). The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000) Biotin) were both purchased from Avanti Polar Lipids (Alabama, U.S.A.). Alexa Fluor 488 streptavidin conjugate and DiI was obtained from life technologies (Oregon, U.S.A.). Mineral oil, chloroform, dimethyl sulfoxide (DMSO), glucose oxidase enzyme type VII (GOx), horseradish peroxidase type VI (HRP), and Ampliflu Red were obtained from Sigma-Aldrich (Missouri, U.S.A.). Sodium
Figure 4.1: **Top:** Microfluidic device filled with black ink. Scale bar 5 mm. **Bottom:** Section of the trapping array (in total 89 traps) filled with droplets containing blue food dye for better visualisation. Four images of the same experiment were stitched together to create a high-resolution panoramic image. Scale bar 500 µm.

Figure 4.2: **a)** Design of the chip. Scale bar (red) 1 cm. **b)** Enlarged section of the trapping channel (in total 89 traps). Scale bar (red) 100 µm.

Phosphate, bovine serum albumin (BSA), Methanol, D(+)-glucose, and D(+)-sucrose were all purchased from Acros Organics (Geel, Belgium). The biocompatible PFPE-block-PEG-block-PFPE-copolymer surfactant introduced by Holze *et al.*[201] was synthesized according to a protocol by Chen *et al.*[202].
4.3.3 **Microfluidic chip fabrication**

First the master mould for the microfluidic chips was fabricated in a clean room. A standard 100 mm diameter silicon wafer (Si-Mat, Germany) was dehydrated for 10 min at 200 °C and after cooling it down it was spin coated with SU-8 2050 at 3250 rpm for 30 s. The photoresist layer was soft baked for 180 s at 65 °C and for 360 s at 95 °C before it was exposed with 160 mJ cm\(^{-2}\) at 365 nm through a transparency photomask (Micro Lithography Services, UK) on a MA 6 mask aligner (Süss MicroTec, Germany). After a post exposure bake for 60 s at 65 °C and for 360 s at 95 °C the wafer was develop in mr-Dev 600 developer for 5 min. As the final step the wafer was placed in a \(1H,1H,2H,2H\)-Perfluorodecyltrichlorosilane atmosphere for 12 h at 100 mbar to avoid adhesion of the PDMS to the wafer and the 40 µm high features.

For fabricating the PDMS part of the microfluidic chip, the oligomer and curing agent were mixed at a ratio of 10:1 and afterwards degassed. An aluminium casting mould was placed on top of the wafer and the PDMS mix was poured to a height of 6 mm. After curing the PDMS on a hot plate for 8 min at 150 °C it was cooled down and peeled of the wafer. Fluid inlets and outlets were punched with a 1.5 mm outer diameter biopsy puncher (Miltex, PA, U.S.A.). The finished PDMS part was bonded to a glass cover slip by exposure to air plasma in a plasma cleaner PDC-32G by Harrick Plasma (New York, U.S.A.). Prior to use the channels on the chips were flushed for 15 min with an dry nitrogen stream caring \(1H,1H,2H,2H\)-Perfluorodecyltrichlorosilane to ensure a stable droplet generation and prevent wetting of the channel walls by the aqueous phase.

4.3.4 **Microscopy and data evaluation**

The wide-field fluorescence microscopy experiments were performed on an Olympus IX71 inverted microscope (Tokyo, Japan) equipped with an iXon Ultra 897 EMCCD camera by Andor Technology (Belfast, Ireland), a mercury lamp as well as appropriate optical filter sets for Alexa Fluor 488 (exciter HQ 470/40 X, dichroic 500 DCX, and emitter 500 LP), DiI (exciter D 535/50 X, dichroic 566 LP, and emitter 604 LP), and Ampliflu Red (same filter set as DiI). For bright-field microscopy a UK1117 camera by EHD imaging (Damme, Germany) and a Miro M110 high-speed camera (Vision Research, New Jersey, U.S.A.) were used. The recorded fluorescence signals were evaluated using ImageJ 1.45 (National Institutes of Health, U.S.A.) and OriginPro 9.1 (OriginLab Corp., 241 Massachusetts, U.S.A.).
4.3 Experimental

The average fluorescence intensities of the membrane or lumen of the GUVs were background corrected by subtracting the average fluorescence intensity of a location next to the channel.

4.3.5 Microfluidic chip operation

All fluids were supplied to the microfluidic chip through fluorinated ethylene propylene (FEP) tubing type 1548 by IDEX health & science (Washington, U.S.A.) by attached 1 mL syringes BD Plastipak (Heidelberg, Germany) with a nemesys syringe pump (Cetoni, Korbussen, Germany). On the chip droplets were generated by flow focusing and captured in a trapping channel (see Figure 4.1 bottom). After a couple of minutes the trapping channel was filled with droplets and a by pass solenoid valve (Cetoni, Korbussen, Germany) was opened. Thereby, the flow was diverted and the flow of droplets through the trapping channel is stopped. The captured droplets were analysed by fluorescence microscopy before the valve was switched again and new droplets were flushed into the traps with a higher flow rate. Typically flow rates of the each aqueous phase and the carrier phase were 0.25 – 1.0 µL min\(^{-1}\) and 2 – 4 µL min\(^{-1}\) respectively. See Figure 4.3 for a scheme of the microfluidic setup.

Figure 4.3: Scheme of the setup: Not to scale. The setup comprises four syringe pumps, which supply the aqueous and carrier phase to the chip. On-chip droplets are generated and trapped in a trapping array. Once the traps are filled with droplets a by pass valve is opened to stop the flow of droplets through the trapping channel.
4.3.6 Solutions

All aqueous solutions were prepared in ultrahigh-purity water (Merck Millipore, Massachusetts, U.S.A.). The GUV medium, referred to as hosting solution (HS) and intravesicular solution (IS) for DiI and streptavidin-labelling assays contained A-HS 1 Osmol L$^{-1}$ glucose and A-IS 1 Osmol L$^{-1}$ sucrose, respectively. Membrane labelling of the GUVs were initiated by adding (on- or off-chip) either solution A-R1 (13 µM DiI and 1 Osmol L$^{-1}$ glucose) or A-R2 (Alexa Fluor 488 streptavidin conjugate 8 mg L$^{-1}$ and 1 Osmol L$^{-1}$ glucose) to the prepared vesicle suspension (ratio 1:1). The enzyme cascade assay required the following set of solutions: B-HS (500 mOsmol L$^{-1}$ glucose and 50 mM sodium phosphate buffer pH 7.4) and B-IS (500 mOsmol L$^{-1}$ sucrose, and 50 mM sodium phosphate buffer pH 7.4 and 0.2 U L$^{-1}$ HRP) for the vesicle preparation. The enzyme cascade assay itself was conducted by adding (on- or off-chip) B-R1 (1 Osmol L$^{-1}$ glucose, 0, 3, 15, 30, 50 or 150 µM Ampliflu Red and 15 mg L$^{-1}$ BSA) and B-R2 (4 U L$^{-1}$ GOx and 100 mM sodium phosphate buffer pH 7.4) to the prepared vesicle suspension (ratio 1:1:1).

FC-40 containing 2 w% of the biocompatible PFPE-block-PEG-block-PFPE-copolymer was used as immiscible carrier phase for droplet generation. This solution was saturated with H$_2$O by mixing and subsequently stored under a layer of water, until use.

4.3.7 GUV preparation

The GUVs were prepared with the water/oil emulsion transfer method[133, 134, 203]. First POPC and DSPE-PEG(2000) Biotin both dissolved in chloroform were mixed at a molar ratio of 98:2. Subsequently, the chloroform was removed by vacuum evaporation and the dried lipids were redissolved in mineral oil to a total phospholipid concentration of 200 µM. This phospholipid solution (PS) was sonicated for 60 min at 50 ºC in a Sonorex Super sonicator (Bandelin, Berlin, Germany).

The following steps of the GUV preparation were performed in 1.5 mL polypropylene micro tubes (Sarstedt, Nümbrecht, Germany). The GUVs for DiI and streptavidin-labelling assays were prepared using A-HS and A-IS. For the preparation of GUVs for the enzyme cascade assay the solutions B-HS and B-DI were used. In the following section these solutions are referred to in general terms: HS and IS. First, a water in oil emulsion of 50 µL of IS and 500 µL of PS was produced by mechanical agitation. This emulsion was transferred to a micro tube containing of 500 µL HS, layered with 200 µL PS. The droplets were forced through the interface between PS and HS by centrifugation in a miniSpin plus centrifuge by Eppendorf (Hamburg, Germany) for 3 min at 1500 × g at room tem-
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4.4.1 Encapsulation process

Encapsulation of GUVs in droplets requires a careful selection of the carrier phase. Hydrophobic carrier phases like the mineral oil are not suitable due to their ability to leach the phospholipids from the GUVs which can lead to a destruction of the GUVs. Therefore, we chose perfluorinated FC-40 containing 2 w% of a biocompatible PFPE-block-PEG-block-PFPE-copolymer as carrier phase[201]. Monodisperse droplets in the size range between 45 and 50 µm were produced by planar flow-focussing using flow rates of 0.25 – 1.0 µL min\(^{-1}\) and 2 – 4 µL min\(^{-1}\) of vesicle suspension and carrier phase, respectively. Since the formation of droplets by planar flow-focussing exposes the GUVs to strong shear forces,[25] the vesicles might be damaged during the encapsulation process. In order to assure vesicle integrity, the encapsulation process was investigated by bright field high-speed microscopy (Figure 4.4). Here, the vesicles remain intact during the droplet formation which was manually evaluated from high-speed recordings (in total 500 vesicles with a diameters above 5 µm were observed) (see video 2 in the supplementary information). The number of vesicles per droplets followed a Poisson distribution (see Figure 4.5). With a low concentration of the vesicle suspension MVDs containing mostly one or zero GUVs per droplet (Figure 4.4) are formed. High concentrations can be used to generate MVDs entirely filled with vesicles (Figure 4.6).

4.4.2 Capturing of MVDs

In order to inspect GUVs in a large number of droplets we used a trapping array based on a design by Shi et al.[43] (see Figure 4.1 and 4.2). The traps in the array fix the droplets in place. Compared to stopping the flow in a simple straight channel the design here has the advantage to allow a much more precise control of the droplet location without making the chip more complex. We additionally added an off-chip by-pass valve to
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Figure 4.4: Bright-field image of the GUV encapsulation. In the junction the vesicle suspension (left channel) and the carrier phase (top and bottom channel) are used to form droplets by flow focussing. One GUV can be seen in the forming droplet as bright dot. Scale bar 25 µm.

Figure 4.5: Frequency distribution histogram of the number GUVs per droplet. To illustrate the resemblance the Poisson function was fitted and overlaid (red). A sample size of 100 droplets was manually analysed. Data processing was performed in Origin 9.1 (OriginLab, Massachusetts, U.S.A.). The MVDs were produced using flow rates of 0.5 µL min⁻¹ of GUVs suspension and 3.0 µL min⁻¹ of the carrier phase.
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Figure 4.6: a) Fluorescent image of a droplet entirely filled with GUVs. Prior to the encapsulation the GUVs were off-chip incubated with Alexa Flour 488 conjugated streptavidin. b) Bright field image of the same droplet. Scale bar 25 µm.

the design to speed up the sequential loading and unloading of the traps. The off-chip valve diverts the droplet stream around the trapping channel. If it is opened it immediately stops droplets from going through the trapping array (see Figure 4.1 and supplementary video 1). Closing the valve directs the droplet flow again through the trapping channel and the droplets are exchanged. A cycle of droplet capturing, analysing, and reloading with fresh droplets takes about 5 – 15 min, depending on the flow rate settings. It allows for sequential inspecting of droplets. The time between droplet generation and the earliest possible observation is about 10 s. Altogether this setup enables a batch wise examination of MVDs by fluorescence microscopy.

Depending on the flow rate settings a low flow of the carrier phase through the array remains when the by-pass valve is open (less that ~5 % of the total flow). This causes the GUVs to slowly rotate inside the droplets (see supplementary video 3). By stopping the droplet generation this movement can be brought to a halt.

The carrier phase can dehydrate the droplets over time which causes the droplets in the traps to shrink in size.[204] To reduce this shrinkage the carrier phase was saturated with water. Nevertheless, the maximum time a MVD can typically be observed is 15 min (see supplementary video 4). After this time a droplet gets small enough that it can be flushed out of the trap (~74 % of the original diameter). For the assays in this study which all take less than 1 min this observation time is more than sufficient.
4.4.3 Vesicle integrity

Using the previously described capturing array the membrane integrity of the membrane and lumen of the encapsulated GUVs were investigated. Figure 4.7 a) shows a bright-field image of a MVD. The vesicles stay intact in the droplets for up to 15 min limited by the maximum possible observation time.

Figure 4.7: Off-chip stained vesicles in droplets: a) Bright-field image of a trapped droplet containing multiple GUVs. Fluorescent images of GUVs in droplets with b) DiI or c) Alexa Flour 488 conjugated streptavidin stained membranes. d) The lumen of the encapsulated GUVs was fluorescently stained using the enzyme cascade assay. Dashed line indicating extends of the droplets, determined by bright-field images. Scale bar 25 µm.

