Sample Handling in Nanoscale Chemistry

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“The year is divided in three periods: before, after and during the moose-hunt!”
Abstract

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Miniaturization is a strong on-going trend within analytical chemistry. This has led to an increased demand for new technologies allowing smaller volumes of samples as well as reagents to be utilized. This thesis deals with the use of open chip-based reactors (vials); a concept that offers an increased flexibility compared to the use of closed reactors. The vials are manufactured by anisotropic etching of silicon.

First, a short introduction is given on the benefits of performing chemistry in miniaturized formats. Different types of reactors useful for performing chemistry in nanoscale are described and the advantages and disadvantages of using a closed contra an open system are discussed.

Precise dosing of nanoliter-sized volumes of liquids in contact mode is performed by using miniaturized pipette tips or capillaries (Paper I, III & IV). Also, non-contact dosing using piezo-electric dispensers is demonstrated by performing nanoliter sized acid-base titrations (Paper II). Standard deviations on the order of 1% were obtained.

Several strategies for handling the evaporation of water, while performing tryptic digests of native myoglobin in low nanoliter sized vials, are demonstrated. An increased conversion rate of the protein to peptides was observed when a nanovial (15 nL) reactor was used compared to the use of a conventional plastic vial (100 µL). Principles based on reducing the driving force for evaporation (Paper III), continuous compensation of evaporated material (Paper IV) as well as covering the reaction liquid with a volatile liquid lid of solvent (Paper V) are used. The volatile liquid lid is also used when performing PCR in volumes as low as 50 nL (Paper VI).

Short descriptions of the analytical methods utilized in the thesis; capillary electrophoresis (Paper III, IV, V & VI), matrix assisted laser desorption/ionization time of flight mass spectrometry (Paper I) and fluorescence measurements (Paper II & VI) are presented.

Finally, an outlook of the developed technologies is given together with a discussion concerning possible future requirements in miniaturized chemistry.

Keywords: capillary electrophoresis, continuous compensation, enzymatic degradation, evaporation, lab-on-a-chip, liquid lid, MALDI, miniaturization, myoglobin, nanoliter, nanoscale chemistry, parallel, picoliter, piezo-dispenser, polymerase chain reaction, reactor, titration, tryptic digest, vial

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List of Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.


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Table of Contents

1. General introduction concerning miniaturization .......................................................... 1

2. Reactors ......................................................................................................................... 3
   2.1 Reactors based on closed systems .............................................................................. 3
       2.1.1 Capillary and column based reactors ................................................................. 3
       2.1.2 Chip based reactors .......................................................................................... 3
   2.2 Open systems ............................................................................................................. 5
       2.2.1 Surface based reactors ..................................................................................... 5
       2.2.2 Chip based reactors .......................................................................................... 5

3. Dosing of liquids into open vials .................................................................................... 7
   3.1 Contact dosing .......................................................................................................... 7
   3.2 Non-contact dosing .................................................................................................. 8
   3.3 Piezo-electric nanoliter titration (Paper II) ............................................................... 9

4. Reactions in open chip-based vials ................................................................................ 13
   4.1 Nanorobot ................................................................................................................ 14
   4.2 Methods for control of evaporation of water .......................................................... 15
       4.2.1 Use of mechanical lids ................................................................................... 15
       4.2.2 Addition of non-volatile matrices .................................................................. 15
       4.2.3 Increase of humidity (Paper III) .................................................................... 16
       4.2.4 Continuous compensation (Paper IV) .............................................................. 17
           4.2.4.1 Filling procedure ...................................................................................... 19
           4.2.4.2 Evaporation theory for continuous compensation ................................ 20
           4.2.4.3 Evaporation measurements during continuous compensation .......... 22
       4.2.5 Use of liquid lid (Paper V&VI) ....................................................................... 23
   4.3 Influence of increased surface/volume ratio .............................................................. 28

5. Analysis of nL-pL sized volumes of sample ................................................................ 31
   5.1 Capillary electrophoresis .......................................................................................... 31
       5.1.1 Principle ............................................................................................................ 31
       5.1.2 EOF ................................................................................................................ 32
       5.1.3 Wall adsorption .............................................................................................. 33
   5.2 Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry .... 33
       5.2.1 Principle ............................................................................................................ 33
       5.2.2 Sample preparation (Paper I) ......................................................................... 34
   5.3 Fluorescence measurements ..................................................................................... 35

6. Future outlooks ............................................................................................................. 37

7. Acknowledgements ....................................................................................................... 41

8. References ..................................................................................................................... 43

Appendix: Paper I - VI
List of Symbols

A   area
D   diffusivity or diffusion coefficient
d   diameter
d_{\text{drop}}   droplet diameter
d(\kappa)   difference of humidity
E   electric field
h   height
h_D   mass transfer coefficient
\kappa_1   vapor pressure over a surface
\kappa_2   vapor pressure in bulk air
L   length
\mu_e   electrophoretic mobility
\eta   viscosity of the separation media
q   ion charge
r   radius
Sh   Sherwood number
v   ion velocity
W   rate of evaporation

List of Abbreviations

ABS   acrylnitrile butadiene styrene
C   \alpha\text{-chymotrypsin}
CCD   charged coupled device
CD   compact disc
CE   capillary electrophoresis
CEC   capillary electrochromatography
CGE   capillary gel electrophoresis
CIEF   capillary isoelectric focusing
CITP   capillary isotachophoresis
CZE   capillary zone electrophoresis
DNA   deoxyribonucleic acid
DOD   drop-on-demand
EOF   electroosmotic flow
ESD   electrospray deposition
G   endoproteinase Glu-C
HPLC   high performance liquid chromatography
LIF   laser induced fluorescence
MALDI   matrix assisted laser desorption/ionization
MECC   micellar electrokinetic capillary chromatography
M1 method for measuring the evaporation rate based on measuring the time for one vial volume of solvent to evaporate
M2 method for measuring the evaporation rate based on measuring the flow rate in a solvent feeding capillary
MS mass spectrometry
myo myoglobin
µTAS micro total analysis systems
PCR polymerase chain reaction
PDMS poly(dimethylsiloxane)
pI isoelectric point
PMT photon multiplier tube
SVR surface area/volume ratio
T trypsin
theo theoretical values
TOF time of flight
1. General introduction concerning miniaturization

During the last few years, the need for new technologies to perform miniaturized chemistry has increased dramatically. Typical applications are in areas such as high throughput screening of drug candidates [1-3], combinatorial chemistry [4-6], genome analysis [7], clinical chemistry and drug development [8] and proteomics [9]. For performing a massive number of parallel reactions, it is obvious that miniaturization is necessary and this requires adequate techniques for handling very small sample volumes. Furthermore, in other areas such as material science, there is a growing interest¹ in combinatorial approaches, since a large number of experiments can be carried out in a relatively small-sized scale [10, 11].

While the merits of miniaturized systems have been pointed out extensively [12, 13], perhaps one particular advantage needs to be highlighted. In situations where chemical reactions are to be performed, and the amount of material is limited, volume reduction is very advantageous since high sample concentrations can be maintained in this way. Under limited sample conditions, a dilution step will facilitate the sample handling but consequently, it will result in slow reaction kinetics since the latter is dependent on the concentration of sample and reagents. Furthermore, an excessive dilution will result in poor detection sensitivity when the reaction is monitored with a concentration dependent detector. Hence, the development of miniaturized systems, utilizing technologies to handle small volumes of fluids are of considerable interest.

An additional aspect of miniaturization is the reduced cost for reagents. Chemistry performed in small volumes allows the use of exclusive and very expensive reagents at a reasonable cost. For example, an important procedure for protein identification and characterization is to perform protein digests. The cost for performing one digestion reaction, with trypsin in conventional volumes (50 µL) using protocols recommended by the supplier (Sigma-Aldrich), is ca 30 SEK. A volume reduction down to 15 nL will reduce the cost for the enzyme dramatically to ca 0.01 SEK.

Moreover, the increased surface area/volume ratio (SVR) associated with e.g. volume reduction can provide an extra benefit if the solid surface is engaged in the reaction, as a ligand carrier or as a catalyst. It is also preferable to enlarge the surface even further when for example working with immobilized enzymes [14].

In addition, the development of miniaturized techniques has found an interest within the area of industrial production of bulk chemicals [15] and impressive results have been presented at the IMRET conferences². However, such techniques will not be discussed within the frame of this thesis since the devices utilized are designed for much larger volume throughput than in the techniques presented here.

¹ COMBI 2000, 2nd Annual Combinatorial Approaches for New Materials Discovery, January 23-25, 2000, San Diego, CA, USA
² http://www.imm-mainz.de/content.html
In pioneering work, the manufacturing procedures for miniaturized reactors were limited to etching procedures in mono-crystalline silicon [16]. The etching procedures for producing three-dimensional structures (micromachining) were developed from the methods used within the electronic industry that manufactured integrated circuits. Since then, micromachining has been performed in a large number of materials [17]. Micromachining has also been used for the production of molding masters [18], which widens the range of materials used to include most polymer materials [19, 20]. Additionally, highly specific tools for handling solid materials e.g. nano-tweezers based on carbon nanotubes [21], optical trapping [22] and laser catapulting [23]; have been suggested.

