

Nanofluidic tools for bioanalysis : the large advantages of the nano-scale Janssen, K.G.H.

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CHAPTER **1**

Introduction and Outline

In this thesis new and miniaturized bioanalytical tools for the life sciences are presented. Two interrelated paths are described in this introduction: on one hand how metabolomics can have an important impact in drug development and treatment, and why this requires the analysis of sample volumes as small as those from single cells. To analyze these minute sample volumes, new separation and detection methods are reported in this thesis: isotachophoresis in nanochannels, depletion zone isotachophoresis, and a surface enhanced Raman spectroscopy sensor. On the other hand, working with nanochannels, has led to the discovery of new unexpected fundamental phenomena, also reported in this thesis for the first time: in nanochannels acidification of buffer solutions up to 1 mol/L occurs, and extreme pressures > 1000 bar can be induced. This introduction provides the rationale, concepts and terms as well as (theoretical) background for this research.

Section 1.1 introduces the potentially large impact of the systems biology approach, and metabolomics in particular, on drug research and treatment. In section 1.2, a gap is identified between the requirements of metabolomics and current separation and detection methods, to analyze samples as small as an aliquot of a single cell. Miniaturization of equipment platforms including Lab-on-a-Chip, are also discussed. A potential solution to bridge this gap is discussed in section 1.3, namely the use of nanochannels for further downscaling. Special properties and challenges in the use of nanochannels are described. Section 1.4 describes electrophoretic separations, which are compatible with minute volume analysis, particularly isotachophoresis. The principles of the new technique of depletion zone isotachophoresis are also described. Section 1.5 explains the optical detection technique of surface enhanced Raman spectroscopy, a technique compatible with the minute volumes and low abundance at which analytes of interest occur in minute samples. The goal and outline of the thesis are described in section 1.6.

1.1 Systems Biology, a new approach in drug research and treatments

In conventional drug research the target for an intervention was often reduced to a single receptor or pathway, which was then modulated with a drug. Although successful for various diseases in the past, this reductionist approach has not proven sufficient to tackle more complex diseases such as cancer¹, multiple sclerosis², migraine³ and rheumatoid arthritis⁴ and osteoarthritis⁵. The complexity of these diseases is attributed to the involvement of many pathways and multiple potential targets. This is reflected in not fully understood efficacy variations and various side-effects when studying a group or population of patients when using one single pharmacologically active compound. In drug development, this increases the risk of failing the demanded validation studies in a late stage, as even for an active compound which initially may look promising the efficacy in a wider population is much harder to predict. This higher risk of drugs that will not reach the market and return their investment, directly results in increasing drug development costs. In turn, this makes it less attractive to develop new drugs, so that in the end fewer new drugs reach the market⁶. A new approach to drug research is therefore critically needed.

In answer to this need, the vision of systems biology has recently gained momentum⁶. In contrast to the reductionist approach mentioned in the previous paragraph, the philosophy of systems biology suggests a holistic approach: if the relevant multiple biological processes and their interplay involved are mapped, the disease mechanism can be better understood. In a drug development approach, when starting from a systems perspective, better efficacy and toxicity predictions are expected, resulting in an increase in drug development research efficiency, and corresponding decrease in costs; this approach was recently termed systems pharmacology^{7,8}.

Another important contribution to what makes a treatment intrinsically complex are differences between individual patients, in genotype and from environmental changes during development. As a consequence there is a variation in efficacy between individuals, affecting not only the development of new treatments, but the application of many existing pharmaceutical treatments as well. A drug might not be effective for certain patients while in yet others it may require a different dosage, be ineffective, or induce unexpected side effects that may even be severely harmful. The treatment of rheumatoid arthritis in the Netherlands is an example of this current approach where the same regime is applied to all patients: first the common drug methotrexate is applied. When not effective in disease modification, the dosage is increased and other drugs (sulfasalazine, leflunomide or hydroxychloroquine) are tried. When still not effective a combination of several drugs is tried (abatacept, rituximab or tocilizumab), before finally drugs of a new generation called biologicals (one of the TNF-alpha blockers) are applied⁹. This trial and error process can take many months, with potentially no improvement in patient condition or unnecessary worsening of the disease and additional patient suffering. This again demonstrates the need for a more direct, evidence-based way for selecting the proper pharmaceutical interventions, to maximize efficacy and minimize side effects, in the clinical practice for the individual⁴.