To further examine the membrane of the GUVs two membrane staining were performed. The vesicles were incubated off-chip with either a solution containing DiI or Alexa Fluor 488 streptavidin conjugate. The DiI intercalates in the vesicles membrane, while Alexa Fluor 488 labelled streptavidin binds to the biotinylated phospholipids in the vesicle membrane. After a washing step with HS, the labelled/stained vesicles were
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encapsulated in droplets, and trapped in the array (see Figures 4.7 b) and c)). Both assays, the synthetic DiII and bio-chemical biotin-streptavidin labelling, show that the complete membrane of the vesicles remains intact. Additionally, the lumen of the GUVs in droplets was stained with fluorescent resorufin (using enzyme cascade assay followed by three washing steps with HS, off-chip). This suspension of stained GUVs was supplied to the chip and encapsulated in droplets. Figure 4.7 d) shows a MVDs with the lumen stained, proving that also the lumen remains intact inside the MVDs. However, the background fluorescence inside the droplet indicates that some of the vesicles might have been damaged before they were encapsulated in droplets. On-chip the solution shows similar background fluorescence even before encapsulation. A sample of vesicle suspension was inspected after it was aspirated the syringe an shows a similar background fluorescence. These findings do not only demonstrate that the vesicles can be successfully encapsulated, but also that membrane and lumen stay intact and are not damaged during the trapping of the droplet.

To demonstrate that MVDs can be loaded to perform labelings within them, the chip is equipped with three inlets for aqueous phases (Figure 4.3). In a first proof-of-concept for on-chip labelling of membranes an unlabelled GUV suspension and a DiII solution were encapsulated in droplets. Inside the droplet the solutions are mixed within milliseconds[205] and the DiII intercalates into the vesicle membrane. In the trapped droplet the stained membranes of the vesicles can be observed (see Figure 4.8 a)). Since no increase in fluorescent intensity was observed we concluded that the DiII staining was already completed at the time of trapping. Even though all channel walls are coated with perfluorosilane to reduce wetting by the aqueous phase DiII is deposited on channel walls in the time course of the experiment. Overall this results in a higher background inside and outside the droplets (see Figure 4.8 a)).

In the same way droplets of an Alexa Flour 488 conjugated streptavidin solution, and a GUV suspension with biotinylated phospholipids in the vesicles membrane were mixed and encapsulated on-chip. The streptavidin binds to the biotin on the membranes outer surface, thereby making it fluorescent (see Figure 4.8 b)). Again no increase in fluorescence of the vesicles was detected. Our platform offers the possibility to perform on-chip variations of the concentration in the MVDs. During the encapsulation, the flow rate of the streptavidin solution (8 mg L$^{-1}$) was kept constant (0.5 µL min$^{-1}$) while the flow rate of the vesicle suspension was varied in steps (0.5, 0.75, 1 and 2 µL min$^{-1}$). It should be noted that not only the concentration of the streptavidin changes, but also the concentration of GUVs in the droplet changes. Also the droplet formation frequency was increased the size of the trapped droplets remained constant. The final fluorescent intensities of vesicle membranes in the size range 5 to 10 µm were measured and corrected by
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Figure 4.8: Fluorescent images of on-chip labelled GUVs in droplets. a) DiI, and b) Alexa Flour 488 conjugated streptavidin stained membranes. Dashed line indicating extends of the droplets, determined by bright-field images. Scale bar 25 µm.

the background fluorescence of the droplet (see Figure 4.9). As expected, it was found that with decreasing concentrations background fluorescence is reduced (less unbound streptavidin) and the relative signal of GUVs increases. A concentration of 1.6 mg L\(^{-1}\) was found to give the highest signal among the tested concentrations. For all tested concentration the relative standard deviation between the individual signals of each concentration was less that 8 %. Experiment shows the successful optimisation of the streptavidin concentration.

4.4.4 Compartmen talised enzyme cascade reaction

An enzyme cascade reaction assay was performed to demonstrate the capability of this setup to perform complex biochemical reactions inside MVDs. Two solutions (R-B1 and R-B2), and a vesicle suspension are encapsulated and mixed in droplets.

Inside the MVD GOx (contained in solution B-R2) converts the glucose from solution B-R1 to gluconolactone and hydrogen peroxide. The produced hydrogen peroxide can penetrate into the vesicle. Additionally, solution B-R1 contains Ampliflu Red, which also diffuses into the vesicle. In the GUVs HRP is encapsulated. It converts the Ampliflu Red and hydrogen peroxide to oxygen and the fluorescent resorufin. The negative charge of the fluorescent resorufin prevents it from escaping through the lipid membrane. Resorufin is accumulated in the GUVs (see Figure 4.10 a)). In side the MVD the cascade reaction is completed at the time of trapping (Figure 4.10 b)). No further increase of the fluorescence of the vesicles was observed.
4.4 Results and discussion

Figure 4.9: **Labelling optimisation by on-chip concentration variation.** Graph of the (final) fluorescence intensities of the GUVs and droplets plotted against the Alexa Flour 488 conjugated streptavidin concentration in the droplet. The streptavidin solution ($8 \text{ mg L}^{-1}$) was supplied at a constant flow rate of $0.5 \mu\text{L min}^{-1}$ and the flow rate of the vesicle suspension was varied to $0.5, 0.75, 1$ or $2 \mu\text{L min}^{-1}$. Sizes of the analysed GUVs were between 5 and 10 µm. All samples were taken with the same acquisition setting, 10 samples for each concentration.

MVDs of five different concentrations of Ampliflu Red were generated and the fluorescence intensities of the lumen of GUVs with sizes between 3 to 6 µm were measured (see Figure 4.11). As a control MVDs containing $17 \mu\text{M}$ Ampliflu Red but lacking GOx were formed. The vesicles act as a container in which a substance can be enriched like in lysosomes or mitochondria. The measured values suggest that the intensities vary depending on the concentration of Ampliflu Red. In this set of experiments, a linear relationship is assumed as depicted in Figure 4.11. However, the variable size of the vesicles, their movement and background potentially caused by burst vesicles rendered a detailed analysis of the behaviour in the current setup impossible. An advanced system is needed to solve these problems. The background should be reduced using for example enzyme inhibitors like Amplex Red stop reagent by life technologies that inhibits the enzyme reactions. The remaining movement of vesicles hampered the accurate image acquisition of the lumen. It could be controlled in a next generation microfluidic device that protects the vesicles from flow using on-chip valves. Finally, a 3D acquiring system would solve the problem of out-of-focus movement of vesicles. If the improvements help to rule out
the current limitations, a more resilient set of data could be acquired to confirm the linear relationship of fluorescence intensity and concentration of Ampliflu Red. To date, initial tests using the stop reagent did not lead to a reduced background and have to be optimized. In addition, first attempts to measure reaction kinetics failed due to the previously described reasons and the fast reaction, which was already completed before imaging. Reducing the GOx concentration by 4-fold to slow down the reaction rate was not sufficient to monitor the dynamics of the reaction process. Nonetheless, the measurement of time-dependent enzyme reactions in an improved setup would be very interesting. It would open a new possibility of studying dynamics of compartmentalised enzymatic reactions in a controlled manner.

Figure 4.10: a) **Scheme of the enzyme cascade assay in a MVD.** Outside the vesicle GOx converts the substrate glucose to gluconolactone and hydrogen peroxide. Ampliflu Red and hydrogen peroxide penetrate through the lipid bilayer into the vesicle where HRP the two substrates react to oxygen and the fluorescent resorufin. Due to its negative charge the resorufin is trapped in the vesicle. Not to scale. b) Fluorescent image of a MVD containing three GUVs, which are fluorescently labelled, using the enzyme cascade assay. Scale bar 25 µm.
4.5 Conclusions

GUVs can successfully be encapsulated in monodisperse droplets to form MVDs. This is achieved by using a modular, flexible and straightforward droplet-based microfluidic device. This setup enabled us to monitor the MVDs for up to 15 min. Furthermore, we could show using fluorescence microscopy in combination with lumen and membrane staining assays that membrane and lumen stay intact. GUVs can be encapsulated with additional solutions to stain them or initiate the reaction. As a proof of concept study for complex compartmentalised biochemical reactions in MVDs an enzyme cascade reaction was performed. This assay demonstrates the capabilities of the MVDs in terms of loading of the GUV and loading of droplets. Additionally, this assay clearly shows the wide scope of possibility that our MVD based system offers to study the effects of compartmentalisation on biochemical reactions in a cell like model system.

MVDs are suitable model systems to study the effects of lipid membrane compartments such as inter vesicle reactions. In comparison to the MVVs the MVDs offer a convenient and versatile tool to study the effects of compartmentalisation in cell like system when the outer membrane is not of particular interest.

Figure 4.11: Graph showing the fluorescence intensities of GUVs plotted against the Ampliflu Red concentration in the droplet. All sample images were taken with the same acquisition setting, 8 samples for each concentration. The data was fitted with a linear function.
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The presented droplet microfluidic technology is biocompatible because the carrier phase and surfactant used in this study are known not to harm cells.[201] We suggest that cells could be co-encapsulated with GUVs inside a droplet. The vesicles might be used to supply nutrients or reagents to the cells. Due to the lipid membrane the GUVs could offer a beneficial release characteristic.
5 A New microfluidics-based droplet dispenser for ICPMS

A detailed protocol for the preparation and operation of the presented system can be found in the appendix chapter 7.1. Adapted from P. E. Verboket#, O. Borovinskaya#, N. Meyer, D. Günther, P. S. Dittrich: A New Microfluidics-Based Droplet Dispenser for ICPMS, Analytical Chemistry 86 (2014) 6012-6018. with permission from the American Chemical Society. The figure numbers, citation numbers and placement of the supplementary information have been modified since publication. #: These authors contributed equally to this work. Link to the article: http://pubs.acs.org/doi/abs/10.1021/ac501149a P.E.V., O.B., D.G. and P.S.D. designed the research. P.E.V. and O.B. performed experiments and analyzed the data. O.B. designed the droplet transport system. P.E.V. designed and developed the LADE-chip and the concept of liquid-assisted droplet ejection. N.M. performed bulk controls and evaluated the data. All authors discussed the results and approved the manuscript.
5. A New microfluidics-based droplet dispenser for ICPMS

5.1 Abstract

In this work, a novel droplet microfluidic sample introduction system for inductively coupled plasma mass spectrometry (ICPMS) is proposed and characterized. The cheap and disposable microfluidic chip generates droplets of an aqueous sample in a stream of perfluorohexane (PFH), which is also used to eject them as a liquid jet. The aqueous droplets remain intact during the ejection and can be transported into the ICP with >50% efficiency. The transport is realized via a custom-built system, which includes a membrane desolvator necessary for the PFH vapor removal. The introduction system presented here can generate highly monodisperse droplets in the size range of 40 – 60 µm at frequencies from 90 to 300 Hz. These droplets produced very stable signals with a relative standard deviation (RSD) comparable to the one achieved with a commercial droplet dispenser. Using the current system, samples with a total volume of <1 µL can be analyzed. Moreover, the capabilities of the setup for introduction and quantitative elemental analysis of single cells were described using a test system of bovine red blood cells. In the future, other modules of the modern microfluidics can be integrated in the chip, such as on-chip sample pretreatment or parallel introduction of different samples.

5.2 Introduction

Conventionally, elemental analysis of liquids in the inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICPMS) is realized by means of pneumatic nebulizers in combination with spray chambers.[206] The operating principle of such a sample introduction system is based on the conversion of a liquid into a polydisperse aerosol (nebulizer) and subsequent filtering out of large droplets (spray chamber). These systems exist in various geometries, are robust, and are routinely used in many applications.[207] Their main drawback, however, is a high sample consumption (designed to run with the liquid flow of >0.3 mL min\(^{-1}\))[208] and an incomplete sample transport, which limit their applicability for the analysis of micro sample volumes commonly available in biological, forensic, toxicological, and clinical studies.[209] Reducing the nebulizer nozzle dimensions has decreased the effective sample consumption from a few milliliters per minute to microliters per minute and even nanoliters per minute[210] and has significantly enhanced the aerosol transport efficiency (TE) thanks to a much lower sample uptake.[209] These days, there are many variations of microflow nebulizers, which can operate with close to 70 – 100% sample TE with or without spray chambers[211, 212] and desolvation systems; these include the microcon-
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centric nebulizer,[213] micromist nebulizer,[213] high-efficiency nebulizer,[152] and direct injection high-efficiency nebulizer.[153] Reduction of the nebulizer opening dimensions, however, leads directly to the increased risk of clogging whenever samples containing highly concentrated salts or undigested biological fluids have to be analyzed.[209] In addition, the highest stability of the aerosol production in microflow nebulizers is achieved by passive liquid uptake,[214] whose rate depends on the pressure difference at both ends of the nebulizer capillary that is directly determined by the nebulizing gas flow rate. Since the range of the optimum flow rate of this gas is normally very narrow, the flexibility of the system in terms of the sample uptake is also limited.