In this thesis, the discussion will be focused on devices with applications within the area of nanoscale chemistry (nanochemistry) which herein is defined as technologies for handling and performing chemistry, using nanoliter to femtoliter sized volumes of reagents.
2. Reactors

Chemistry, employing nanoliter volumes of samples and reagents can be performed by using several different principles. It seems natural to classify the reactors in open or closed systems when different unit operations are discussed. Alternatively, the reactors can also be divided in reactions performed in continuous flow systems or reactions performed in confined batch volumes [24].

In this thesis, the accessibility to a system and its reaction mixture, before (dosing of reagents), during (“incubation”) and after (analysis) the reaction, is a most important theme. In the present chapter, a short presentation is given of different closed systems e.g. capillaries or etched chip-based channels as well as different open systems. Advantages as well as disadvantages regarding the use of different reactor types are discussed. This thesis presents work primarily carried out with open chip-based vials as reactors since this concept fulfills important requirements of flexibility.

2.1 Reactors based on closed systems

2.1.1 Capillary and column based reactors

Capillaries are suitable to use as reactors, an inherent advantage since the reactor volume is easily determined. Further, capillary-reactors can readily be made in different materials e.g. glass, plastic, metal, and a wide range of diameters is available. Enzymatic reactions have been successfully performed in capillaries with the enzyme in free solution [25-29], with the enzyme immobilized on the capillary wall [30-35] and with the enzyme immobilized onto a support medium [36, 37]. Capillary reactors have also been used for PCR reactions in free solution [38-40] or with immobilized templates in a capillary reactor [41]. Recently, Manz et al. presented a system where a flow channel, made by etching a glass chip, was used for flow PCR [42].

A drawback of the capillary system is a limited flexibility in terms of accessibility, since these are closed systems. Furthermore, even although a reaction can be carried out in nL - pL sized volumes, sample transfer and handling procedures usually require µL sized volumes of solution.

2.1.2 Chip based reactors

The use of systems based on microchips is an obvious approach in view of the rational processes (photolithography, wet or dry etching methods etc.) for fabrication, which can be used to obtain a large number of identical functional microstructures positioned at highly defined coordinates [16]. In order to produce cheap disposable devices in other materials than glass or silicon, polymer replicas have been introduced [19, 20]. The first micro/miniatuorized total analysis system (μTAS) on a silicon chip, was a
micro gas chromatograph [43] that was presented in the late seventies. In 1992, Harrison and Manz et al. introduced the Lab-on-a-Chip concept [44, 45] with a chip-based device for capillary electrophoresis. Since then, many different devices have been presented in the literature and at conferences e.g. µTAS³. Commercial chip-based devices are now also available [46]. Stacked devices have been presented where layers are connected to each other in order to construct a more complete µTAS device [47, 48].

Most of the µTAS chips have been designed for chip based capillary electrophoresis and an overview of the development within this area can be found in the following review articles [17, 49-52]. However, only a few of these devices have a reactor included [53-62]. Other application areas for µTAS chips are e.g. capillary electrophoresis (CEC) [63] with packed reactor beds and immobilized reagents or systems combined with electrospray mass spectrometry [64-70], cell handling [71-76] and immunosorbert assays [77]. PCR reactors [78-80] without electrophoretic separation of the amplified DNA have also been presented. The SVR in these chip-based systems allows efficient and rapid heating and cooling during the PCR thermo cycling.

A major drawback with chip-based devices is the difficulty of interfacing the real world and the miniaturized lab. The microchips need to be integrated in a system with both sample preparation and analysis in order to accomplish the potentional savings of volumetric reduction [13]. Also, as for the capillary based reactors, the sample transfer and handling procedures usually requires µL sized volumes of solutions, even though a reaction can be carried out in nL – pL sized volumes. The integration of different components onto a chip is not trivial [81] but once the sample is introduced into the chip device, electrokinetic control of the fluid flow [82-85] enables the handling of pL sized volumes of sample [86]. Not only water based fluids can be electrokinetically controlled; organic solvents can be handled as well [48, 87, 88]. Distribution of fluids and reagents using centrifugal forces is a different approach for sample handling in chip-base devices. This has led to the development of a device in a chip-based format very similar to a music compact disc (CD) [89]. Also, pneumatic pumping of pL volumes of reagents has been presented using hydrophobic microcapillary vents fabricated in poly(dimethylsiloxane) (PDMS) [90]. Pneumatic as well as hydrostatic pumping has been used in a microfabricated device of PDMS used for dynamic DNA hybridization using paramagnetic beads [91].

³ http://www.mesaplus.utwente.nl/mutas2000/
2.2 Open systems

2.2.1 Surface based reactors

The simplest form of an open miniaturized reactor is to deposit drops of reagents onto a surface with pre-immobilized chemicals to perform an assay [92]. Devices have been produced e.g. gene-chips for DNA hybridization [93, 94] where oligonucleotides are synthesized in situ using a photolithographic technique. The resulting chip contains all combinations of an octanucleotide in a high-density array. Alternative manufacturing methods for DNA chips are electrodirected copolymerization of pyrrol [95], where covalently attached primers or templates are dispensed onto a functionalized gold surface [96, 97] and immobilization of oligonucleotides within miniature gel-pads arranged on a microchip [98, 99]. For protein characterization, trypsin immobilization directly onto a matrix assisted laser desorption ionization (MALDI) target can be used to produce a surface based reactor [100]. Patterns of proteins have also been produced by microcontact printing using PDMS stamps [101]. The surface based reactor format is very useful for performing assays where the test solution is in contact with several reactors at the same time. Such reactors have been used for growing cells (different cells are regarded as different reactors) in a common medium [7] for phenotypic macroarray analysis.

2.2.2 Chip based reactors

For a reaction that requires a long reaction time in free solution, the use of open microreactors or vials is very useful. Some of the attractive features are:

- A large number of individual reactors (as many as 500,000) can easily be manufactured on a single chip (glass, silicon, quartz or plastic).
- Random access to individual vials is possible, using a precision robotics system. Moreover, open systems can be accessed any time before, during and after a reaction (for e.g. addition of reagents, withdrawal of sample or detection).
- The sample is kept in a confined space. Sample dispersion and/or dilution, which is an inherent problem in continuous flow systems, is avoided.
- No carry-over problems are experienced, as is due to dispersion and lagging (adsorption) of sample in sequential flow reactions.

However, there are also drawbacks of the open vial concept, notably:

- Contamination from the outer environment (dust, including biological material) can be a severe problem.
- When dealing with a large number of different samples, the transfer of small sample volumes demands relatively complicated systems, such as arrays of micro-capillaries. However, this sample transfer problem is not trivial in flow systems either.
- Evaporation of the reaction solvent.
The concept of miniaturized open chip-based nanovials was introduced in 1992 by Jansson et al. [102]. The nanovials were used as sample containers for electrokinetic injection of DNA samples prior to a CE separation. By using photolithography and anisotropic etching techniques, a large number of such vials could be manufactured on a monocrystalline silicon wafer, Figure 1. Later on, the usefulness of the open chip-based vials, with nano- and picoliter sized volumes, was demonstrated as targets for MALDI-TOF MS analysis of proteins (Paper I), and as reaction vessels for tryptic digests of myoglobin followed by an electrophoretic separation of the obtained peptide map (Paper III, IV&V).

Figure 1: A schematic of the chip-based vials used. The manufacturing procedures are 1: transfer of a pattern from a photomask to a monocrystalline oxidized silicon wafer by using photoresist and photolithography, 2: development of the photoresist pattern and creation of an etching mask (SiO$_2$), 3: anisotropic etch of the wafer, 4: deposition of a thin conductive film of gold to enable an electrical contact during electrokinetic injections or MALDI-TOF MS analysis. The vials have an inverted pyramidal shape with the angle 54.74° between the flat surface and the vial wall. The vials used have side lengths ranging from 50 - 400 µm (corresponding volumes: 30 pL - 15 nL).

The concept of using open nanovials has also been taken up by several other workers for electrochemical studies [103-108], bioluminescence measurements [109] and as MALDI-TOF MS targets for analysis of oligonucleotides [110], peptides [111, 112] and organic molecules [113]. Fabrication technologies that have been employed to produce the vials are etching [103, 104, 110], laser ablation [108, 109], replica imprinting from micromachined masters [105-107], as well as etching optical fibers [114, 115].

The trend towards miniaturization is very clear. Although, the 96-well plate is still the most commonly used format for open reactors, systems for e.g. PCR [116, 117] have been miniaturized from the 96-well format to an 864-well format [118] and screening assays have been miniaturized (200 nL vial volume), using a plate design with 9600 wells in each plate [8]. Other non-standard miniaturized formats have been presented where molding techniques have been used for producing wells in polydimethyldisiloxane and polypropylene (2304 wells of 8 µL each) [119, 120]. Drilling has also been used to produce arrays of nanowells in acrylnitrile butadiene styrene (ABS) plastic sheets (2025 wells of 0.37 µL each) [5].
3. Dosing of liquids into open vials

There is a rapidly growing need in analytical chemistry for devices, which can dispense small volumes of fluid. It is very difficult to handle sample volumes in the nL – pL range using conventional pipettes or syringes which are commonly employed in most automated systems. The tendency of small droplets to adhere to surfaces also increases the difficulties of dosing when the volumes are reduced. In this section, different strategies for dosing small volumes of liquid are divided in contact and non-contact principles. Depending on the application, combinations of the presented principles might be advantageous to utilize.