A new concept of injecting the liquid sample into the ICP in the form of monodisperse discrete droplets was introduced by Olesik and Hobbs.[215] Uniform \( \approx 50 \mu m \) droplets could be produced on demand by a micropump,[216] which ejected a defined liquid volume after capillary contraction, and transported via a laminar flow oven into the ICP with 100% efficiency. This system did not find wide application but has significantly contributed to the fundamental understanding of analyte behavior in analytical ICPs.[154]

A system for controlled generation of monodisperse microdroplets consisting of a piezoelectrically driven dispenser head, a control box, and a droplet visualization system is commercially available (Microdrop Technologies GmbH, Norderstedt, Germany). The droplets can be produced in sizes of 30, 50, 70 and 100 \( \mu m \) in a frequency range from 100 Hz to a few kilohertz with only 1% droplet variation in volume, and liquid volume flow rates of picoliters per minute to nanoliters per minute can be measured. The transport of these relatively large droplets into the ICPMS was realized using ambient temperature desolvation with helium[155] instead of argon[217] as a carrier gas.[218, 219] The droplets can be transported into the plasma either vertically or horizontally, depending on their size, with close to 100% efficiency.[220] This system has already shown its potential in quantitative analysis of single nanoparticles[220, 221] and characterization of individual biological cells by ICPMS,[222] which has recently gained great interest in the field of nanosafety[223] and in cell biology.[224] Another similar system based on the thermal inkjet technology has been recently introduced[225] and successfully applied to the elemental analysis of urine using dosing frequency calibration.[226]

Even though the single-droplet introduction is very efficient and promising for the analysis of micro sample volumes or single entities such as nanoparticles and cells, the currently available microdispenser modules have several drawbacks. They provide droplets with a fixed volume, which is set by the nozzle diameter and can only slightly be varied by dispenser settings (unless custom settings are used[218]), are sensitive to the
changes of the physical properties of the liquid (salt content, pH), are prone to clogging, difficult to clean, and rather expensive. In addition, efficient and nondestructive introduction of cells might be problematic due to the passive liquid uptake through the capillary and the application of a high voltage.

In this work a different approach to generate microdroplets for ICPMS analysis using droplet microfluidics is presented. Droplet microfluidics has been intensively used for studying (bio)chemical reactions [38, 227–229] and for single-cell studies.[6, 230] Recently, it has been proposed as a valuable system for sample preparation in matrix-assisted laser desorption/ionization mass spectrometry[29] and as sample introduction system for electrospray mass spectrometry.[30, 31] In our interface, monodisperse aqueous droplets are produced using the highly volatile carrier phase perfluorohexane (PFH) in a custom-designed liquid-assisted droplet ejection (LADE) chip, which is made entirely of poly(dimethylsiloxane) (PDMS). The chip is cheap, disposable, can produce droplets in a broader volume range and is more robust to changes of liquid sample properties. Interfacing of the new chip with the ICPMS was accomplished via a custom-built transport system including a membrane desolvator, which permitted the PFH vapor removal. This paper summarizes characterization and optimization of the system and its application for the analysis of single bovine blood cells.

5.3 Experimental section

5.3.1 Materials

SU-8 2002 and SU-8 2050 photoresists were purchased from Microchem Corp. (Massachusetts, U.S.A.). 1H,1H,2H,2H-Perfluorodecyltrichlorosilane was purchased from ABCR-Chemicals (Karlsruhe, Germany). PDMS and curing agent (Sylgard 184) were obtained from Dow Corning (Michigan, U.S.A.). Perfluorohexane, 95+ % and 99 % (used for the ICPMS tests), were purchased from Alfa Aesar (Karlsruhe, Germany) and Sigma-Aldrich (Missouri, U.S.A.), respectively. Merck IV multielement standard solution was purchased from Merck Millipore (Massachusetts, U.S.A.). The test solutions were prepared from single-element standard solutions (Inorganic Ventures, Virginia, U.S.A.) and contained nitrate salts of either Ce or Na, Mg, Ca, Mn, Fe, Mo (referred as multielement solution) at concentrations of 1 mg kg\(^{-1}\). Merck IV and the test solutions were diluted to the required concentrations with 2% sub-boiled HNO\(_3\) prepared in ultrahigh-purity water (Merck Millipore, Massachusetts, U.S.A.).
5.3.2 Cell preparation and digestion

Washed pooled bovine/calf red blood cells in phosphate-buffered saline (PBS) were obtained from Rockland Immunochemicals Inc. (Pennsylvania, U.S.A.). Additional PBS for dilution of the cell suspension was purchased from Life Technologies (Paisley, U.K.). The concentration of cells was manually determined using a hemocytometer (five replicates). Three aliquots of 1 ml of the cell suspension were microwave digested (Ethos Plus, Milestone Inc., Connecticut, U.S.A.) in 8 ml of 60% sub-boiled HNO$_3$ and 21 of 30% H$_2$O$_2$ (TraceSelect, Fluka Analytical, Buchs SG, Switzerland) adding Co to determine the digestion recovery. After 5000× dilution of the dissolved cell suspension, the quantification of Fe was realized by ICPMS using external calibration and Mn as internal standard. Acid and PBS blanks were monitored in the same way.

5.3.3 Master mold fabrication

The master mold for the preparation of the patterned PDMS half was fabricated on the top of polished 100 mm diameter silicon wafers (Si-Mat, Kaufering, Germany). First, a 2 µm high adhesion layer was applied to the wafer as follows. The wafer was dehydrated for 10 min at 200°C and after cooling down spin coated with SU 8 2002 (micro resist technology, Berlin, Germany) for 30 s at 2000 rpm. After a soft bake for 60 s at 95°C the wafer was flood exposed with UV light (80 mJ cm$^{-2}$ measured at 365 nm) on a MA 6 mask aligner (Süss MicroTec, Garching, Germany). A post exposure bake was performed for 120 s at 95°C. To form the microfluidic features the wafer was spin coated with a layer of SU8-2050 (micro resist technology, Berlin, Germany) for 30 s at 3250 rpm. A soft bake was performed for 180 second at 65°C and for 360 s at 95°C. The wafer was again exposed with UV light (160 mJ cm$^{-2}$ at 365 nm) on a MA 6 mask aligner using a transparency photomask (Micro Lithography Services, South Woodham Ferrers, UK). After a post exposure bake for 60 s at 65°C and 360 s at 95°C, the wafer was developed in mr-Dev 600 developer for 5 min exposing the 40 µm high features. To prevent adhesion of the PDMS the patterned wafer was treated in a 1H,1H,2H,2H-Perfluorodecyltrichlorosilane (ABCR-Chemicals, Karlsruhe, Germany) atmosphere at 100 mbar for 12 h. For the flat PDMS, half a blank polished 100 mm diameter silicon was treated with the silane using the same method.
5. A New microfluidics-based droplet dispenser for ICPMS

5.3.4 Microfluidic chip fabrication

The microfluidic chip consists of two PDMS pieces that are bonded together. One half is patterned with the microfluidic channels and the other half is flat and used to seal the channels. The PDMS was prepared by mixing the oligomer and the curing agent at a ratio of 10:1. After degassing the PDMS mixture it was poured into a custom-made casting form placed on top of a silicon wafer. The casting form has an opening allowing filling with PDMS and provides the semicircular shape. The chips for use on the microscope were fabricated in a rectangular shape with an open-top casting form. The PDMS was cured at 150°C for 6 min. Holes were punched in the patterned PDMS half using a 1.5 mm outer diameter biopsy puncher (Miltex, Pennsylvania, U.S.A.) to form the inlets. The layers were then bonded together by adhesive bonding using the PDMS curing agent.[122] A blank 100 mm carrier wafer was spin-coated with PDMS curing agent for 30 s at 6000 rpm. Subsequently, the patterned PDMS half was stamped onto the coated carrier wafer and then manually aligned on top of the flat PDMS half. After curing for 24 h at room temperature the tip of the chip was cut off with a utility knife opening the outlet nozzle. It has a rectangular shape with the dimensions of approximately 40 µm × 25 µm (Figure 5.1). Finally, the microfluidic channels were silanized by flushing for 20 min with a stream of dry N₂ carrying 1H,1H,2H,2H-perfluorodecyltrichlorosilane to achieve a more robust droplet generation. All steps of the chip fabrication are shown in detail on the Figures 5.2 and 5.3. The entire chip design including all feed lines is shown in Figure 5.4.

Figure 5.1: SEM image of the nozzle. Scale bar 20 µm.
5.3 Experimental section

Figure 5.2: **Fabrication process of the patterned PDMS half.** A partially round casting form b) is placed on top of a patterned silicon wafer a). c) PDMS poured in to the casting form and is cured by heat. d) The casting form is carefully removed and the PDMS slab is peeled off the wafer.

Figure 5.3: **Assembly of the microfluidic chip.** Holes are punched b) in the patterned PDMS half a). d) The patterned PDMS half is stamped onto the coated carrier wafer and then manually aligned on top of the flat PDMS half c). The chip is cured for 24 h at room temperature to complete the bond. f) In the last step the tip of the chip is cut off, opening the outlet nozzle.

Figure 5.4: **Scheme of the chip design.** Channels for PFH and aqueous sample are shown in orange and blue, respectively. Scale bar 500 µm.
5. **A New microfluidics-based droplet dispenser for ICPMS**

5.3.5 **Microfluidic chip operation**

All fluids were supplied using a neMESYS syringe pump (Cetoni, Korbussen, Germany). The aqueous sample solutions were added with a 1 mL Primo syringe (Codan, Lensahn, Germany), at flow rates of 0.3 – 1 µL min\(^{-1}\). PFH for generation and acceleration of droplets was delivered at flow rates between 30 and 100 µL min\(^{-1}\) with 5 µL syringes (B. Braun, Melsungen, Germany). Fluids from the syringes were transferred to the LADE chip using PTFE tubing (PKM SA, Lyss, Switzerland). After the flows were started, 3 – 5 min was required to fill the microfluidic channels with liquid and to stabilize the droplet generation.

5.3.6 **Optical droplet measurements**

Bright-field microscopy was performed on an Olympus IX71 inverted microscope (Tokyo, Japan) with a Miro M110 high-speed camera (Vision Research, New Jersey, U.S.A.). The LADE chips for microscopic use were mounted horizontally on a custom-made microscope insert. This insert holds the chip in place above a plastic Petri dish, which collected the ejected liquids. The high-speed recordings were analyzed using the droplet morphometry and velocimetry software[231] to obtain droplet size and droplet frequency statistics. All images were recorded at 10000 frames s\(^{-1}\) with an exposure time of 10 µs. Before each measurement the system was given at least 3 min to stabilize. The droplet size and frequency for various flow rates of deionized H\(_2\)O and PFH were measured twice for 1 s on two different chips (in total four measurements). The average of the frequency and droplet size of these four measurements was calculated. The experiments with deionized H\(_2\)O, Merck IV solution, and PBS were performed on three different chips. Each solution was measured once for 1 s, and the average droplet size and frequency were calculated.

5.3.7 **Droplet transport system**

The setup of the droplet transport system is shown in Figure 5.5. The droplet jet was ejected vertically into a custom-built adapter made of poly(methyl methacrylate) (PMMA). A cyclonic gas flow of He supplied through the adapter transported the droplets further into a vertically arranged stainless steel tube with 6 mm inner diameter and 50 cm length. A cartridge heater placed in the middle of the steel tube was used to accelerate the solvent evaporation and to reduce the total droplet size permitting its further
transport. A poly(vinyl chloride) (PVC) tubing was used to connect the steel tube with a membrane desolvator (CETAC6000AT\(^+\) (only the desolvator unit), CETAC Technologies, Nebraska, U.S.A.), whose operating parameters are summarized in Table 5.1. The dry droplets were introduced into the ICP after admixing with Ar via a laminar flow adapter to maintain stable operating conditions within the ICP. Besides the microfluidic chip, a commercially available microdroplet dispensing system consisting of a dispenser head, a control unit, and a CCD camera for the droplet visualization (MD-K-150 with control unit MD-E-201-H, Microdrop Technologies GmbH, Norderstedt, Germany) was used with the same transport setup for comparison experiments.
5. A New microfluidics-based droplet dispenser for ICPMS

Table 5.1: Optimized operating parameters of the transport system and ICPMS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartridge heater</td>
<td>30 W</td>
</tr>
<tr>
<td>He gas flow rate</td>
<td>0.6 – 0.8 L min⁻¹</td>
</tr>
<tr>
<td>Desolvator membrane temperature</td>
<td>160 °C</td>
</tr>
<tr>
<td>Desolvator sweep gas flow rate</td>
<td>3 – 4 L min⁻¹</td>
</tr>
<tr>
<td>Ar gas flow rate</td>
<td>0.1 L min⁻¹</td>
</tr>
<tr>
<td>ICP plasma power</td>
<td>1300 W</td>
</tr>
</tbody>
</table>

5.3.8 ICPMS

A commercial quadrupole-based ICPMS (ELAN6000, PerkinElmer, Massachusetts, U.S.A.) was employed in this work. Its operating conditions are summarized in Table 5.1. The data was read out every 10 ms with 3 ms interval in between, resulting in the measurement duty cycle of 77 %.

5.3.9 Data evaluation

The MS-signals generated by droplets were evaluated using OriginPro 8.6 (Origin-Lab Corp., Massachusetts, U.S.A.). The signals were plotted as frequency distribution histograms, which were subsequently fitted with the Gaussian function. The mean and $\sigma$ of the fit represented the mean signal intensity and its standard deviation (SD), respectively. The transport efficiency was derived from the total number of droplet events detected per total effective measurement time (only the dwell time) and the droplet production frequency. The total number of droplets was estimated summing up all the transient signals. The intervals for signals produced by one, two, and more droplets were chosen based on minima of their frequency distribution histogram.