3.1 Contact dosing

In the early development of equipment for dispensing, most devices were based on the use of syringes. One of the drawbacks with the use of syringes is their relatively large dead volumes. In order to reduce the volumes needed to dispense reagents, devices based on miniaturized pipette tips (Paper I, III & IV) or pressurized capillaries (Paper IV & V) can be used. For a detailed description of the dosing principle using capillaries, see section 4.2.4.1. The smallest dead volume can be obtained when miniaturized pipette tips are used, but the necessary flow regulation is more difficult to achieve compared to the use of a capillary. This is (probably) due to an increased pressure drop caused by the small dimensions of the drawn tip. Filling of the tip is performed by capillary action through the nozzle of the tip. Dosing is performed by applying an overpressure to the tip while it is positioned inside a well or a vial. The dosed reagent will form a droplet attached to the tip until contact with the wall of the well (or a liquid inside the well) is established. Commercial pressure driven micropipettes are available and have been used for dosing 10 pL sized volumes [121].

Contact dosing can be performed by using a tip with a slot (compare the action of an old fashioned ink pen), which soaks in a certain volume of liquid by surface tension when it is dipped into a solution. When the tip is brought in contact with a solid surface, a reproducible volume of the reagent is dispensed onto the surface [122]. This technique is only suitable for surface based reactors and is not so useful for dosing a reagent into an open microvial. However, if the bottom of a vial is flat, contact dosing of a small reagent volume should be possible to perform. A complete description of how to build your own “micro-array printer” can be found on the Brown Lab’s homepage.

An important drawback with the different contact-mode dispersion techniques is the risk for cross-contamination.

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4 http://cmgm.stanford.edu/pbrown/mguide/index.html
3.2 Non-contact dosing

Methods for non-contact dosing can be divided according to the driving force utilized. Electrospray deposition (ESD) has been proposed for dosing reagents [123, 124] onto surfaces. The devices are very similar to the nanospray tips [125] commonly used for sample introduction in electrospray ionization mass spectrometry. The amount of deposited material is determined by weight using a quartz microbalance [123]. Dosing is performed by applying a potential between the spray nozzle and the target and thus creating a local electrostatic field. An interesting feature is that parallel dosing can be performed using a dielectric mask that addresses the reagents to predetermined positions (defined by the mask) [124]. ESD is also useful for sample preparation in MALDI-MS due to the formation of very fine crystals of the MALDI matrix [126, 127].

Non-contact dispensing can also be performed by applying a pressure pulse to a confined volume of solution, allowing an aliquot of the solution to leave the larger bulk volume via a small exit hole. A fast pressure increase of the pulse is needed to overcome surface tension forces and to expel a small droplet of the solvent. The pulse can be created by a solenoid valve mounted between the exit hole and the bulk volume. This technique is preferable to use for dispensing of µL-sized volumes [120] but can be used for volumes down to 100 nL5.

The pressure pulse can also be created by using piezo-ceramic materials. Devices for controlled piezo-electric dosing of nL - pL sized volumes of chemicals are now being developed at rapid pace due to the presence of a well-developed technology of inkjets, commonly used in high-resolution printers. Great precision can be obtained and the usefulness of piezo-electric dispensers has already been demonstrated in different applications [110, 128-131]. The piezo-electric dispenser utilized in Paper II for performing nanoliter titration [129] employs a glass capillary tube with a constricted outlet. A glued-on piezo-ceramic body surrounds the capillary tube. When a voltage pulse is applied to the body, a contraction occurs, which generates a steep pressure pulse within the capillary. If the capillary is filled with liquid, a small portion of the liquid will discharge from the outlet. Under optimized conditions, the expelled liquid can form a single droplet with an almost perfect spherical shape. The piezo-ceramic material can also be positioned at one side of a flow channel with the dispensing nozzle on the opposite side [132, 133]. Also, dispensers using flat chambers with the piezo-electric material mounted on one side, perpendicular to the nozzle, are available6.

Droplet formation during piezo-electric dispensing is governed by a number of interdependent parameters, such as the nature (viscosity, surface tension) of the liquid, the shape and size of the nozzle, the characteristics of the applied electric pulse (voltage and pulse length), etc. Frequently, small satellite droplets are formed along with the

6 Gesellschaft für Silizium-Mikrosysteme mbH (GeSiM), http://www.gesim.de/
main droplet, which is detrimental for the dosing precision. However, it is rather straightforward to obtain single droplets by adjusting the voltage and the duration of the electric pulses sent to the piezo-ceramic body. In this context, it is important that the nozzle of the dispenser is clean and not wetted by the solvent. For dispensing aqueous solutions, we found that silanization of the dispenser tip is necessary. However, organic solvents such as iso-propanol are very difficult to dispense, even with silanized tips. An initial experiment with a surface coating using a perfluorinated compound has shown promising results.

3.3 Piezo-electric nanoliter titration (Paper II)

A controlled and incremental addition of solutions from a piezo-electric dispenser can be utilized for determination of the absolute amount of a substance by titration. Hieftje et al. constructed a device, which made it possible to generate a continuous stream of electrically charged reagent droplets of ca 350 nL volume [134]. Individual droplets were directed into a titration vessel, by applying an electric field. One major drawback of this procedure is the need for a continuous supply of titrant, which results in large waste volumes. Another device for the generation of smaller droplets was developed using a vibrating glass needle [135]. In this way, a continuous stream of small droplets, having volumes down to ca 3 nL, was generated. However, a generation of single droplets on demand was not possible, and therefore the minimum volume, which could be dispensed with accuracy, was ca 40 nL [136, 137]. We have used the piezo-electric dispenser to perform automated titrations of low nL analyte volumes (Paper II). The incremental addition of titrant is easy to control by a drop-on-demand (DOD) addition. The system includes a detection system, based on wave-guided laser-induced fluorescence [138]. The use of precision robotics makes it possible to rapidly perform a large number of titrations in a consecutive order. The titration set-up is evaluated by performing repetitive fluorometric titrations of ca 9 nL of H$_2$SO$_4$ with NaOH.

A correct calibration procedure of the system is very important. This can be achieved “on-the-fly” by determining the volume of the droplets by measuring their diameter [139-141] e.g. by using a microscope and image analysis. However, to obtain high precision data is not straightforward due to difficulties in defining boundaries of curve-shaped objects, like droplets or fibers, the latter can be used as calibrating standards for measurements with a charged coupled device (CCD) camera. Moreover, errors in observed droplet diameter propagate to the third power in errors of volume. An alternative method would be to dispense a number of droplets on a microbalance. From the weight of the dispensed material, the volume of an individual droplet could be calculated, if the density of the solution is known. However, weighing procedures also inherit certain disadvantages. The density of a solution is not always available when dealing with unknown samples and volume determination by weighing will be affected by the evaporation of solvent during the time between dosing and weighing.
A titration is started by pooling a predetermined number of sample droplets (including the indicator), on the surface of a polyethylene support using a first piezo-electric dispenser. Next, the pooled sample is repositioned, using an X-Y table, under the nozzle of a second piezo-electric dispenser filled with reagent. The titration process is started by discharging single droplets of reagent into the sample at a predetermined rate and simultaneously, the data collection from the fluorescence detection is started. After a preset number of titrant droplets have been added, data collection is discontinued and the collected titration data are exported to a PC to perform the endpoint evaluation. Automated repetitive titrations (100 titrations) are performed by repeating the entire procedure but at a new individual titration position, determined by the programmed control unit.

*Figure 2* shows the first results from automated fluorimetric piezo-electric titrations. The calculated volume of titrant, necessary to reach the equilibrium point is represented by the height of the vertical lines for the corresponding samples. Initially (for the first 30 titrations), the reproducibility was satisfactory, while a substantial deterioration was observed for the subsequent titrations. We found this problem to be related to a wetting of the nozzle and the problem was solved by treating the tip with Repel-Silane. The effect of the treatment lasted for about one working day. The greatly improved results are shown in *Figure 3*. The excellent reproducibilities obtained for different titrations are presented in *Table 1* and *Table 2*.