5.4 Results and discussion

5.4.1 Microfluidic device

Microfluidic Device. A photograph of the LADE chip is shown in Figure 5.6a. It is made entirely of PDMS by means of standard soft lithography. One part of the chip was
5.4 Results and discussion

(a) Photograph of the LADE chip next to a one Swiss franc coin for scale. (All fluidic channels are filled with blue food dye for visualization. The round part of the chip can be directly inserted into a socket of the adapter. The rectangular part of the chip contains the three access holes for sample and PFH introduction. (b) Rendering of the two components of the LADE chip.

Figure 5.6: (a) Photograph of the LADE chip next to a one Swiss franc coin for scale. (All fluidic channels are filled with blue food dye for visualization. The round part of the chip can be directly inserted into a socket of the adapter. The rectangular part of the chip contains the three access holes for sample and PFH introduction. (b) Rendering of the two components of the LADE chip.

designed to be cylindrical so that it fits tightly into the inlet of the droplet transport system. To achieve this round shape a custom-made casting form was produced. The chip is composed of two halves (Figure 5.6b). Due to the low cost of the material (approximately $2 per chip) and the fast fabrication time (about 15 min hands on time per chip, excluding the wafer fabrication) the use of a new chip for every experiment is feasible, which eliminates cross contamination and the need for time-consuming cleaning. Furthermore, the fabrication technique allows for fast changes of the design and the integration of additional microfluidic components.

Figure 5.7a shows a scheme of the key microfluidic features of the LADE chip. In the first channel junction, monodisperse droplets of an aqueous sample solution are generated. The aqueous phase is segmented by flow focusing using the immiscible and highly volatile PFH (boiling point 58 – 60 °C).[232] Size and frequency of the droplets can be controlled by the flow rates of the aqueous phase and the PFH. The second junction is used to add more PFH in order to increase the flow speed to at least 1 m s⁻¹, which is necessary for the ejection of the liquid in a stable and straight jet (Figure 5.7b and the Supplementary Information movie). This double-junction design allows controlling the jet stability independent from droplet generation, enabling the production of a broader range of droplet sizes. Furthermore, this concept simplifies the integration of further microfluidic components. It is also an advantage that the liquid sample droplets do not get into direct contact with the nozzle, which prevents clogging of the nozzle by dried residues. After ejection, the PFH carrier phase breaks into small droplets, whereas the aqueous droplets remain intact inside a PFH shell (Figure 5.7c). Fragmentation of the aqueous droplets during the ejection was not observed under the microscope.
5. A New microfluidics-based droplet dispenser for ICPMS

Figure 5.7: (a) Scheme of droplet generation and acceleration. In the first junction monodisperse aqueous droplets are generated in the stream of PFH. To accelerate the droplets, further PFH is added at the second junction. Subsequently, the droplet stream exits the LADE chip through the nozzle. Arrows indicate the direction of the flows for liquid streams. (b) Micrograph showing the on-chip droplet generation and acceleration as well as the ejection from the chip. Scale bar 500 µm. (c) Micrograph of an aqueous droplet and its surrounding PFH after ejection from the LADE chip. Scale bar 100 µm.

5.4.2 System characterization

The droplet sizes and frequencies were characterized with a high-speed camera and an automated image-processing program. The results (shown in Table 5.2) from the two chips demonstrate the droplet monodispersity and a low chip-to-chip variation. The size of the on-chip produced aqueous droplets ranges from 40 – 60 µm in diameter (30 – 110 pL). However, the droplet size range can easily be extended by changing the channel height or chip design. The influence of the dissolved substance in the aqueous phase on the droplet generation was insignificant (see Table 5.3). This indicates that the system can potentially be used for various aqueous solutions without the need for an individual measurement of the frequency and size.

A transient MS-signal generated by one single droplet is very short and lasts only a few hundreds of microseconds.[233] These signals could not be temporally resolved with the MS used (10 ms dwell time). The transient signals of single droplets containing Ce nitrate solution and their frequency distribution histogram are shown in Figure 5.8, parts a and b. The double distribution (not including the first tailing peak) is the result of a high temporal jitter in droplet arrival at the ICP (>10 ms) and unsynchronized signal
Table 5.2: **Droplet size and frequency for various flow rates measured twice for 1 s on two separate chips, with H$_2$O as aqueous phase.**

<table>
<thead>
<tr>
<th>Flow rate pure H$_2$O [µL min$^{-1}$]</th>
<th>Flow rate PFH droplet generation [µL min$^{-1}$]</th>
<th>Flow rate PFH droplet acceleration [µL min$^{-1}$]</th>
<th>Average droplet diameter [µm]</th>
<th>Average droplet frequency [Hz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>35</td>
<td>35</td>
<td>57 ± 2</td>
<td>89.4 ± 3.8</td>
</tr>
<tr>
<td>0.3</td>
<td>40</td>
<td>40</td>
<td>54 ± 1</td>
<td>104.1 ± 16.7</td>
</tr>
<tr>
<td>0.3</td>
<td>50</td>
<td>50</td>
<td>47 ± 1</td>
<td>158.8 ± 22.6</td>
</tr>
<tr>
<td>0.3</td>
<td>60</td>
<td>60</td>
<td>44 ± 1</td>
<td>181.1 ± 24.1</td>
</tr>
<tr>
<td>0.5</td>
<td>35</td>
<td>35</td>
<td>57 ± 1</td>
<td>158.2 ± 10.1</td>
</tr>
<tr>
<td>0.5</td>
<td>35</td>
<td>40</td>
<td>58 ± 2</td>
<td>158.8 ± 31.0</td>
</tr>
<tr>
<td>0.5</td>
<td>35</td>
<td>50</td>
<td>57 ± 1</td>
<td>155.8 ± 5.6</td>
</tr>
<tr>
<td>0.5</td>
<td>40</td>
<td>40</td>
<td>55 ± 1</td>
<td>168.7 ± 17.6</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>50</td>
<td>49 ± 1</td>
<td>251.7 ± 36.8</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>60</td>
<td>43 ± 2</td>
<td>288.1 ± 24.7</td>
</tr>
</tbody>
</table>

acquisition. It corresponds to the signal of one (630 ± 50) or two (1250 ± 70) droplets detected within the dwell time of 10 ms. Table 5.4 summarizes the relative standard deviation (RSD) of signals of single droplets for several other isotopes measured using a multielement solution at a concentration of 1 mg kg$^{-1}$.

The signal precision is not only a function of the variation in droplet volume but also of the multiplicative noise related to the transport system and the ICP and of the Poisson noise.[234] An additional source leading to a broadening of the signal distribution is the incomplete signal detection due to splitting of single-droplet signals between the dwell time (10 ms) and quadrupole settling time (8 ms). This can be eliminated employing continuous, time-resolved detection.[155, 235] A signal RSD as low as 8% was achieved, which is comparable to the RSDs obtained using the commercially available microdroplet generator system and indicates high monodispersity of the aqueous droplets generated by the chip.

The first peak of the intensity distribution histogram (Figure 5.8b from 0 to 25 counts/10 ms) cannot be attributed to the background only because of its longer tailing in comparison to the signal measured with the aqueous phase switched off, which amounted to only 2.8 ± 1.9 counts. The ratio of the mean of this peak to the mean of
Table 5.3: Droplet size and frequency for three different solutions. Flow rate of PFH for droplet generation and acceleration was 35µL min$^{-1}$ each.

<table>
<thead>
<tr>
<th>Flow rate aqueous sample [µL min$^{-1}$]</th>
<th>Pure H$_2$O droplet frequency [Hz]</th>
<th>Pure H$_2$O average droplet diameter [µm]</th>
<th>Merck IV droplet frequency [Hz]</th>
<th>Merck IV average droplet diameter [µm]</th>
<th>PBS droplet frequency [Hz]</th>
<th>PBS average droplet diameter [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>90.7</td>
<td>55 ± 2</td>
<td>90.1</td>
<td>57 ± 1</td>
<td>90.2</td>
<td>57 ± 1</td>
</tr>
<tr>
<td>0.5</td>
<td>145.5</td>
<td>56 ± 1</td>
<td>144.9</td>
<td>58 ± 1</td>
<td>146.7</td>
<td>58 ± 1</td>
</tr>
</tbody>
</table>

Table 5.4: RSDs of the signals generated from single droplets consisting of the multi-element solution. The droplets were produced using 0.5, 60 and 60µL min$^{-1}$ of aqueous solution, generating PFH and accelerating PFH, respectively.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{23}$Na</td>
<td>11%</td>
</tr>
<tr>
<td>$^{55}$Mn</td>
<td>8%</td>
</tr>
<tr>
<td>$^{56}$Fe</td>
<td>11%</td>
</tr>
<tr>
<td>$^{95}$Mo</td>
<td>13%</td>
</tr>
</tbody>
</table>

The next peak, which is produced by the single droplets, was element-specific and varied with operating conditions of the chip and the He gas flow rate. The appearance of the first peak can be mainly explained by disruption and fragmentation of the droplets during the transport and, additionally, by washout of a remaining aqueous solution by PFH plugs, or other memory effects within the transport system. Further studies are required to investigate this phenomenon in more detail.

The TE of the droplets depended strongly on the He gas flow rate and reached its maximum at $\approx$65% (Figure 5.9). The 58% maximum TE achieved using the commercial dispenser, which produced aqueous droplets in the size of 72.1 ± 0.5µm at 10Hz, suggests that the number of droplets delivered to the ICP is strictly limited by the transport assembly and not by the chip itself. The temporal jitter of droplets generated by the microdroplet dispenser was also significantly higher (>10 ms) than that reported using horizontal and vertical transport assemblies operated at ambient temperature.[220] Only a minor change in the droplet TE was observed varying the flow rate of the Ar sweep gas.
5.4 Results and discussion

Figure 5.8: (a) Transient signals generated by droplets consisting of the Ce nitrate solution and (b) their frequency distribution histogram. The first and the second fitted peaks correspond to the signals of one and two droplets acquired within 10 ms dwell time, respectively. The Gaussian function was fitted to find the mean and SD of the signals. The droplets were produced using 0.3, 35 and 35 µL min\(^{-1}\) of aqueous solution, generating PFH, and accelerating PFH, respectively.

(Figure 5.9). The current TE and high variation in arrival times of droplets at the plasma can be improved by optimizing the geometry of the transport system, shortening the total distance between the position of droplet ejection and the ICP, and implementing the desolvation at a very early stage.

To increase the analysis throughput a discrete sampling strategy is of an advantage. Similar to the cartridge sampling technique,[236] small volumes of different samples can be introduced into a capillary between PFH plugs which will prevent their intermixing. A series of droplets of each sample plug produced on the chip can be sequentially measured.
Figure 5.9: Droplet transport efficiency dependence on the flow rate of He carrier gas and Ar sweep gas. The droplets were produced using 0.3, 35 and 35 µL min\(^{-1}\) of aqueous solution, generating PFH and accelerating PFH, respectively. For the experiment with He the flow rate of Ar was set at 3 L min\(^{-1}\) and for the experiment with Ar the flow rate of He was set at 0.6 L min\(^{-1}\).

Approximately 0.5 µL of the multielement solution was measured in this way, and the signal of \(^{56}\)Fe was monitored. The signal distribution histogram is shown in Figure 5.10. Even such a small liquid aliquot could still be measured and produced signals with an RSD of 11 %. Approximately 8 % of this RSD can be assigned to the contribution from counting statistics.[237]

5.4.3 Cell analysis

The new system can be employed for introduction and subsequent analysis of single biological cells. First, tests were carried out using 6 – 7 µm in diameter bovine/calf red blood cells suspended in PBS. The suspension was diluted in PBS to a concentration of \(1 \times 10^7\) cells mL\(^{-1}\) ensuring that only 7 % of the droplets carry more than 1 cell. The sample was introduced at a rate of 0.5 µL min\(^{-1}\), and the flow rates of the PFH generating and accelerating streams were 40 µL min\(^{-1}\). Transient signals generated by single cells and their frequency distribution histogram are shown in Figure 5.11, parts a and b. A frequency distribution histogram of PBS signals without cells can be found in Figure 5.12. The TE of the cells was significantly lower (4.5 %) than the TE obtained with droplets.
5.4 Results and discussion

Figure 5.10: **Frequency distribution histogram of signals generated by single droplets.** Approximately 0.5 µL of the multielement solution was injected. The Gaussian function was fitted to find the mean and SD of the signals. The droplets were produced using flow rates of 0.5, 40 and 40 µL min\(^{-1}\) of aqueous solution, generating PFH, and accelerating PFH, respectively.

containing multielement solution and degraded during the measurement due to the cell precipitation in the syringe and the capillary. No clogging of the microfluidic channels by cells after operation was detected under the optical microscope.

The size of the red blood cells is larger than the size of the completely desolvated salt particle, which would be \(\approx 1 \mu m\) for a 50 µm droplet generated from 1 mg kg\(^{-1}\) multielement solution used in this work. Additionally, the high salt content of the PBS will result in an even larger residue of the droplet carrying the cell after liquid evaporation. Therefore, the low TE is most likely a result of losses in the transport assembly, whose length was relatively long and should be further optimized specifically for applications involving cells. Nevertheless, the RSD of the \(^{56}\text{Fe}^+\) signals produced by single cells from the detected cell population amounted to 23% (Figure 5.11b).