![Figure 2: Results from titrations performed with non-silanized piezo-electric dispensers. Sample: 10 drops (each drop with a volume of 0.90 nL, 50 mM H₂SO₄), Titrant: 80 drops (each drop with a volume of 0.52 nL, 50 mM NaOH). Time between each added droplet of NaOH: 1000 ms for sample 1-100, 500 ms for sample 101-200, 250 ms for sample 201-300 and 3000 ms for sample 301-400. Calculated volume of added titrant: 18.1 nL.](image-url)
Figure 3: Three sequences of consecutive titrations performed at different titration rates. The tips of the piezo-electric dispensers were treated with Repel-Silane before starting the measurements. Sample: 10 drops (each drop with a volume of 0.90 nL, 50 mM H$_2$SO$_4$), Titrant: 80 drops (each drop with a volume of 0.52 nL, 50 mM NaOH). Time between each droplet: sample # 1 – 100: 1000 ms, sample # 101-200: 500 ms, Sample # 201-300: 250 ms. Calculated volume of added titrant to reach the equilibrium point: 18.1 nL. Note the anomalous results of sample 1, 101, and 201 (marked with +), which is due to initial evaporation of water from the sample in the piezo-electric dispenser tip.

Table 1: Calculated average titration volumes with standard deviations for titrations performed at different titration rates. The results are compared with the calculated titration volume (18.1 nL NaOH).

<table>
<thead>
<tr>
<th>Titration rate – time between droplet addition (ms)</th>
<th>Average – first ten titrations (nL NaOH)</th>
<th>Stdev – first ten titrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>15.9</td>
<td>1.5</td>
</tr>
<tr>
<td>500</td>
<td>16.6</td>
<td>1.4</td>
</tr>
<tr>
<td>1000</td>
<td>17.1</td>
<td>0.78</td>
</tr>
<tr>
<td>2000</td>
<td>16.5</td>
<td>0.86</td>
</tr>
<tr>
<td>3000</td>
<td>18.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Table 2: Calculated standard deviations for titrations performed with the same titration rate (1000 ms between droplet addition) at different concentration levels. The standard deviation of the results obtained at the lowest concentrations (marked *), is calculated only from three titrations.

<table>
<thead>
<tr>
<th>Concentration OH (mM)</th>
<th>Concentration H⁺ (mM)</th>
<th>Stdev – first ten titrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>100</td>
<td>0.78</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1.03</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>1.23</td>
</tr>
<tr>
<td>0.05</td>
<td>0.1</td>
<td>1.63*</td>
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</tbody>
</table>
4. Reactions in open chip-based vials

Miniaturized systems for performing reactions can offer many advantages, such as reduced costs for reagents, possibilities to maintain high reaction concentrations, possibilities to perform chemistry when a limited amount of sample is available etc. In the work with open chip-based vials, we have chosen enzymatic degradation of proteins as a model application since there is an increasing need for tools and methods within e.g. proteomics where many proteins are available only in limited amounts. Normally, proteins are denatured prior enzymatic degradation in order to open up the structure and thus exposing the cleavage sites. This results in an increased conversion rate from protein to peptides. We have chosen native myoglobin as a substrate since the globular structure often requires long digestion times. Native myoglobin can therefore be regarded as a suitable model substance to use in a general method development for digests.

Controlled enzymatic digestion of proteins (or peptides) is one of the standard procedures in biochemistry [142]. In summary, a proteolytic enzyme is added to the protein in a buffer solution. During the incubation (digestion), the enzyme cleaves the protein at cleavage sites typical for the enzyme used. From a specific fragment, the order of the amino acids can be determined in further investigations using Edman degradation [142]. The use of several assays based on enzymes with different cleavage sites is advantageous. In many cases, peptide fragments produced (using e.g. trypsin) contains specific cleavage sites typical for a second enzyme (e.g. endoproteinase Glu-C) and vice versa. Thus, many of the fragments can be correlated to each other using the determined overlapping amino acid sequences. This results in valuable information of the amino acid sequence for structure elucidation of the entire protein.

The most frequently used enzyme for performing digests is trypsin with cleavage sites on the carboxylic side of the amino acids lysine and arginine in the chain of amino acids. After the digest, the peptide mixture can be analyzed by liquid chromatography [143, 144], capillary electrophoresis (CE) [145], electrospray-MS [146] or matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) [147, 148]. The result of the analysis is presented as a peptide map (a “fingerprint”) of the investigated protein. Such peptide maps are very useful for determination and characterization of primary structures of proteins. Protein digest-protocols are also often used in purity verification and control of precision and reproducibility in recombinant processes. Comparison between similar proteins can reveal minor structural differences. Moreover, preliminary identification of proteins can be performed by comparing peptide maps with maps of known substances.

A fundamental problem of the open vial concept is the evaporation of solvent (water) from the vials. Without any countermeasure to prevent or reduce evaporation, nL - pL sized volumes evaporate in a few seconds when left in the ordinary atmosphere. Several strategies have been proposed for dealing with this problem but the use of one single method is not always suitable for all different situations.
4.1 Nanorobot

Due to the requirements to precisely position tools and other equipment needed for adding reagents, for performing reactions and to carry out subsequent analysis, a computer-controlled robot was constructed. Figure 4 shows a schematic of the robot used in the work with parallel-operated reactions (Paper IV).

Figure 4: A schematic top-view of the nanorobot constructed for performing nanochemistry. It consists of a computer controlled XY-table with 1 µm addressable precision. Several vertical mounted micro-positioners are used for positioning along the z-axis. The current schematic shows the set-up utilized for performing parallel reactions in 15 nL sized vial volumes (Paper IV). Depending on the application, different tools are mounted onto the positioners e.g. a cutting wheel for capillaries (Paper IV), piezo-electric dispensers (Paper II), optical fibers (Paper VI), solvent supplying capillaries or capillary tips (Paper IV&V), a CE separation capillary (Paper IV&V). Since the XY-platform is rather large (20*20 cm), a manual XYZ-manipulator can be mounted onto the XY-table together with different containers or chip-holders. All operations are observed using a long distance working microscope equipped with a CCD camera and an image analysis system.
4.2 Methods for control of evaporation of water

4.2.1 Use of mechanical lids

The most common method to prevent evaporation is to cover the reaction mixture with a solid or mechanical lid [119, 149]. Plastic chips can be effectively sealed by welding a thin foil of plastic over the wells [150]. This is straightforward for µL sized volumes but not easy to apply on nL–pL sized volumes. A problem that typically occurs is that part of the reaction mixture adheres to the lid.

A very innovative application of a solid lid of gold covering a reservoir has also been presented [151]. The lid was used to control the release of a component into a mixture by dissolving the lid electrochemically but has not been used for covering a reactor during incubation.

4.2.2 Addition of non-volatile matrices

Evaporation can be reduced by addition of non-volatile matrixes to the reaction mixture, Figure 5, which reduces the vapor pressure of the solvent and thus reduces the driving force for evaporation (see section 4.2.4.2). This has successfully been used by Ewing et al. and others [104, 106, 119] for nL sized reaction volumes in open vials.

Matrix added to the reaction mixture

Figure 5: The vapor pressure is reduced by adding a high-boiling matrix to the reaction mixture.

However, when reactions are carried out under an extended period, an additional humidified environment seems to be necessary. The main drawback of matrix addition is a possible interference of the additives with the reaction and its analytes. In addition, the additives may be detrimental contaminants in subsequent analytical measurements (such as CE separations, mass spectrometry etc.).
4.2.3 Increase of humidity (Paper III)

Another approach to reduce the evaporation is to enclose the sample in a humidified environment [8]. In principle, working at 100% relative humidity would completely prevent an evaporation of water from the sample, Figure 6.

![Diagram](image)

**Figure 6**: The evaporation of water from an open vial can be reduced by increasing the humidity in the surrounding environment.

However, operation at this humidity level is not possible without introducing severe problems. At a very high humidity level, water starts to condense onto irregularly shaped surface areas, like scratches, dust etc., leading to overflow to adjacent vials and cross contamination. The behavior is related to differences in surface wettability (contact angle), the strive of surfaces to minimize their free energy and the associated well-known phenomenon of capillary condensation [152]. Another problem is that the evaporation rate of water from a sample is affected by the presence of salts and other components, which can cause an excessive condensation of water into the sample vials. In practice, the concept is useful if the humidity is kept at a level where condensation does not occur, but a periodic water addition is then necessary (Paper III). Operation becomes increasingly difficult at elevated temperatures.

In Figure 7, the result from a tryptic digest of native myoglobin operated in a humidified environment and performed in a nanovial (15 nL) is compared with a digest performed in a conventional plastic vial (100 µL). Considerable differences can be seen between the electropherograms but the mid regions show a notable similarity. The performance of the digest (the rate of protein conversion into peptides) seems to be dependent on the type of vial used. This can be measured as the ratio between the height of a peak representing a peptide (A or B) and the original myoglobin peak (myo). The peak ratio is higher when the digest is performed in a nano-vial compared to a digest performed in a conventional plastic vial. It is suggested that this is the result of the higher SVR in the nanovial, which is further discussed in 4.3.
Figure 7: Two electropherograms obtained after CE analysis from tryptic digests of native myoglobin performed in a chip-based nanovial (left) and in a conventional plastic vial (right). The conversion from protein to peptides can roughly be calculated from the ratio between the peak height of a representative peptide clearly notable in both electropherograms (A and B) and the peak height of the remaining undigested myoglobin (myo).