The average number of Fe atoms/cell of 5.3 \(\pm 1.2 \times 10^8\) was determined based on calibration of the instrument with droplets consisting of the multielement solution. The cells and the standard solution were measured using two different chips. The mean signal intensity of the first background peak was subtracted from the mean signal generated by the single cells. The means were determined by fitting the distribution histograms with the Gaussian function. The first peak originates mostly from the ArO\(^+\) polyatomic species interfering on the measured mass-to-charge ratio (m/Q); however, the contri-
Figure 5.11: $^{56}$Fe$^+$ transient signals generated by single red blood cells and (b) their frequency distribution histogram. The droplets were produced using flow rates of 0.5, 40 and 40 µL min$^{-1}$ of aqueous solution, generating PFH, and accelerating PFH, respectively. The distribution of Fe released from the cells to its tailing cannot be excluded. Employing the non-matrix-matched standard, the content of Fe per cell was underestimated by 25% compared to $6.2 \pm 0.6 \times 10^8$ atoms/cell calculated from the cell concentration and bulk concentration of Fe. This under-estimation can be caused by incomplete vaporization of droplets residues carrying cells in the plasma[238] or by cell hemolysis.

It has already been demonstrated that the element content of individual cells can be quantitatively determined using calibration with solid particles[239] and monodisperse aerosols.[240] Recently, monodisperse microdroplets consisting of an inorganic salt standard solution were utilized for quantitative mass determination of single metallic nanoparticles.[220] This approach can be directly transferred to the quantification of naturally occurring or exogenous elements in single cells and quantification of the proteins after
chemical and biospecific labeling.[241] The recent developments in metal-based labeling of the cell proteome[242] and in the field of mass cytometry[243] have enabled multiplexing in the single-cell analysis and extended the use of the ICPMS to cell biology. The single-droplet-based calibration approach can expand the capabilities of the mass cytometry toward the absolute protein quantification in the cell, provided the labeling can be performed quantitatively. The results obtained with the microfluidic system demonstrate its potential toward quantitative metallomics and proteomics in bulk or on the single-cell basis. For a more accurate quantification, the system can be further modified in the way that the sample and standard can be introduced from the same chip in parallel or sequentially.

5.5 Conclusions

A novel droplet microfluidics-based sample introduction system for the ICPMS analysis of micro samples is described. The droplets generated in the microfluidic chip are highly monodisperse and can be produced in the size range from 40 – 60 µm. This size range, however, can be further extended by modifications of the microfluidic channels. The aqueous droplets ejected from the chip in the stream of PFH remain intact and can
be transported into the ICPMS via a custom-built transport system with >50% efficiency. The ongoing work in our lab is currently focused on the improvement of this system to achieve complete and more stable sample transport. The proposed sample introduction system demonstrated its potential for the analysis of liquid volumes of <1 µL and for the quantitative elemental analysis of single cells. In this respect, the development of a chip capable of generating the droplets of sample and standard sequentially or in parallel[244] would be highly beneficial for the accurate quantification. In addition, the integration of a multichannel sample introduction can potentially be used for the internal standardization or to increase the measurement throughput by running more than one sample in parallel. This parallel approach will be valid only if isotope signatures of the samples are different. Furthermore, such an approach would require a simultaneous mass spectrometer. This new introduction technique has also a potential for the integration of further microfluidic modules for sample pretreatment, e.g., separation,[45, 245, 246] dilution,[247–249] fast mixing,[38] chemical reactions,[250] or cell sorting.[251, 252] In addition, cells encapsulated inside the droplets can be lysed to reduce the volume of the cell residua and to possibly improve their transport efficiency.
5.6 Improvements and further developments of the LADE-chip

The following sections contain preliminary studies (not part of the original publication) focused on further advancements and improvements of the LADE-chip as sample introduction system for ICPMS. These studies exploit the fact, that the LADE-chip is a versatile microfluidic platform, which can easily be adapted to various needs. Changes to the designs of the chip and the integration of further microfluidic functionalities enable an improved sample preparation and supply. Here, four concepts to broaden the scope of applications for the LADE-chip are investigated.

The first concept explores the maximum throughput of aqueous sample and maximum frequency of droplet generation on the initial LADE-chip. In a second approach a modified chip with an on-chip mixing function for sample pretreatment is presented. Another concept investigates the reduction of the PFH consumption by downsizing of the nozzle cross-section. In the fourth approach the simultaneous introduction of two different droplet species to the ICPMS is tested. The following sections introduce these four concepts in detail. Unless otherwise stated, all experiments were performed using the equipment and techniques described in chapter 5.3.

5.6.1 High frequency droplet generation

One method to increase throughput of the LADE-chip is to increase the droplet frequency by raising the sample flow rate. In the previous studies (see chapter 5.2) the droplet frequency was limited by the MS. It was tried to match the droplet frequency to the acquisition frequency (approximately 100 Hz) to record signals of single droplets. At higher frequencies it was not possible for the instrument to distinguish between individuals droplets. But reports about instruments operated at acquisition rates of up to $10^5$ Hz show that there is a need for a higher dispensing frequency.[253]

The previously introduced chip (see chapter 5.2), was used with modified flow rate settings to generate droplets of ultra pure water at the highest possible frequency. It was found that the highest flow rate settings at which the chip works reliable for a longer period is 6, 60 and 60 µL min$^{-1}$ of aqueous solution, generating PFH, and accelerating PFH, respectively. Droplets with an average diameter of $35 \pm 1 \mu m$ are formed at a frequency of $4601 \pm 61$ Hz (measured on-chip). This is an $16 \times$ increase of droplet throughput compared to the previous maximum of $288.1 \pm 24.7$ Hz. Additionally, it was observed that
with these settings only monodisperse PFH droplets with a diameter of 100 ± 1 µm, containing the monodisperse aqueous droplets are ejected from the chip (see Figure 5.13). At lower flow rates typically a straight jet is formed in between the droplets (see Figure 5.7). The droplets have a profound impact on the jet formation. It seems that the aqueous droplets cause an interruption of the jet and accumulate the PFH around them. At a sufficiently high droplet frequency, there is not enough time to re-establish the jet before the next droplet is ejected. So far, little is known about the behavior of multiphase droplet jets and they should be studies in more detail.

Figure 5.13: Monodisperse droplets ejected from the LADE-chip at a frequency of 4601 ± 61 Hz. The droplets were generated at flow rates of 6, 60 and 60 µL min⁻¹ of aqueous solution, generating PFH, and accelerating PFH, respectively. Scale bar 50 µm.

For short times even higher flow rates are possible (up to 9, 60 and 60 µL min⁻¹ of the aqueous phase, PFH for droplet generation and acceleration, respectively). Frequencies of up to 7000 Hz can be achieved, but the majority of chips delaminates after a couple of minutes at these settings. Other chip fabrication methods or materials might help to overcome this limitation. Also the integration of multiple ejection nozzles on one chip could help to increase throughput even further (see chapter 5.6.4).

This preliminary study clearly shows the potential of the LADE-chip as high frequency droplet dispenser. The LADE-chip can generate and eject droplets at kilohertz rates and would be a suitable system for spectrometers with high acquisition rates.

5.6.2 On-chip sample preparation

Mixing of a sample with another substance is one of the most common sample preparation steps. Standards or reagents can be added to a sample or the sample is simply diluted for the following processing step. Conventional this is performed offline by manual or automated pipetting, but also online dilution methods are known.[144, 254] Here, microfluidics with the ability of fast mixing can help to increase throughput and reduce complexity of experiment.[38] Therefore, it is of great interest to implement this processing step by the means of microfluidics into the LADE-chip. It could be used to
5.6 Improvements and further developments of the LADE-chip

increase throughput and simplify the online dilution of samples exceeding the maximum of allowed total dissolved solids, for example from digests or high matrix samples. Furthermore, it could also be used to add an internal standard or to perform online isotope dilution experiments to enhance the performance.[254]

For this purpose, an on-chip mixer design (see Figure 5.14) which incorporates a second inlet for another aqueous phase was developed. It is based on a mixing design by Song et al.[38]. Just before the junction for the encapsulation in a droplet, the channels of the two aqueous phases merge into a single channel. Inside the droplet, the aqueous phases are mixed within milliseconds. Compared to the original LADE-chip design (see Figure 5.4) the droplet generation junction was moved further away from the nozzle. The droplets stay for a longer time on-chip to give sufficient time for the mixing prior to the ejection. Also it is supposed to reduce the influence from disturbances of the jet to the droplet formation, for example when excess of PFH is removed from the tip of the chip. Additionally, the part where the droplets are formed is not covered by the cyclonic adapter and an observation of the droplet formation is possible.

![Design of the on-chip mixing LADE-chip](image)

Figure 5.14: Design of the on-chip mixing LADE-chip. Scale bar (red) 500 µm

The on-chip mixing LADE-chip (channel height 40 µm) was successfully tested to mix two streams of ultra pure water on-chip (see Figure 5.15) and to eject the droplets from the chip. Preliminary measurements of the droplet frequency and size showed that they are comparable with those of the original LADE-chip (see Table 5.2). So far only a 1:1 mixing ratio of liquids was tested. Theoretically the mixing ratio should be limited by the pumping precision and diffusion. The design was also tested for the sequential introduction of two samples, where only one of the two channels is supplying aqueous sample at a time. Individual channels can be used to generate and eject droplets, but currently there is no information about a possible cross-contamination of samples.

The on-chip mixing capability was successfully integrated into the LADE-chip. First experiments indicate that chip can be used to perform dilutions or to add standards.
5. A New microfluidics-based droplet dispenser for ICPMS

Figure 5.15: Droplet generation on the on-chip mixing LADE-chip, with two aqueous phases. Scale bar 25 µm.

5.6.3 Reduction of the PFH consumption

The reduction of the PFH consumption offers several benefits for the LADE-chip system. First, it can help to reduce the cost of the experiment. Additionally, less PFH ejected from the chip means that less has to be extracted by the desolvation system and potentially results in a more complete removal (if it is not limited by the desolvation system).

To achieve a stable ejection an exiting velocity of at least 1 m s\(^{-1}\) is required. By reducing the nozzle cross-section, the same exiting velocity can be achieved at lower flow rates. This reduction can be achieved by lowering the height of the channel that forms the nozzle. Three different wafer for channel heights of 5, 10 and 20 µm were fabricated (see chapter 2.1.2) and tested. The resulting chips had rectangular nozzle cross-sections of 25 µm \(\times\) 20 µm, 25 µm \(\times\) 10 µm and 25 µm \(\times\) 5 µm. Only the 25 µm \(\times\) 20 µm nozzle cross-section was reliably working at flow rates of 0.1, 35 and 35 µL min\(^{-1}\) aqueous phase, PFH for droplet generation and acceleration, respectively. In principle lower flow rates are possible for the droplet ejection, but the jet is not sufficiently stable for use with the ICPMS. Compared to the 25 µm \(\times\) 40 µm original nozzle with 40 µL min\(^{-1}\) of PFH for droplet ejection and acceleration, a total reduction of 10 µL min\(^{-1}\) was achieved and the jet stability was improved. It should be noted that the height reduction also results in the formation of smaller droplets on the chip. Due to the high flow resistance of the smaller channels the designs, 5 and 10 µm in height, showed delamination of the PDMS parts upon use. To overcome this issue, other chip materials and fabrication techniques should be considered, such as, glass etching or injection molding. Very recent advancements in the formation of aqueous droplets in gas as the continuous phase seem to be a promising approach to completely avoid the use of a liquid carrier phase, such as PFH.[255] For such a device no desolvation to remove the PFH would be required.
5.6 Improvements and further developments of the LADE-chip

The preliminary study demonstrates that the consumption of PFH can be reduced with smaller nozzle cross-sections. However, the high flow resistance of the smaller channels is challenging for LADE-chip fabricated from PDMS.

5.6.4 Dual-LADE-chip

The parallel introduction of two or more droplet species into the plasma is another approach to increase throughput. Multiple samples are introduced and measured at the same time. However, this is only possible if the isotope signatures are different. Parallel introduction also enables the measurement of a sample and the calibration solution simultaneously. This online calibration could also be used for the correction of instrument drifts. For this kind of experiment simultaneous measuring mass spectrometers, such as time of flight ICPMS instruments, are of interest with their ability to obtain a full spectrum of every droplet.[235, 256] Here, a modified LADE-chip with the capability of generating and injecting droplets of two aqueous samples simultaneously is introduced.

A chip design which incorporates two times a miniaturized version of the original design (compare Figure 5.4) was developed (see Figure 5.16). This chip is referred to as dual-LADE-chip in the text. The rear side of the chip was designed wider than the original design to accommodate the additional inlets. This provides sufficient distance between the inlets and prevent tearing out of inlet ports during fabrication. Hence, an adapted mold was developed (see supplementary Figure 5.17). At the tip of the chip two individual droplet generation junctions and two nozzles in a distance of 175 µm were implemented. To reduce the amount of PFH that is required to operate the two nozzles the design was fabricated in a height of 20 µm (see chapter 5.6.3). Figure 5.18 shows a image of an dual-LADE-chip.