### 4.2.4 Continuous compensation (Paper IV)

Instead of reducing the rate of evaporation, another principle can be applied, where water is added continuously to the vials to compensate for evaporation (Paper IV). When a continuous supply of solvent (e.g. via capillaries, connected with the reaction vials and a solvent supply vessel) is kept in balance with the evaporation, reactions can be sustained over many hours or even days. However, it is important to utilize very pure solvent, since impurities from the solvent are accumulated in the sample.

Figure 8: The amount of evaporated solvent from a nanovial is continuously compensated for. The other end of the capillary is connected to a solvent supply vessel (not shown in the figure).
Continuous compensation of water can be performed in parallel by arranging the solvent feeding capillaries in a holder with V-shaped grooves. Figure 9 shows the result of the CE-analysis of the content in eight separate vials wherein enzymatic degradations of myoglobin have been performed.

Figure 9: Eight electropherograms obtained after the CE analysis of eight individual enzymatic digests of native myoglobin (7 pmol in each vial) performed in parallel in chip-based vials. The electropherograms are labeled according to the enzymes used: trypsin (T), α-chymotrypsin (C) or endoproteinase Glu-C (G).

Reaction conditions: chip-based vials with 15 nL volumes, 8 mg/mL myoglobin, 8 mM NH$_4$HCO$_3$ pH 7.9, 0.2 mg/mL enzyme concentration, 37°C, reaction time 4 hrs (prior analysis of the content in the first vial).

CE conditions: separation capillary ID 50 µm, OD 150 µm, total length 102 cm (72 cm to the detector), 0.01 M phosphate pH 7.0, 100 µg/mL FC 134, +15 kV 5 µA.
We have also constructed an array of 32 solvent feeding capillaries with dimensions, suitable for parallel reactions in 1 nL chip-vials, *Figure 10*.

*Figure 10: A photograph showing a nanodispenser fitting 1 nL chip-based vials. The size of each individual vial is 200*200 µm and the outer diameter of the capillaries is ca 70 µm. The width of the entire dispenser is ca 15 mm.*

This device is manufactured by precise positioning of the feeding capillaries using a holder with V-shaped grooves matching the spacing between the chip-based vials. The holder is a plastic replica made from a micromachined master. After gluing the capillaries in position, a small scratch is made on all the capillaries in order to enable careful breaking of the capillaries [153]. A cutting wheel (tungsten carbide), normally used for cutting optical glass fibers, was utilized for this purpose. The fabrication of larger arrays as well as capillaries, designed for still smaller reactor volumes, should be quite feasible in this way.

4.2.4.1 Filling procedure

The filling procedure of a vial starts with applying a very low overpressure in the solvent vessel in order to form a small solvent meniscus at the end of the capillary, *Figure 11A*. When the meniscus is brought in contact with the vial wall, the surface tension is broken and a flow of solvent starts to fill the vial, *Figure 11B*. Simultaneously, the capillary is moved upwards until the solvent level inside the vial has reached a level where the evaporation from the solvent surface counterbalances the flow. In this respect, the inverted pyramidal vial has a beneficial shape since the surface area of the solvent meniscus increases rapidly as the volume of the liquid increases. Due to this increase in surface area, the evaporation also increases rapidly to a point where it counterbalances the flow from the capillary. The vial can even be overfilled without overflow (c.f. “topping” a glass of water), *Figure 11C*. This is due to
the effect of the surface tension and the abrupt change of angle between the sidewall of the vial and the horizontal surface. When the vial is completely filled, a steady state can be maintained for several hours with the solvent supply in balance with the evaporation (Figure 11D).

Figure 11: Schematic of the procedure of filling a vial. The schematic only shows the capillary end that is in contact with the vial. The other end is connected to a solvent supply vessel not shown in the figure. A: An enlargement of the end of the solvent supplying capillary showing the formation of an initial solvent meniscus caused by overpressure in the solvent supply vessel. B: When the liquid touches the vial surface, the surface tension of the meniscus is broken and the filling of the vial is initiated. C: The solvent supplying capillary is moved upwards, while filling the vial. D: The evaporation of solvent from the vial is counter-balanced by a continuous flow of solvent through the capillary. N.B. The capillary is withdrawn from the vial after event C, in the case where a reagent solution is added to a vial.

4.2.4.2 Evaporation theory for continuous compensation

The driving force for the evaporation of solvents into air is the difference between the vapor pressure of the solvent just above the surface and the vapor pressure in the bulk air. If the air is slowly passed over the surface, a boundary layer is formed with a concentration gradient of the solvent within it. Since the dominating resistance against mass transport is in the diffusion boundary layer [154], focus will only be given on this.

The diffusivity of vapors in different gases, from a planar surface to a gas stream, can conveniently be determined with a method developed by Winkelmann [155]. In short, the method is based on the formation of a boundary layer (using a controlled diffusion distance and thus with a known thickness of the layer) between a solvent vessel and a stream of dry air or gas. The amount of evaporated material is straightforward to measure when large volumes of solvent (several mL) are used, and therefore the evaporation rate can easily be calculated. However, the method is not straightforward to use for the determination of evaporation rates in the range of nL/min since small
volume changes are difficult to measure. In addition, the thickness of the boundary layer formed is difficult to determine when the evaporation of solvents to stagnant air is to be examined (the diffusion distance is not exactly defined). To circumvent these difficulties, we have based our calculations on a mass transfer theory, where the shape of the solvent surface is defined as a hemisphere. In order to be able to apply this theory in a straightforward way, the surface area of the square entrance of our vials was recalculated to the surface area of half a droplet with a corresponding surface area.

Mass transfer from a single water droplet to stagnant air follows the law of molecular diffusion. The rate of evaporation ($W$) can be calculated from the diffusivity (temperature dependent) of water in air ($D$), the surface area of the droplet ($4\pi r^2$), and the difference of humidity ($d(\kappa)$), at a given distance from the surface ($dr$) [154]:

$$W = -D \cdot 4\pi r^2 \cdot \frac{d(\kappa)}{dr} \quad (1)$$

After integration from the radius of the droplet ($r_1$) over the distance out to the bulk air (at the distance $r_2$) with the corresponding temperature dependent vapor pressure over the surface ($\kappa_1$) and the vapor pressure in the bulk air ($\kappa_2$), the expression results in:

$$W = \frac{4\pi D(\kappa_1 - \kappa_2)}{(\frac{1}{r_1} - \frac{1}{r_2})}$$

Since the thickness of the diffusion boundary layer ($r_2$) is infinitive, the expression results in eq (2).

$$r_2 \Rightarrow \infty$$

$$\frac{W}{4\pi rD(\kappa_1 - \kappa_2)} = 1 \quad (2)$$

The evaporation rate of solvent from a liquid surface to the surrounding bulk air can also be expressed as the mass transfer coefficient ($h_D$) [154],

$$h_D = \frac{W}{4\pi r^2(\kappa_1 - \kappa_2)} \quad (3)$$

$$2r = d_{drop} \quad (4)$$
where \( r \) is the radius and \( d_{\text{drop}} \) is the droplet diameter. After substitution of eq (3) and eq (4) into eq (2), the mass transfer coefficient, the diameter of the drop and the diffusivity can be related to each other according to eq (5).

\[
\frac{h_D d_{\text{drop}}}{D} = 2 = Sh
\]  

(5)

This expression is called the Sherwood number (Sh) [154]. For a spherical droplet, the Sherwood number is always equal to two and describes the mass transport from the droplet into stagnant air. In our case, we regard the liquid surface area of a square vial as a corresponding half droplet. The droplet diameter used \((d_{\text{drop}})\) is calculated from a droplet which half area \((2\pi r^2)\) with the same area \((A)\) as the planar liquid surface in the vial. The evaporation rate of solvent from a vial at different temperatures can now be calculated utilizing the calculated, eq (5), mass transfer coefficient \((h_D)\), the area of the vial \((A=L*L, \text{Figure 1})\), and the difference of vapor pressure, \((\kappa_1-\kappa_2)\):

\[
W = h_d \times A \times (\kappa_1 - \kappa_2)
\]  

(6)

4.2.4.3 Evaporation measurements during continuous compensation

During the reaction, a large amount of water is evaporated from the vials. The amount can be measured using two different methods (Paper IV). The first method (M1) is based on measuring the time for one vial volume of solvent to evaporate and the other method (M2) is based on measuring the flow of solvent in a solvent feeding capillary. In Figure 12, the measured evaporation rates obtained for different solvents (water, cyclohexane and ethanol) in a chip-based 15 nL sized vial are obtained. The rates are measured with the two methods and are compared with the calculated theoretical values using equation (6).

![Figure 12: Measured evaporation rates for water, cyclohexane and ethanol using methods based on measuring the time for one vial volume to evaporate (M1) and the flow in the solvent feeding capillary (M2) compared with theoretical values (theo) calculated using equation (6).](image-url)
In Figure 13, the turnover rates (number of vial volumes of water evaporated) for water evaporated from vial volumes (15 nL – 30 pL) at different temperatures are presented. The evaporation rates were measured using the method that was based on measuring the solvent flow in the feeding capillary (M2).