The droplet generation and ejection was investigated on a microscope (see Figure 5.19). Frequency and droplet size were determined by automated image analysis. Diameter and frequency were measured on one chip for 2 s per channel. At a flow rate of the aqueous sample of 0.1 µL min$^{-1}$ and 35 µL min$^{-1}$ of PFH for droplet generation and acceleration (per channel), the average droplet diameter in both channels was 23 ± 1 µm and the average droplet frequency of both channels was 157 ± 7 Hz. The low variations of the frequency and droplet size indicate a low variation between the channels. Theses setting were used throughout the following ICPMS experiments. The ejection of the droplets occurs as two parallel jets (see Figure 5.20).

As proof of principle experiment one channel of the chip was used to generate droplets of a 1 ppm solution of cerium, while the other channel generated droplets of
5. A New microfluidics-based droplet dispenser for ICPMS

Figure 5.16: **Dual-LADE-chip design.** Scale bar (red) 500 µm.

Figure 5.17: **Aluminum casting forms for the dual-LADE-chip.** Scale bar 1 cm.
5.6 Improvements and further developments of the LADE-chip

Figure 5.18: **Image of a dual-LADE-chip.** Channels are filled with blue food dye for better visibility. Scale bar 1 cm.

Figure 5.19: **Micrograph of the dual channel on-chip droplet generation and acceleration.** Scale bar 500 µm.
a 1 ppm praseodymium solution (both solutions contained the nitrate salts of the respective elements and were supplied by Inorganic Ventures, Virginia, U.S.A.). With the previously described setup (see chapter 5.3) these droplets were introduced to the plasma of the ICPMS.[257, 258] The following settings were used: A He gas flow rate of 0.6 L min$^{-1}$, a cartridge heater power of 30 W, a desolvator membrane temperature of 160 °C, a desolvator sweep gas flow rate of 3 L min$^{-1}$ and a plasma power of 1300 W. The $^{140}$Ce or $^{141}$Pr signals were acquired in an alternating fashion with a dwell time of 10 ms and a 3 ms settling time in between. The frequency distribution histogram of the recorded signals is shown in Figure 5.21.

By fitting with the gaussian function the mean signal intensity and SD were obtained. For $^{140}$Ce it was $133 \pm 25$ counts/10 ms and for $^{141}$Pr it was $201 \pm 39$ counts/10 ms. Assuming a similar ionization and detection efficiency for both element signals, one expects that the ratio of the signal intensities corresponds to the ratio of the isotopic abundances. For the natural isotopic abundance of $^{140}$Ce (88.48 %) and the natural isotopic abundances $^{141}$Pr (100 %) the expected ratio is 0.88. However, the measured ratio of the two isotopes of $0.66 \pm 0.25$ is significantly lower, but it is still within the error. It should be noted that the background signal on the $^{141}$Pr was significantly higher than on the $^{140}$Ce (see Figure 5.21 first peak, around 0 to 75 counts/10 ms), which might have caused a shift of the mean signal intensity in the Pr channel. For this measurement an estimated TE of 15 % for $^{140}$Ce and 14 % for $^{141}$Pr was calculated. While these values are lower that those of the original LADE-chip, improved settings could help to achieve similar TE.

In general this first study indicates that the multi channel introduction with the dual-LADE-chip is possible, but optimization and further tests of the system are required.
5.6 Improvements and further developments of the LADE-chip

![Frequency distribution histograms of the signals of two different droplet species.](image)

Figure 5.21: **Frequency distribution histograms of the signals of two different droplet species.** One droplet species contains 1 ppm Ce (white) and the other species contains 1 ppm Pr (grey). Each channel was fitted with a Gaussian function to find the mean and SD of the signals. For the droplet generation, 0.1, 35 and 35 µL min⁻¹ of aqueous solution, generating PFH, and accelerating PFH, were used respectively.

5.6.5 **Conclusion**

These preliminary studies show that the LADE-chip is versatile and can be improved and adapted for various needs. The droplet generation frequency of the chip was increased by sixteen times. This makes the LADE-chip suitable for modern instruments with high acquisition frequencies. Furthermore, a 12% reduction of the PFH consumption and a more robust jet formation was achieved by downsizing of the nozzle cross-section. The functions of the chips have been extended by implementing designs for on-chip mixing and the parallel generation and ejection of two droplet species. Although only the last of these concepts was successfully demonstrated in experiments with the ICPMS, it clearly shows the potential that this system offers for future implementation of the other microfluidic modules. Therefore, it is indispensable to perform further optimizations and experiments.
6 Conclusion and Outlook
6. **Conclusion and Outlook**

In this thesis, microfluidic platforms for the manipulation and analysis of microcompartments were presented. With the help of a tailor-made microfluidic device the study of vesicle fusion was performed. A model system for the inner compartmentalization of cells was developed and a discrete sample introduction device for ICPMS was built. The unique control over these compartments that microfluidics offers enabled applications spanning from bioanalytics and biomimetics to elemental analysis.

6.1 **Platforms for artificial cell studies**

The novel microfluidic platforms set new benchmarks for artificial cell studies. One device allowed the exact spacial and temporal control over electrofusion of GUVs in a microfluidic chip and the other device introduces MVDs as a straightforward model system for cellular compartmentalization.

The electrofusion platform combines for the first time the positioning of GUVs by hydrodynamic traps with a set microelectrodes to initiate the fusion events. Therefore, a short pulsed electric field was applied perpendicular to the interface between the vesicles. The overall setup has been optimized to monitor the mixing of the membrane lipids with a FRET assay and the kinetics of a complexation reaction, initiated by vesicle fusion. In comparison to conventional micro-pipetting techniques for vesicle fusions the platform offers a superior throughput and is less complex to operate.

The system could be used to perform more detailed studies of lipid mixing in fused membranes. Of special interest are here the kinetics of lipid mixing and how various lipids or membrane proteins influence it. Furthermore, the platform should used to study biochemical reactions in biomimetic containers, as it was found in this study that the reactions in the vesicles happen at higher rates than in the bulk. Currently it not fully understood what causes this increase in reaction rate. Studying this effect could help us to a better understanding of processes in the cell membrane and inside the cell. The pairing of the GUVs, which in the current system happens statistically gives room for improvement. A double trap system specifically designed for pairing, as they are known for droplets, could be adapted to improve the throughput of the experiments.[173] This would also require to be able to address all electrodes individually by implementing more pads to connect the off-chip electronics. In the current design the electrodes can be addressed in sets of 9 traps at a time. With an improved system, kinetics of membrane mixing and reactions in ultra small biomimetic compartments could be measured. It is also feasible to perform and investigate fusions of cells with vesicles or cells with cells. Another ap-
6.1 Platforms for artificial cell studies

An approach to improve the system would be the integration of microfluidic techniques for the formation of GUVs directly on the chip. This could be done by the emulsion transfer[66] or electroformation method[259]. Such a device would be able to perform all tasks from sample preparation to analysis in a single LoC improving reproducibility and throughput by minimizing human interaction. The electrodes, currently only used for the initiation of the fusion events could also take over other tasks. The hydrodynamic traps for example could be substituted with a dielectrophoretic trap using electric fields to align the GUVs for electrofusion.[260] Also the monitoring of a reaction initiated by fusion could be done electronically. Impedance spectroscopy could be used to measure changes in vesicle size and reactions within them. Such a fully electronically setup could be operated with no need for a microscopy setup.

The second platform was developed to produce and analyze MVDs, which are a novel model system to study cellular compartmentalization. MVDs are generated in a straightforward process by encapsulating GUVs in monodisperse droplets. The platform also enables the mixing with various compounds to perform compartmentalized enzyme cascade reactions. In comparison to approaches that employ MVVs as model system, the MVDs offer a better control of loading and are easier to generate. For an analysis of the GUVs in droplets by fluorescence microscopy a trapping array was adapted. The presented setup is modular, flexible, and can be adapted to perform many kinds of biochemical reactions. When the outer membrane is not of particular interest, the MVDs are a convenient and versatile tool to study the effects of compartmentalization of cellular systems.

This cell model system could in future be used to study all kinds of cellular processes that are subjected to compartmentalization by lipid bilayers like the production of glucose in chloroplasts or the storage of substances in vacuoles. Reactions that span across multiple vesicles, each performing a different part of the reaction, could be conducted. The individual vesicles could be equipped with functionalities, to make them act like organelles. One vesicle for example could produce adenosine triphosphate like a mitochondrion, while in another vesicle this adenosine triphosphate is consumed to build up proteins as the golgi body. Therefore, it is desirable to increase the time for observations by reducing the shrinkage of the droplets. Shrinkage could be minimized by avoiding PDMS as material for the microfluidic device. Apart from the use as a cell model system, the vesicles could also be co-encapsulated with cells inside of droplets. The used carrier phase and surfactant are known to be compatible with cell encapsulation.[201] The vesicles could supply the cells with nutrients or other substances, where they could release these substance when they get stimulated by a cellular signal or an external stimulus. Additionally, the vesicles could serve as sensor probes responding, for example, with...
an increase of fluorescence to substances excreted by the cells.

6.2 The synergy of droplet microfluidics and ICPMS

The novel droplet microfluidics sample introduction device for ICPMS opened new possibilities for the analysis of droplet contents and has several advantages over conventional introduction systems. A cheap and disposable microchip generated highly monodisperse droplets of sample solutions or suspension over a wide range of sizes and frequencies. The sample droplets were ejected from the chip and by a custom transport system delivered to the plasma (>50% efficiency). Elemental analysis of single cells and liquid sample volumes <1 µL was demonstrated. Concepts for advanced on-chip sample pretreatment and multiplexed introduction were tested.

In the future this introduction system could be used to introduce cells for mass for mass cytometry experiments, where current systems struggle when complex matrices are used. Also it has the potential for the introduction of nanoparticles, where the disposable chip could help to eliminate laborious cleaning of the setup. To overcome the currently incomplete sample transport, an improved transport system should be developed. This could be achieved by reducing the length of transport and implementing the desolvation system directly into the falling tube. Also the adapter which connects the chip with the transport system should be modified to tolerate a wider range of ejection angles and to reduce the loss of sample. As shown in the preliminary studies in chapter 5.6 the chip functions can be enhanced and the integration of further microfluidic modules is possible. In follow-up experiments these chips should be tested in detailed concept studies. Furthermore, other sophisticated sample treatment steps can be implemented, such as sample separation,[45, 245, 246] chemical reactions,[250] or cell sorting[251, 252]. Substantial improvements of the system could be achieved by the reduction or replacement of PFH as carrier phase. Reduction of the PFH consumption would diminish the experiment costs and lower the amount of PFH that has to be extracted by the desolvation system, which could lead to a more complete extraction. As described in chapter 5.6 a smaller nozzle cross-section can help to achieve a reduction of PFH flow rate required for a stable ejection. For even smaller nozzles other chip fabrication techniques have to be considered. Replacing PFH with another liquid compound, such as hexane, could reduce the polyatomic interferences and eliminate the corrosion of the skimmer cones caused by highly reactive PFH fragments. The material of the current PDMS chip is not compatible with substances like hexane. Hence, other chip materials and fabrication methods like glass etching or injection molding should be considered. The processing of hexane
also requires special precautions, to avoid the danger of explosions. Very recent advancements in the formation of aqueous droplets in gas as the continuous phase, may provide a method to completely avoid a liquid carrier phase.[255] In this way, a direct connection between the microfluidic droplet generation and the ICPMS setup, without a desolvation system could be achieved.
7 Appendix
7.1 A Microfluidic Chip for ICPMS Sample Introduction

Adapted from P. E. Verboket, O. Borovinskaya, N. Meyer, D. Günther, P. S. Dittrich: A Microfluidic Chip for ICPMS Sample Introduction, *J. Vis. Exp.* 97 (2015) e52525. with permission from the Journal of Visualized Experiments. Link to the article: http://dx.doi.org/10.3791/52525 P.E.V. wrote the manuscript. For further contributions see chapter 5. All authors discussed and approved the final manuscript.

7.1.1 Abstract

This protocol discusses the fabrication and usage of a disposable low cost microfluidic chip as sample introduction system for inductively coupled plasma mass spectrometry (ICPMS). The chip produces monodisperse aqueous sample droplets in perfluorohexane (PFH). Size and frequency of the aqueous droplets can be varied in the range of 40 to 60 µm and from 90 to 7000 Hz, respectively. The droplets are ejected from the chip with a second flow of PFH and remain intact during the ejection. A custom-built desolvation system removes the PFH and transports the droplets into the ICPMS. Here, very stable signals with a narrow intensity distribution can be measured, showing the monodispersity of the droplets. We show that the introduction system can be used to quantitatively determine iron in single bovine red blood cells. In the future, the capabilities of the introduction device can easily be extended by the integration of additional microfluidic modules.

7.1.2 Introduction

Elemental analysis of liquid samples by inductively coupled plasma mass spectrometry (ICPMS) is commonly carried out using nebulizers in combination with spray chambers as introduction system[206]. In this sample introduction system the sample is sprayed by a nebulizer to generate a polydisperse aerosol. A downstream spray chamber is used to filter out large droplets. This method is associated with high sample consumption (>0.3 mL min⁻¹)[208] and an incomplete sample transport. Thus, it becomes impractical for applications where only microliter sample volumes are available, as in biological, forensic, toxicological and clinical studies[209]. To reduce the sample consumption, nebulizers with smaller nozzle dimensions were developed[209]. However, the reduced
nozzle size increases the risk of clogging when samples of undigested biological fluids or concentrated salt solutions have to be analyzed [209].