![Figure 13](image)

*Figure 13: The turnover rates (number of vial volumes evaporated over a period of time) for water at different temperatures for vial of different volumes (30 pL – 15 nL).*

### 4.2.5 Use of liquid lid (Paper V&VI)

![Diagram of mineral oil](image)

*Figure 14: The evaporation can be prevented by a complete coverage of mineral oil over the entire chip (left) or in each individual vial (right).*

For applications that require high temperatures, such as PCR, evaporation can be eliminated by covering the reaction mixture with a non-volatile liquid lid *e.g.* mineral oil [103]. The oil can cover the entire chip or be added separately to each vial, *Figure 14*. However, the drawback with mineral oil appears when the lid is to be removed. This is quite difficult since mineral oil tends to stick to most surfaces and residual oil will remain present. Instead, an attractive solution is to cover the reaction liquid with a film of a non-miscible volatile liquid. This method was first utilized by Gratzl *et al.* [141] who carried out diffusional titrations of pL-sized samples, which were submerged in heptane in a Petri-dish.
Evaporation of water from solutions kept in chip-based vials has been prevented by creating a thin layer (0.5-1 mm) of a volatile non-miscible liquid over the chip (Paper V). In order to prevent a premature evaporation of the cover liquid, a continuous supply of the liquid is guided over the surface. Figure 15 shows a schematic of the principle.

![Figure 15: The principle of preventing evaporation of water from the chip-based vials by a complete coverage with a volatile liquid lid. Consequently, solvent has to be continuously added to the lid in order to compensate for the evaporation.](image)

The advantage of this setup is that a similar degree of accessibility as in open vials is obtained, while the vials are always sealed by the liquid lid. Reagents can be added or sample can be withdrawn any time during or after a reaction, by guiding robot-controlled micropipettes or the inlet of a CE-column through the covering film close to or into the sample. Contamination of the pipettes or the CE column by adhered covering liquid is not a problem, since this liquid evaporates as soon as the pipette or the CE column is withdrawn, leaving a clean sample exposed to the environment. Moreover, the entire liquid lid (and also the solvent of the sample) will evaporate completely, when the supply of covering liquid is discontinued.

However, some losses of reaction solvent were noticed after an extended period of operation. This has been investigated by observing a chip-based vial filled with 15 nL of water, kept at 37°C covered with a thin, flowing film of octane (estimated film thickness, ca 0.5 mm). The meniscus of the water sample was monitored by using the microscope and the video camera of the robotics equipment. The results are shown in Figure 16 (left). After 90 min, no loss of water was observed. After 105 min, the first losses were clearly visible, and after 140 min, losses are on the order of 50%. The experiments were also repeated with water-saturated octane, in order to compensate for possible effects of water dissolution, however no improvement was observed. In addition, a significant dependence of the flow rate of octane on the loss of water was not observed. It seems likely that the water loss occurs primarily via diffusion through the thin layer of the covering solvent.
Further, experiments at higher temperatures were also carried out. It showed to be possible to retain most of the water sample in the vial at a temperature close to the boiling point (95°C) during several minutes (Figure 16 right). This performance is unique in the sense that is well beyond the temperature range of the other non-contaminating methods to prevent evaporation, which were discussed earlier in the sections 4.2.3 and 4.2.4.

In order to test the liquid lid concept in practice, a tryptic digest of myoglobin was performed in a 15 nL vial covered with a film of octane. Within a time frame of 90 minutes, most of the myoglobin was enzymatically converted into peptides. An electropherogram of the peptide map is shown in Figure 17 left.

*Figure 16: Photographs showing the loss of water from a 15 nL chip-based nanovial under different conditions (see text).*
Figure 17: Two electropherograms showing peptide maps of myoglobin after tryptic digests, performed in chip-based vials (15 nL).

Left: The reaction was carried out under a liquid lid of octane. Reaction conditions: 95 minutes reaction time at 37°C, myoglobin: 4 mg/mL in 4 mM NH₄HCO₃, trypsin: 0.2 mg/mL. CE conditions: 85 cm capillary length, 60 cm effective length, ID 50 µm, OD 150 µm, 0.01 M KH₂PO₄, FC134 100 µg/mL, +15 kV, 6 µA.

Right: The reaction was carried out while continuous compensation of the evaporating water from the vial was performed. Reaction conditions: 240 minutes reaction time at 37°C, myoglobin: 8 mg/ml in 8 mM NH₄HCO₃, trypsin: 0.2 mg/mL. CE conditions: 102 cm capillary length, 72 cm effective length, ID 50 µm, OD 150 µm, 0.01 M KH₂PO₄, FC134 100 µg/mL, +15 kV, 5 µA.

It is interesting to compare this map with the results obtained from a 15 nL tryptic digest, where the evaporation was counteracted by a continuous supply of water. The main pattern of the peaks is very similar in both cases. However, in the electropherogram of Figure 17 (right), some additional peaks after the last three large peaks are seen. At present, we are not certain about the origin of these peaks. We cannot exclude the possibility that these signals are due to hydrophobic peptides. Such peptides would be extracted or discriminated by the flow of octane. This is an important possible drawback, which has to be considered when using the liquid lid method.

Thus, the choice of the cover liquid is critical. Undesirable interactions and/or losses of hydrophobic components can occur. On the other hand, there may be situations where the removal of such components is desirable. For example, a comparative study, where both methods, utilized in Figure 17 (left) and Figure 17 (right) are used
in a side by side experiment, may reveal valuable chemical information of individual components in complex mixtures.

Combining the liquid lid technology with a piezo-electric dispenser offers a unique possibility for non-contact dosing while solvent evaporation is avoided, Figure 18.

Figure 18: A schematic of non-contact dosing through a liquid lid using a piezo-electric dispenser. Dispensing is possible to perform without splashing of formation of satellite droplets from the dispensed liquid.

Droplets can actually be shot through a thin solvent film and coalesce into the vial without disintegration into smaller droplets. The droplets keep intact because the energy needed to overcome the surface tension for creating new smaller droplets is higher than the kinetic energy within the droplet leaving the dispenser. In this way, a non-contact dispensing of very small volumes of sample can be performed without problems of matrix evaporation. In fact, a “closed system” is obtained but the flexibility of an “open system” is maintained. However, it is very important that the thickness of the lid is thin (ca 0.5 mm) and the dispensing parameters are optimized. In addition, parameters such as viscosity and density of the dispensed solution as well as the solvent lid will affect the success of the operation.
4.3 Influence of increased surface/volume ratio (SVR)

An important parameter that is affected by miniaturization of the reaction volume is the SVR, Figure 19. When the volume is reduced below 0.5 µL, the SVR increases rapidly.

Figure 19: A plot showing the change of SVR when the volume is reduced for different geometric structures compared with a chip-based nanovial (according to Figure 1). The size of the cylinder and cone are determined using the length (l), height (h) and diameter (d); cylinder l=4d, cone h=4d. The ratio increases rapidly for all structures when the volume is reduced to ca 0.05 µL or less!

In the experiments with the open nanovials where tryptic digests of myoglobin were performed, an increased reaction performance was observed for reactions carried out in nanovials compared with plastic vials of conventional size (Paper III). It is reasonable to assume that these observed differences are related to the different surface properties and the SVR since it is well known that surfaces can adsorb proteins or peptides [104, 156-161]. During surface adsorption, native myoglobin molecules mainly form a random coil structure (a type of a surface-induced denaturing state of the myoglobin molecules) [157, 161], which is likely to result in an increased accessibility of the enzyme to the protein. For a continued digestion process via surface-adsorbed myoglobin, the digestion products have to be exchanged with new myoglobin at the vial surface. This is likely to occur since it has been shown in terms of surface binding energy, that proteins are preferentially adsorbed compared to peptides [162]. In addition, Vroman has suggested that a consecutive replacement of adsorbed proteins to more surface-active ones occurs, even though the latter are present in lower concentrations in the solution [163, 164]. Other factors such as the shorter diffusion length in the nanovials could also enhance the digest due to faster mass transfer-related association of protein molecules with the vial surfaces.
In order to investigate the influence of the difference in SVR, an additional experiment was performed. Two identical digests in 100 µL volumes, using conventional plastic vials, were carried out during 6 hours. In order to simulate the reaction conditions in a nanovial, a thin gold-wire (l = 5 m, d = 100 µm) was bundled and inserted into one of the vials, which roughly resulted in the same surface-to-volume ratio as in the case where the digest was performed in a nanovial (nanovial: ~15000 m⁻¹, conventional vial: ~900 m⁻¹). The experiment showed that significantly more peptide material was produced in the vial with the gold-wire, Figure 20.

![Figure 20: Two electropherograms showing peptide maps of myoglobin after tryptic digests, performed in a conventional plastic vial (right SVR ~900 m⁻¹) and with an inserted gold wire (length 5 m, diameter 100 µm) to simulate the conditions in a nanovial (left SVR ~15000 m⁻¹). The conversion from protein to peptides can roughly be calculated from the ratio between the peak height of a representative peptide clearly notable in both electropherograms (A and B) and the peak height of the remaining undigested myoglobin (myo). The yield improvement of peptide was a factor 3.5 (expressed as relation between peak height ratios), which was consistent with the results achieved from the earlier experiments, using nanovials and conventional vials, showing a corresponding peak height relation of 3.3.](image)

Reaction conditions: 100 µL reaction volume, 8 mg myoglobin/mL, 0.2 mg trypsin/mL, 8 mM NH₄HCO₃ pH 7.9, 6 hrs 37°C.