A different approach for sample introduction was proposed by Olesik et al. [215]. The authors injected a liquid into ICPMS in the form of monodisperse discrete micro-droplets, which were produced by a piezo-electrically driven micropump. Even though this very system did not find wide application, it initiated the further development of the concept of discrete droplet introduction in ICPMS. Today, piezo-electrically driven dispensing systems, which can generate droplets in size of 30, 50, 70 and 100 µm and at frequencies of 100 – 2000 Hz, can be purchased. The droplets can be transported into ICPMS with close to 100 % efficiency [220]. These microdroplet dispensers have been applied for quantitatively measuring single nanoparticles [220, 221] as well as characterizing individual biological cells [222]. A similar system based on thermal inkjet technology [225] was tested for analysis of biological samples [226]. Although the available single droplet introduction systems are very efficient, can be used for small sample volumes and are promising for the analysis of nanoparticles and cells, they have several limitations. For a fixed nozzle size, the droplet size can be varied only slightly (unless custom settings are used [218]). Changes of the physical properties of the liquid (pH, salt content) can alter the droplet characteristics (size, injection speed). Also, these devices are rather expensive, prone to clogging and are difficult to clean.

Another method to generate droplets is known in the field of droplet microfluidics [27]. In recent years droplet microfluidics has gained interest for (bio-)chemical reactions [228, 229, 261, 262] and for single cell studies [6, 230]. Additionally, this technique was applied for introducing samples in electrospray ionization mass spectrometry [30, 31] and for preparing samples in matrix-assisted laser desorption/ionization mass spectrometry [29, 47].

Recently, we introduced a microfluidic based system for sample introduction in ICPMS [257]. The key component of our introduction system is the liquid assisted droplet ejection (LADE) chip. This chip consists completely of poly(dimethylsiloxane) (PDMS). In the first channel junction flow focusing is used to generate monodisperse droplets of an aqueous sample solution (Figure 7.1). For this purpose the highly volatile (boiling point of 58 to 60 °C [232]) and immiscible carrier phase perfluorohexane (PFH) is used (Figure 7.1). These PFH properties enable a stable droplet generation and fast removal of the carrier phase. Changes in the properties of the sample liquid influence this generation method less, compared to other droplet generators. The droplet size is adjustable over a wide range by changing the flow rates of the aqueous phase and the PFH. In a downstream secondary junction, more PFH is added to increase the flow speed to at least 1 m s⁻¹. At this
Figure 7.1: Micrograph of droplet generation, acceleration and ejection. In a flow focusing junction, monodisperse aqueous droplets are generated in the stream of PFH. Additional PFH is added at a second junction. Subsequently, the liquid stream is ejected from the LADE chip through a nozzle. Arrows indicate the direction of the flow. Scale bar is 500 µm. Inset: Micrograph of an aqueous droplet in a PFH shell after ejection from the LADE chip. Scale bar 100 µm. Adapted with permission from[257]. Copyright 2014 American Chemical Society. This figure has been modified with data from research conducted since publication in the laboratory of P. S. Dittrich.

speed the liquid can be ejected from the chip in stable and straight jet (Figure 7.1) without droplet destruction (Figure 7.1 inset). This double-junction design allows controlling the jet stability independent of droplet generation. The droplets are transported to the ICPMS with a customized transport system. This system comprises a falling tube and a membrane desolvator to remove the PFH. The dried residues of the aqueous droplets are subsequently ionized in the plasma of the ICPMS and a mass detector measures the ions. The front part of the chip is barrel-shaped to ensure a tight connection with the droplet transport system. The ejection of the aqueous sample as droplets in PFH is beneficial, because contact with the nozzle is avoided. This considerably lowers the risk of nozzle clogging, which can be a problem when working with cell suspensions or concentrated salt solutions. The LADE chips, fabricated by PDMS soft lithography, are cheap (material cost approximately $2 per chip), disposable and easy to modify. In combination with the fabrication that requires only a small amount of manual work each experiment can be performed with a new chip. Therefore, a laborious cleaning is not needed and cross contamination is minimized.

Here, the fabrication of the LADE chip by soft lithography and its application for ICPMS are described. Examples of measurements with an aqueous solution and a cell suspension are presented.
7.1.3 Protocol

1. SU-8 Master Fabrication (Figure 7.2)

![Diagram showing SU-8 processing steps]

Figure 7.2: **Schematic of the SU-8 processing.** First an adhesion layer is coated. A silicon wafer is spin coated with SU-8 2002, soft baked, flood exposed with ultraviolet light and post baked. On top of this layer the microfluidic structures are produced. The wafer is spin coated with SU-8 2050 and soft baked. The design of the microfluidic structures is transferred to the wafer by exposing it with ultraviolet light through a photomask. After a postexposure bake, the photoresist is developed and a hardbake is performed.

**NOTE:** Perform the fabrication of the SU-8 master molds in a clean room to prevent defects caused by dust particles. Two wafers are needed for the fabrication, one wafer with microfluidic features and one without.

1. Prepare the master molds for the microfluidic chip. First apply an adhesion layer to the silicon wafer.

   1. Dehydrate a silicon wafer for 10 min at 200 °C. Cool the wafer down to RT and load it on to a spin coater and spin coat it with SU-8 2002 with the following protocol.

   2. Dispense about 3 mL resist onto the wafer.

   3. Spin the wafer at 500 rpm for 10 s to spread the resist over the whole wafer.

   4. Spin the wafer at 2000 rpm for 30 s to achieve a resist height of approximately 2 µm.

2. Remove excess resist from the edge of the wafer with an acetone soaked swab, to prevent sticking of the wafer to the hot plate in the next step. Bake the wafer for 60 s at 95 °C on a hot plate.
Figure 7.3: Design of the photomask for the LADE chip containing the following features: a) Guiding structures for the casting form, b) an inlet for the PFH for droplet acceleration, c) an inlet for PFH for droplet generation and d) an inlet for the aqueous sample. e) Indicator line for cutting off the tip of the chip. Channel widths f) = 40 µm, g) = 20 µm and h) = 25 µm.

3. Expose the whole wafer with ultraviolet light (80 mJ cm\(^{-2}\) at 365 nm). Post-bake the wafer for 120 s to 95 °C.

4. Cool the wafer down and immediately spin coat the wafer again using the following protocol for SU-8 2050:

   1. Spin the wafer at 100 rpm for 20 s (dispense about 3 mL SU-8 resist during this step).
   2. Spin the wafer at 500 rpm for 10 s to spread the resist over the whole wafer.
   3. Spin the wafer at 3250 rpm for 30 s resulting in a resist thickness of approximately 40 µm.

5. Again, remove excess resist from the edge of the wafer with an acetone soaked swab and soft bake the wafer on a hot plate for 180 s at 65 °C and for 360 s at 95 °C.

6. Prepare the photomask by sticking it to a soda-lime glass. See Figure 7.3 for the mask design. Use a mask aligner to expose the resist with ultraviolet light (160 mJ cm\(^{-2}\), measured at 365 nm) through the prepared mask. Bake the exposed wafer again on a hot plate for 60 s at 65 °C and for 360 s at 95 °C.

7. After cooling down the wafer to RT, immerse it in a glass Petri dish filled with developer for 5 min to develop the resist. Gently agitate the petri dish to remove unexposed SU-8. Rinse the wafer with isopropanol and blow it dry with
7.1 A Microfluidic Chip for ICPMS Sample Introduction

a nitrogen gun.

8. Examine the wafer under a microscope. In case undeveloped resist remains on the features, develop the wafer again for a few minutes, as described in step 1.7.

9. Remove any residual solvent by baking the wafers for 2 h at 200 °C. Check the height of the features with a step profiler. In case the measured height differs from the desired height begin with this protocol again and adapt the spin speed in step 1.1.4.

10. To prevent sticking of the PDMS to the wafer silanize it by placing it in desiccator together with 50 µL of 1H,1H,2H,2H-perfluorodecyltrichlorosilane in a small porcelain dish. Reduce the pressure in the desiccator to 100 mbar and incubate the wafer for 12 h.

1. For the blank PDMS parts silanize another silicon wafer using the method of step 1.10. To save time silanize both wafers at the same time in a single desiccator.

2. LADE Chip Fabrication

NOTE: The LADE chip is made out of two PDMS pieces that are bonded together by adhesive bonding[122]. The first part contains the microfluidic features. The other part is flat and used to seal the channels. Bonded together, they form the round shape necessary to interface the chip with the droplet transport system. Here, the fabrication of the two parts and their bonding is described. All process steps are shown in Figure 7.4.

1. Prepare 44 g of PDMS by mixing 4 g of prepolymer with 4 g of the PDMS curing agent (this will result in up to 6 chips). Degas the PDMS in a desiccator until it is bubble free (this will take about 20 min).

2. Replica molding of the structured halves.

   1. Place the casting form on top of the wafer and snap it into place using the guiding structures around the design (see Figure 7.5). Skip the snapping into place for the flat PDMS halve.

   2. Pour approximately 3 to 4 g of the degased PDMS in the casting form and place it for 6 min on a hot plate at 150 °C. Cool down the cured PDMS in the casting form and carefully lift the casting form wafer using a spatula.

   3. In order to prevent any contamination of the microfluidic channels cover
Figure 7.4: Process chart of the LADE chip fabrication. First the structured and the flat PDMS parts are fabricated by replica molding. The two pieces are bonded together by adhesive bonding. Finally, the tip of the chip is cut off and the microfluidic channels are silanized. Adapted with permission from[257]. Copyright 2014 American Chemical Society. This figure has been modified since publication.
3. To fabricate the flat PDMS halves repeat the above-mentioned steps 2.2.1 to 2.2.3 with the blank silanized wafer.

4. Peel of the tape and punch fluid connection holes into the structured halves with a biopsy puncher. Protect the structures with tape during storage.

5. Bond the PDMS parts together by adhesive bonding using the PDMS curing agent[122].

1. Take an untreated silicon wafer and spin coat it with PDMS curing agent for 30 s at 6000 rpm. Take the wafer out of the spin coater.

2. Remove the tape from the structured halves and place them with the structures facing downwards onto the wafer. Gently push on top of the PDMS to remove air bubbles.

3. Remove the tape from the blank PDMS halves. Take a structured halve from the wafer and manually align it on top of the flat PDMS halve. Gently squeeze the part together to remove air bubbles and let the assembled chip
cure for 24 h at RT. Do not push the parts together with force as this can cause the channels to collapse.

6. Cut the tip of the chip along the indicator line orthogonal to the nozzle channel with a utility knife to open the outlet nozzle. Use an alignment device to ensure a straight cut, which is necessary for a straight liquid ejection. Inspect the chip under a microscope for defects in the microfluidic channels and dust particles. Put a tape over the inlet holes to protect the chips during storage.

7. Connect a Woulff bottle with tubing to a dry nitrogen source and to all inlets of the LADE chip. Deposit 50 µL of H,1H,2H,2H-perfluorodecyltrichlorosilane at the bottom of the Woulff bottle and close it.

8. Silanize the microfluidic channels by flushing all channels for 20 min with the nitrogen stream carrying H,1H,2H,2H-perfluorodecyltrichlorosilane at a flow rate of approximately 1 mL s⁻¹. The chips are ready for experiments and can be stored for at least several weeks at RT.

3. Preparations for Measurement / Droplet Transport System

NOTE: Build the whole droplet transport system on top of an optical table, since it is necessary to construct a stable supporting structure for the setup. A scheme of the whole droplet transportation system is depicted in Figure 6.

1. Install a custom cyclonic poly(methyl methacrylate) (PMMA) adapter with an 50 cm attached stainless steel tube vertically. Attach the adapter to a helium source with a mass flow controller. Attach a (high-speed) camera and a lamp to the adapter on the opposite sites for droplet visualization.

2. Place a cartridge heater in the middle of the steel tube and use poly(vinyl chloride) (PVC) tubing and a Legris tube connector to connect the end of the steel tube with the inlet of the membrane desolvator.

3. Connect the outlet of the desolvator with another PVC tubing to a laminar flow adapter, which is directly connected to the ICPMS inlet. Connect the laminar flow adapter to an argon source with a mass flow controller and later on use it to admix Argon to achieve a stable operation condition.

4. Align the adapter as well as the steel tube vertical with a spirit level. If the alignment is not accurate, it may lead to significant losses of droplets. Insert a plug into the adapter to prevent gases leaking out during the system warm up time.
5. Start all above-mentioned gas flows and devices using the settings from Table 7.1. Allow the system to warm up for 15 min. The cartridge heater needs 2 h to stabilize the temperature, switch it on in advance.

6. Place the syringe pumps on a rack at the height of cyclonic helium adapter. Keep the distance between the syringe pumps and the adapter as short as possible.