CE conditions (Beckman P/ACE system 5500): separation capillary ID 50 µm, OD 375 µm, total length 57 cm (50 cm to detector), 10 mM phosphate buffer pH 7, 100 µg FC134/mL, +30 kV 9.5 µA, 5 s pressure injection.
In this typical case, an increased surface is beneficial for the reaction. In other cases, surface interaction can be detrimental and surface treatment is very important for a successful reaction \( e.g. \) for PCR \([165, 166]\). However, in our work with miniaturization of the PCR using a liquid lid of octane (\textbf{Paper VI}), the sample formed a spherical droplet at the bottom of the octane phase due to the hydrophobic character of the surface. Consequently, surface contact with the vial is minimal. Using octane as a barrier against evaporation of water, amplification of DNA was performed in 50 nL PCR volumes. We noticed that the polymerase concentration needed to be increased when the reaction volume was reduced. This might be a result of an enrichment of polymerase molecules at the water/octane interface due to hydrophobic interaction. This would decrease the number of polymerase molecules free to participate in the amplification. It could also be that the presence of octane decreases the polymerase activity.
5. Analysis of nL-pL sized volumes of sample

The analytical methods described in this chapter are limited to the techniques used in the thesis.

5.1 Capillary electrophoresis

5.1.1 Principle

Electrophoresis was introduced by Tiselius [167], and is a separation technique well suited for the analysis of biomolecules in a miniaturized system. CE was first demonstrated by Hjertén [168, 169], and was further developed by Everaerts et al. [170] and Jorgenson et al. [171-173]. Today, CE is an established separation tool and several commercial machines are available. This has resulted in many published articles with applications such as separations of proteins, peptides, DNA, nucleotides, amino acids, small inorganic ions, whole cells, particles, and several review articles have been published [174-177]. Books describing the theory of the technique are also available [178, 179].

The separation of analytes in free solution using electrophoresis is based on differences in mobility for charged species in an electric field. The ion velocity, $v$, is given by equation (7)

$$ v = \mu_e \cdot E $$

where $\mu_e$ is the electrophoretic mobility and $E$ is the electric field. Using the ion charge, $q$, the viscosity of the separation media, $\eta$, and the radius of the solute, $r$, the electrophoretic mobility can be calculated according to equation (8).

$$ \mu_e = \frac{q}{6\pi \eta r} $$

Sample injection into the separation capillary can be performed electrokinetically or hydrodynamically using only a few nanoliter of the sample. The conductivity of the sample will greatly affect the quality of the electrokinetic injection. When the sample conductivity is lower than that of the separation buffer, the analytes will be injected in a narrow band, which is necessary for good separation efficiency. However, during the electrokinetic injection, a discrimination of the analytes occurs which favors analytes with high electrokinetic mobility. This discrimination can be avoided by performing the injection hydrodynamically but then the band broadening will increase due to Taylor dispersion.
Most detection principles for CE have been developed from HPLC detectors [180]. The most common detector for CE is on-column UV-VIS absorbance. The detector is simple to use but due to the small path length within the capillaries, the concentration sensitivity is moderate (ca $10^{-6}$ M). A more sensitive detection principle is fluorescence; especially laser induced fluorescence (LIF), which offers sensitivity on the order of ca $10^{-12}$ M [181]. Other detection methods based on electrochemical principles can be used e.g. amperometric, potentiometric and conductivity detection. CE has also been interfaced on-line with electrospray mass spectrometry [182].

5.1.2 EOF

Normally, capillaries made of fused silica, with inner diameters in the range 50-100 µm, are used as separation channels. Due to the negatively charged silanol groups ($\text{SiO}^-$ at pH $>$ ca 2-3) on the inner surface of the capillary, positively charged buffer ions in the separation media are attracted and enriched close to the surface. A double layer of ions is formed where the inner stagnant layer is bound to the wall and the outer layer is more diffuse. When a voltage is applied longitudinally over the capillary, the outer diffuse layer moves against the cathode. A bulk flow of the separation media also starts to move against the cathode due to the friction (viscosity) within the fluid. This bulk flow is termed electroosmotic flow (EOF). The flow profile is essentially flat and no additional band broadening is induced due to the bulk flow. For this reason, much higher separation efficiencies can be achieved compared to a pressure driven system with a parabolic flow profile.

There are several other techniques developed from CE or CZE (capillary zone electrophoresis). In micellar electrokinetic capillary chromatography (MECC), surfactant added to the separation buffer creates micelles, which will interact with hydrophobic solutes. Depending on the surfactant used, the micelles will move either towards or with the EOF and thus affect the separation of solutes. DNA fragments are not possible to separate in a CZE system since DNA fragments have the same mass to charge ratio. Therefore, a sieving media or a gel must be created within the capillary [183] - capillary gel electrophoresis (CGE). The sieving effect obtained by the gel results in shorter migration times for shorter DNA fragments. In capillary isoelectric focusing (CIEF), ampholytes with a wide range of pI are used to create a pH gradient. During separation, solutes will migrate through this gradient until the pH is equal to their pI. Capillary isochophoresis (CITP) is a technique commonly used for concentration of the solutes. The separation is performed using a buffer system consisting of a leading buffer with a higher mobility in front of the solutes and a terminating buffer with lower mobility after the solutes. When the separation voltage is applied, the solutes will arrange themselves in consecutive zones behind the fastest moving leading ion. The recent technique is CEC where CE separations are performed within capillaries filled with chromatographic media [184]. In this hybrid between HPLC and CE, the plug-flow of electroendoosmosis is utilized to increase the separation efficiency.
5.1.3 Wall adsorption

The strong negatively charge on an untreated fused silica capillary wall, due to the silanol groups, often attracts proteins during analysis with CE. This can be observed as peaks which become broad or even missing peaks in an electropherogram obtained after separation of proteins. The adsorption can be decreased by a number of methods, such as raising the pH above the pI of the protein (electrostatic repulsion between the protein and the wall) or lowering the pH in order to protonate the silanol groups. Also, an increase of the buffer strength promotes competition between cationic buffer ions and positively charged sites of the protein during interaction with the negatively charged capillary wall. Different buffer additives and modifications of the capillary wall have also been proposed.

A method developed in our laboratory has showed to be very useful to decrease protein adsorption in CE [185-187]. The method is based on the addition of a fluorinated cationic surfactant (FC134) to the separation buffer. The positively charged head group of the surfactant will be adsorbed to the capillary wall due to electrostatic attraction and a first layer is formed. Due to the strong hydrophobic forces between the tails of the surfactant molecules, now bonded perpendicular to the wall, a second layer of surfactants is created of the non-bound surfactant molecules. The bilayer formed will cause a reversal of the EOF due to the positive charge characteristic of this layer. Consequently, the CE analysis should be performed in a reversed potential mode.

5.2 Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

5.2.1 Principle

In 1987-1988, MALDI-TOF MS was introduced by Karas et al. [188, 189] and in 1988 it was reported for the first time to use an UV-laser for desorption of bioorganic compounds with molecular weights above 10 kDa [190].

Prior to analysis, the analyte is mixed with a matrix in a solution using a molar ratio ranging from 1:100 to 1:50000. Several different matrices can be used depending on the type of analyte [111] e.g. for proteins 2,5-dihydroxy benzoic acid or succinic acid are commonly used. The matrix fills several important purposes. First, it dilutes the analyte to prevent association of analyte molecules. Second, it absorbs energy and transfers it gently to the analyte, and thereby protects against analyte decomposition. Finally, it probably enhances ion formation of the analyte by photoexcitation or photoionization of the matrix followed by proton transfer to the analyte molecule [191].
Analyte ions are formed by shooting a short laser pulse against a dried spot of the matrix/analyte mixture. After acceleration of the ions to a fixed kinetic energy by an electric field, the masses of the ions are analyzed in a TOF spectrometer. After determination of the velocity of the ions by measuring the time to pass the flight tube, the mass to charge ratio (m/z) can be calculated. The time frame for an entire analysis after the sample preparation is less than 5 s per sample spot, which opens the possibilities for high throughput analysis.