Table 7.1: **Start settings and recommended measurement settings for the ICPMS and the syringe pumps.**

<table>
<thead>
<tr>
<th>Setting</th>
<th>Start up</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>He gas flow rate</td>
<td>0.6 – 0.8 L min⁻¹</td>
<td></td>
</tr>
<tr>
<td>Cartridge heater</td>
<td>30 W</td>
<td></td>
</tr>
<tr>
<td>Desolvator membrane temperature</td>
<td>160 °C</td>
<td></td>
</tr>
<tr>
<td>Desolvator sweep gas flow rate</td>
<td>3 – 4 L min⁻¹</td>
<td></td>
</tr>
<tr>
<td>Ar gas flow rate</td>
<td>0.1 L min⁻¹</td>
<td></td>
</tr>
<tr>
<td>ICP plasma power</td>
<td>1300 W</td>
<td></td>
</tr>
<tr>
<td>Sample flow rate</td>
<td>1 µL min⁻¹</td>
<td>0.3 – 1.5 µL min⁻¹</td>
</tr>
<tr>
<td>Flow rate of PFH droplet generation</td>
<td>60 µL min⁻¹</td>
<td>35 – 80 µL min⁻¹</td>
</tr>
<tr>
<td>Flow rate of PFH droplet acceleration</td>
<td>60 µL min⁻¹</td>
<td>35 – 80 µL min⁻¹</td>
</tr>
</tbody>
</table>

4. **Measurements**

NOTE: The following protocol is written in general terms because of the variety of solutions and suspensions that can be used. However, cell suspensions should be diluted to a concentration of $<1 \times 10^7$ cells mL⁻¹, when single cell analysis is performed, to ensure that the majority of the droplets carry only one cell. For measurements with cells place the syringe pumps at an angle so that the outlet of the syringes point downwards and install the tubing in such a way that they point downwards.

1. Attach tubing to the syringes. Load two 5 mL syringes with perfluorohexane and one 1 mL syringe with a sample solution or suspension. Remove all bubbles trapped in the syringes and tubing.

2. Install the syringes in a syringe pump and connect them to the inlets of the chip. Start the syringe pumps using the start settings from Table 7.1 (or higher
Figure 7.6: Schematic drawing of the setup (not to scale). The system consists of the LADE chip, a cyclonic adapter, a heated steel tube, a membrane desolvator, and an ICPMS.
flow rates). Give the flows 3 to 5 min to stabilize.

1. Remove excess liquid from the tip of the chip with a tissue. The liquids should now eject from the chip in a straight jet. If a straight ejection cannot be achieved by wiping with a tissue replace the chip and start over with this step.

3. Remove the plug from the adapter and carefully insert the chip into the adapter. Lubricate the chip with FC-40 if necessary. A chip can be used for at least 2 h of experiments.

4. Change the flow rate to be within the recommended measurement settings from Table 7.1. Lower flow rate of the PFH not only saves PFH but also reduces signal background, caused by isobaric interferences.

5. Give the system 2 – 5 min to stabilize (depending on the chosen flow rates). Optimize the ICPMS for the highest signal intensity of the analytes of interest.

6. Set the ICPMS to a dwell time of 10 ms (applied for the very ICPMS used but can be adjusted with other instruments to ensure a time-resolved acquisition). Start recording the signal of a particular m/Q using manufacturer’s protocol.

7. After the measurement, transfer the raw data to data analysis program for evaluation. Bin the data, given in counts per 10 ms, with a built-in-function, and plot resulting bin center values against counts. Fit each peak in the plotted frequency distribution histogram with a Gauss function. The mean and sigma of the fit represent the mean signal intensity and its standard deviation, respectively.

5. Calibration Concept

1. Measure a single or multi-element standard solution containing the element or elements of interest at the same flow rates as the sample.

2. Place a LADE chip in a petri dish on a microscope. For a better image quality use a non-round chip. Fabricate this chip as described in step 2, but using a simple rectangular casting form instead of the partially round shaped one.
3. Follow the steps 4.1 to 4.2.1 to start the droplet generation. Set the flow rates to the flow rates used in step 5.1.

4. Record images of the aqueous droplets with a high-speed camera attached to a microscope (20× objective). Use an image analysis software like the droplet morphometry and velocimetry software by Basu[231] to obtain the average droplet diameter from the recordings.

5. Use average droplet diameter to calculate the droplet volume, assuming the droplet is a spherical object.
   
   1. From this volume and the known concentration of an analyte in the droplet calculate the number of corresponding atoms. Divide the number of measured ions per droplet by the number of atoms to obtain the detection efficiency. Use this detection efficiency to calculate the number of atoms in an unknown sample.

   NOTE: Since the variations between individual chips are small[257], it is not necessary to repeat the measurement of the droplet size for every chip or solution if the flow rates remain the same. A list of the droplet sizes and frequencies according to the specific flow rates is published by Verboket et al.[257].

7.1.4 Representative results

The presented system can be employed to measure small volumes of solutions or suspensions containing cells or nanoparticles. Examples of a measurement of a standard solution and characterization of single cells are shown here. More examples can be found in Verboket et al.[257].

Typically the signal of a single droplet of a solution is a very short event. It usually lasts for a few 100 micros[233]. With the ICPMS used here (dwell time 10 ms) short signals like these cannot be temporally resolved. Figure 7.7a and Figure 7.7b show the signals and frequency distribution histogram of a Na standard solution. The droplets arrive at the plasma with a temporal jitter >10 ms. The detection is unsynchronized. Signals of one (201 ± 24), two (381 ± 34) or three (560 ± 45) droplets are detected within one dwell time. Low variation in signal intensity suggests high droplet monodispersity. The first tailing peak is likely the result of droplet fragments; the cause of this fragmentation is still under investigation.
Figure 7.7: (a) Signals of droplets made from a 1 mg kg\(^{-1}\) Na standard solution. (b) Frequency distribution histogram of these signals. In the 10 ms dwell time signals of one (yellow), two (red) or three (blue) droplets were recorded. Mean and standard deviation of the signals were determined by fitting Gaussian functions. The flow rates used were 0.5, 50 and 60 µL min\(^{-1}\) of aqueous sample, PFH for droplet generation, and PFH for droplet acceleration, respectively.
Figure 7.8: Frequency distribution histogram of $^{56}\text{Fe}^+$ signals generated by red blood cells. Mean and standard deviation of the signals were determined by fitting a Gaussian function. The flow rates used were 2, 80 and 80 µL min$^{-1}$ of cell suspension, PFH for droplet generation, and PFH for droplet acceleration, respectively.

The calibration approach described in 5. (using Fe standard solution) was tested for the determination of Fe content of single bovine/calf red blood cells (6 – 7 µm in diameter) suspended in phosphate buffered saline (PBS). The suspension of $1 \times 10^7$ cells mL$^{-1}$ was used to ensure that the majority of droplets carry only one cell. Figure 7.8 shows the signals from cells as frequency distribution histogram. On average every cell contained $5.3 \pm 1.2 \times 10^8$ Fe atoms (see Verboket et al.[257]).

### 7.1.5 Discussion

Although the fabrication of the chips is very reliable there are some critical points during the fabrication that require special attention. First, cleanliness during the assembly is highly important to prevent contamination of the chip by dust. The dust can block the channels and prevent a stable droplet generation. Second, it is especially important that the tip is cut orthogonal to the nozzle channel. The angle of the cut strongly influences the ejection angle. If the liquid is ejected at an angle it can cause a loss of the ejected droplets.

When building the setup ensure that it is stable. The vertical alignment of the metal
7.1 A Microfluidic Chip for ICPMS Sample Introduction

tube and the adapter are important. Also during the measurements there are some points that need special attention. The insertion of the chip into the adapter has to be performed carefully. It can happen that during the insertion the jet is disrupted and stops. For measurements with cells the position and orientation of the syringe pumps and tubing are important. Their proper placement can reduce settling of the cells in the syringe and the tubing.

The LADE chip presented here has several advantages over existing commercial droplet generators. The system is more robust, provides a wider droplet size range, which can be further extended by modification of the channel geometry, and is disposable. A single use device is of a particular interest for the analysis of samples with a high content of salts or solid residues, as for instance nanoparticles or cell suspensions, which can clog tiny channels and cannot always be easily washed out. The transport of single microdroplets generated by the LADE into the MS is still a limiting step in our system and has to be further optimized. The current droplet transport assembly, although removes the PFH vapor, which would otherwise create additional spectral and non-spectral interferences and cause plasma instabilities, but still results in a high temporal jitter of droplet arrival at the MS and an incomplete droplet transport. In comparison to the commercial available droplet introduction systems the transport system for this setup requires more equipment. The current chip is designed for sample introduction only. However, with slight changes of the design advanced introduction and sample preparation steps cloud be implemented on-chip, e.g., dilution[247–249], ultra fast mixing[38], chemical reactions[250], separation[45, 245, 246], or cell sorting[251, 252]. Under advanced introduction devices we understand for example the introduction of sample and standard droplets sequentially or parallel with a single chip. This would increase the throughput and improve the accuracy of quantitative analysis.
### 7.1.6 Materials

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<th>Catalog Number</th>
<th>Comments</th>
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<td>Silicon wafer 100 mm</td>
<td>Si-Mat (Kaufering, Germany)</td>
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<td>Microchem Corp. (Massachusetts, U.S.A.)</td>
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### 7.1 A Microfluidic Chip for ICPMS Sample Introduction

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<tr>
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<td>1.5 mm biopsy puncher</td>
<td>Miltex (Pennsylvania, U.S.A.)</td>
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<td>5 mL syringe</td>
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<td>Microscope</td>
<td>Olympus (Tokyo, Japan)</td>
<td>IX71</td>
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Acknowledgement

Introduction

Often it is not easy to find a fair way to acknowledge people. In particular finding an appropriate sequence in which to list the people can be difficult. Commonly, it is not possible to reconstruct how this order was created. This can lead to upsets among the acknowledged people. Therefore, a quantifiable value to specify the order in which the people are to be listed is desirable. To overcome these issues the dimensionless number Acknow is presented to specify the order.

Results

The dimensionless number Acknow is defined as the ratio of the estimated average time per week that the person spend with the author of the list in the considered period ($t_{atpw}$) to the duration of one week ($t_{week}$) (see Formula 7.1).

$$Acknow = \frac{t_{atpw}}{t_{week}} \tag{7.1}$$

Table 7.3 shows an example list of people and a short explanation why the persons are acknowledged. The items are listed by descending order Acknow. This list clearly proves that a group of people can be ordered using Acknow.
Table 7.3: List of acknowledgments arranged by the dimensionless number $Acknow$. 

<table>
<thead>
<tr>
<th>Acknow</th>
<th>Name / Group</th>
<th>Explanation</th>
</tr>
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<tr>
<td>$7.38 \times 10^{-1}$</td>
<td>Dr. Susanna M. Früh</td>
<td>You were almost literally always there for me. Thank you very much.</td>
</tr>
<tr>
<td>$2.68 \times 10^{-1}$</td>
<td>Simone A. Stratz</td>
<td>Thank you for being a good office mate and your awesome movie nights.</td>
</tr>
<tr>
<td>$5.21 \times 10^{-2}$</td>
<td>Dr. Olga Borovinskaya</td>
<td>Thank you for introducing me to the ICPMS and the many wonderful hours in which we tried to make everything work :-). I would also like to thank the whole Günther group for their assistance and support.</td>
</tr>
<tr>
<td>$4.93 \times 10^{-2}$</td>
<td>Dittrich group</td>
<td>I thank all group members for the helpful discussions and scientific support. Especially, I would like to thank Phillip and Ben for introducing me to the clean room, Maik and Eva for their help with GUVs.</td>
</tr>
<tr>
<td>$2.14 \times 10^{-2}$</td>
<td>Dr. Tom Robinson</td>
<td>I thank you for entertaining me the countless hours we spent together at LMC.</td>
</tr>
<tr>
<td>$1.61 \times 10^{-2}$</td>
<td>Family</td>
<td>Thank you for always supporting me in every possible sense.</td>
</tr>
<tr>
<td>$1.43 \times 10^{-2}$</td>
<td>Onur Bakirman and Maximilian J. Doppelbauer</td>
<td>Thank you both for all your efforts and your good ideas.</td>
</tr>
<tr>
<td>$6.29 \times 10^{-3}$</td>
<td>Prof. Dr. Petra S. Dittrich</td>
<td>Thank you Petra for your supervision and the possibility to perform my thesis in your group.</td>
</tr>
<tr>
<td>$4.65 \times 10^{-3}$</td>
<td>Christoph Bärtschi, Christian Marro and Heinz Benz</td>
<td>I am very grateful for all the fantastic devices you have built for me.</td>
</tr>
<tr>
<td>$3.14 \times 10^{-3}$</td>
<td>Prof. Dr. Detlef Günther</td>
<td>Thank you for the opportunity to work on a very exciting project and for co-examining my thesis.</td>
</tr>
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</table>

Discussion and Outlook

The dimensionless number $Acknow$ is useful for determining a sequence to acknowledge people. It can be calculated easily and makes it possible to acknowledge multiple people in a reproducible sequence. However, the dimensionless quantity $Acknow$ is not capable to express gratitude to a full extent. Therefore, further parameters to calculate $Acknow$ should be considered. This parameters will be subject of future research.
List of Publications

#: These authors contributed equally to this work.

1. **P. E. Verboket**, P. S. Dittrich:
   Multi vesicular droplets: A cell model system to study vesicle compartmentalised biochemical reactions, in preparation.

2. S. Stratz, **P. E. Verboket**, K. Hasler P. S. Dittrich:
   Long-term cultivation and quantitative single cell analysis of *Saccharomyces cerevisiae* on a multifunctional microfluidic device, submitted.

3. K. Eyer, K. Root, **P. E. Verboket**, P. S. Dittrich:


5. T. Robinson#, **P. E. Verboket#**, K. Eyer, and P. S. Dittrich:

   classified as ACS Editors’ Choice article