5.2.2 Sample preparation (Paper I)

The quality of a mass spectrum is very dependent on the sample preparation [192, 193]. A commonly used method is the dried droplet method, which includes premixing of the analyte and the matrix solution and subsequently deposition on a metal target where the spot is left to crystallize. Unfortunately, the quality of the crystals formed within the spot is not uniform which leads to poor reproducibility within the same spot and between different spots. In many cases, hot areas within a sample spot must be found by shooting the laser at several different positions. Several different sample preparation methods have been proposed to increase the homogeneity and quality of the matrix/analyte crystals e.g. fast evaporation, crystal crushing, spin coated drying, spray methods, recrystallization and seed layer preparation. The goal for all these methods is to reduce the size of the crystals in order to achieve more homogenous composition and thus a better reproducibility. A complement to the listed methods is to prepare a sample spot with a size corresponding with the laser spot. This has been performed using chip-based picoliter vials with an individual volume of 250 pL (Paper I). Further, the fast evaporation of sample solvent, due to the small volume of sample used, also resulted in the formation of very small matrix crystals which is beneficial for good shot to shot reproducibility. Using the 250 pL vials, single-shot mass spectrum of as low amounts as 2.5 amol bradykinin and 25 amol of horse-heart cytochrome c were obtained, see Figure 21.
Recently, targets with hydrophobic surfaces equipped with hydrophilic anchors have been presented\(^7\). The use of such targets results in focused matrix crystals with high concentration of the analyte, which lowers the limit of detection. Also, a new MALDI target modification has been presented to enable the use of liquid matrices in commercial ion sources [194]. This is beneficial for IR-MALDI since the use of solid matrices together with IR ionization leads to increased analyte depletion in the irradiated sample spot. When liquid matrices are used, the irradiated spot can replenish itself by diffusion from the bulk sample solution. This is beneficial in order to increase the homogeneity of the analyte/matrix mixture during collection of several mass spectra without reposition of the target.

### 5.3 Fluorescence measurements

Fluorescence and luminescence detections within miniaturized systems are very beneficial due to the high sensibility and the possibilities to perform measurements in a non-contact mode. One of the most useful features is the possibility to perform parallel measurements in platform [5] and array systems [52, 195-197] using CCD cameras [198] or photon multiplier tube (PMT) detectors.

We have used LIF detection to follow the titration of small (nL) volumes using piezo-pipettes for an incremental addition of reagent (Paper II). In initial experiments,

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\(^7\) AnchorChip, Bruker Daltonics, Germany
methyl-red was used as an acid-base indicator and the progress of the titration was followed by direct monitoring, using a CCD-camera, without any particular optics or illumination arrangements. However, the detection system based on light absorption demanded very high indicator concentration in order to obtain an appreciable signal/noise ratio. This led to large errors, since both the sample and the indicator were titrated. Therefore, a fluorescence detection procedure, using fluorescein as an acid-base indicator was adopted. In this way, a very low concentration (10 µM) of indicator could be employed. The suitability of this approach has earlier been demonstrated by Wolfbeis et al. [138]. These authors used a blue light emitting diode and a bifurcated optical fiber. Although attempts were made to miniaturize the titration cell, their experiments were limited to sample volumes of 500 µL, determined by the size of the optical fiber and the precision of the burette.

The development of intercalating dyes for determination of double stranded DNA has resulted in new sensitive quantification reagents e.g. PicoGreen® [199, 200]. A modified form of the protocol was used to perform a fast determination of successful amplifications in our work with the miniaturized PCR using a liquid lid (Paper VI). The PicoGreen® molecules inhibit the polymerase so the dye must be added after the thermocycling in a separate step prior to the analysis. Also, all DNA molecules present in the sample will be detected, even amplified non-specific DNA and primer-dimer. Therefore, a CGE analysis of the DNA samples should be performed as well in order to confirm the results.

The amplification of DNA can also be followed in real-time by using a fluorogenic target-specific probe (TaqMan®) [166, 201, 202]. The probe is an oligonucleotide with both a reporter and a quencher dye attached. The probe anneals between the forward and reverse primers if the target sequence is present. During PCR thermocycling, annealed probes are cleaved by the 5’ nuclease activity of the polymerase and the reporter dye is separated from the quencher dye. This separation results in a detectable signal from the reporter dye. Additional reporter dye molecules are cleaved from their respective probes within each cycle, resulting in a proportional increase in fluorescence intensity. When working with miniaturized systems, the approach of using target-specific probes is preferable since all reagents can be dispensed prior to the thermocycling.
6. Future outlooks

Nanoscale chemistry can be performed in different kinds of miniaturized reactors. Two different modes are possible: continuous flow reactions or batch-wise operations. Each mode has its own usefulness, advantages and limitations. The final choice of reactor type has to be based on different application criteria, e.g. how reagents can be added (liquids as well as solids), to which extent cross-contamination must be eliminated, and if a removal of by-products is necessary. Problems with solvent evaporation can be critical as discussed earlier. Today’s chemical laboratories are highly automated in order to meet the demands of high-throughput capacity. For example, in the next generation platforms for screening of drug candidates, it is likely that as many as 1,000,000 samples/day will be processed. For simple assays, the capacity available today reportedly is already 245,000 samples/day\(^8\). The amount of sample and reagents needed to perform all these reactions would be enormous without miniaturized systems. Reactions must be performed in parallel to obtain the high-throughput capacity. From this point of view, reactions performed in a batch-wise mode could be preferable compared to continuous flow systems. However, flow systems could also be attractive if a segmented flow technology is utilized with e.g. 1,000 reaction tubes and 1,000 separate fluid segments in each reactor. However, it will not be easy to deal with cross contamination problems. Flow reactors in a format of a CD are already under commercial development. The format enables the use of centrifugal forces to distribute samples and reagents in parallel channel systems. However, even though the parallel capacity within each CD is e.g. 384 reactors, 2,600 CDs would be needed every day to reach the throughput capacity of 1,000,000 samples/day. Miniaturized vials for batch-wise reactions arranged in a high-density format (1,000*1,000 reactors) could therefore be more competitive. The standard format for biochemical assays in today’s laboratories is the 96-well platform. Operations like solid-phase extraction [203], liquid-liquid extraction [204, 205], and filtration can be performed within this format in a highly automated way. Addition of reagents and samples is performed with multiple dispensers arranged in the same format. In a near future, 384- and 1536-well formats will probably replace the 96-well platform. The “high-density-record” demonstrated so far is the 9600-well format [8] and it is just a question of time when platforms with increased density will be utilized.

The degree of miniaturization, which is realistic to utilize in practice is dependent on the amount of reaction product needed. In applications where only an analytical yes/no answer is required, miniaturization can theoretically be pursued until the ultimate detection limit is reached (i.e. single molecule detection). However, in a screening application where the reaction product needs to be used in further studies, miniaturization of the reaction step is feasible only if subsequent steps also can be performed in a compatible miniaturized format. Today, this is not the case. In the future, the primary screening stage as well as lead optimisation and evaluation should all be performed in miniaturized systems to take full advantage of all the benefits a miniaturization offers.

\(^8\) CyBi\textsuperscript{TM}-Screen-Machine, http://www.cybio-ag.com
Many of the dispensing tools suitable for automation *e.g.* piezo-electric dispensers, can deal with very small volumes (down to pL). However, rather large volumes of reagents are needed to fill such dispensers. The development of a new miniaturized inkjet-type device useful for dispensing picoliter samples from volumes less than 1 µL is highly desirable. The dispensers available today are useful for parallel dispensing of only a few rows of sample at a time. This will probably not fulfill the future requirements for high-throughput sample and reagent delivery. Therefore, new dispensers need to be developed to suit the miniaturized reactors in the future high-density platforms. Further, the development of cheap disposable piezo-electric dispensers that could be utilized for sampling of blood, body fluids etc., would be another milestone in miniaturized chemistry. However, a major issue of sampling will always be the logistic problem, where different samples will demand individual storage containers in order to avoid cross-contamination.

The need for parallel analysis is increasing dramatically also in other areas, *e.g.* in the rapidly increasing research area of proteomics where MALDI-MS enables protein identification in an unprecedented way [206]. However, protein separation of low abundant proteins often results in non-visible protein spots in the 2D gel, which is employed to separate the proteins. The spots can be analyzed by a scanning procedure using MALDI-MS [207]. After extraction of the digested proteins from the 2D gel (size 16*16 cm$^2$) 36 days of continuous measurements would be required to collect all mass spectra. When the huge amount of data has been collected, the evaluation of the results will still require a substantial time of work. Proteomics is a typical example, where the reactions should be performed following a more clever strategy in order to reduce the number of samples to evaluate, *e.g.* by using a first sorting procedure of relevant samples, *e.g.* based on a fast fluorescence measurement.

Recently, a break-through concerning LIF detection for DNA separation using parallel capillary gel electrophoresis has been presented [197]. The capillaries are rearranged from a two-dimensional array configuration into a three-dimensional capillary bundle. The fluorescence light from the labeled DNA fragments, at the position for laser excitation, is guided via total internal reflection to the end of the capillaries and imaged onto a CCD camera. With this detector, parallel CGE analysis of PCR products amplified in a high-density open well platform should be possible, offering a higher throughput and separation efficiency compared to current chip-based CGE devices.

In summary, this thesis has described a number of methods for performing nanoscale chemistry. These methods should provide some useful alternatives and/or new inputs to methods presently being used in the research area of chip-based chemistry. The trend today, in my opinion, is that many researchers in the field often give higher priority to technical developments as such than to obtaining performance for real-world applications. My hope is that the presented results (*Paper I-VI*) will give inspiration to develop new ideas to use simple devices in a more creative way.
Sample Handling in Nanoscale Chemistry

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Sample Handling in Nanoscale Chemistry


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