A more direct evidence-based approach can be provided by the systemsbiology approach, not only providing indicators for the disease state that can be monitored during treatment of the individual, but also those that provide an early warning before the actual onset of the disease. This accelerates the process of treatment optimization, to the benefit of the patient. First results have for example identified indicators that predict efficacy of rheumatoid arthritis medication⁴ in individuals. This case illustrates how the benefits of the systems biology approach need not be limited to the development of new drugs, but can also contribute to the prediction of the efficacy of many existing pharmaceutical treatments. The concept of tailoring a treatment to the individual patient is called personalized medicine¹⁰.



Figure 1.1 Schematic view of systems biology components. This very simplified model shows how genetic information within the DNA is transcribed into messenger RNA (mRNA), which is translated into peptides and proteins, which in turn work among others as catalysts in catabolic and anabolic pathways. The set of all metabolites therefore is an important contributor to the phenotype. In practice this system is much more complex as transcription from the genome is in turn regulated by metabolites, peptides, proteins and mRNA and vice versa. Whereas the genome, in combination with the environment during development, contributes to the phenotype on a lifetime scale metabolites reflect the actual phenotype on time scales from days to sub-second¹¹.

Figure 1.1 shows a schematic of the major molecular levels of a biological system and their intricate relations. It shows the intermediate steps between the genotype or genome, the collection of genes of the organism, towards the phenotype. An organism's phenotype is the complex of its observable characteristics, from shape down to sub-second variations in chemical composition. This includes the aspects of health and disease. The interplay between genes, transcripts, proteins and metabolites contributes to this phenotype, providing an indication why diseases can be very complex. The relationships presented in Figure 1.1 illustrate the challenges involved in making the systems biology vision for drug development and personalized health a reality as it requires understanding of many of its mechanisms.

Several parts of the biological system have been extensively studied in previous decades. Enormous progress has been made in the field of genetics, with the human genome now being available. Genes determine lifelong effects such as eve color and are strongly related to the propensity to develop some diseases. including cancer and hereditary diseases. For proteins enormous progress has been made in the understandings of their workings. The understanding of the complex interplay between genes and their transcripts however, remains incomplete. At the other end, an organism's biochemical state on day and shorter time scales, is predominantly reflected in its metabolic state (see Figure 1.1). The entire composition of all metabolites, which includes all small molecules which are intermediates and products of metabolism, is called the metabolome. The metabolome includes communication and regulation compounds such as hormones, neurotransmitters and central energy/carbon metabolites such as adenine triphosphate (ATP). Studying the metabolome as a whole is a relatively new field, whereby the comprehensive quantitative and qualitative analysis of the metabolome is called metabolomics^{11,12}.

Metabolomics is attractive for the evaluation of disease states for two reasons. Firstly, the metabolome reflects the organism's homeostasis, the property of a system to maintain a stable condition and to withstand challenges and perturbation. The metabolome represents an important part of the organism's phenotype as a whole including down to the cellular level, and on a time scale including days down to sub-seconds. This makes the metabolome an attractive indicator for monitoring the influence of medical treatment in personalized health. Secondly, as indicated in Figure 1.1, the metabolome is not only closest to the phenotype, but transcription and protein synthesis are also partly controlled through metabolites. In conclusion, the metabolome is in a unique position to provide an indicator for an individual's health state. Making metabolomics available as a tool for diagnosis of diseases and the prediction of treatment outcome, and thus also enabling personalized health strategies, is the main goal of the research at the Division of Analytical Biosciences (Leiden, The Netherlands) and the Netherlands Metabolomics Centre (NMC). Of course, on the longer term, integration of all available data at different "-omics" levels can only further improve the diagnosis and prediction of the disease outcome.

1.2 Equipment in metabolomics and the impact of miniaturization

1.2.1 Technological challenges in metabolomics measurement equipment

This subsection describes some of the technological challenges in metabolomics research in general, but with the emphasis on minute volume analysis. Discussed is why the field of metabolomics, despite its vast potential, is less far developed than genomics (genome analysis) and peptidomics (protein analysis).

First of all, due to the huge variety in physicochemical properties of metabolites, a full metabolome analysis with a single method or technique is currently not feasible. Today, a full metabolome analysis uses several platforms and takes up to 1-3 hours per sample. Profiling of only the major metabolites in samples where enough volume is available with NMR is faster, and takes only up to 10 minutes per sample. This stands in contrast with genomics, where the target, DNA, is physicochemically relatively homogeneous and can be measured and quantified by e.g. gel electrophoresis. In metabolomics, multiple methods, differing in sample pretreatment, separation and detection are needed. Arguably the result of metabolomics in health and disease may identify clusters of compounds which represent a single biomarker, so that not the entire metabolome needs to be analyzed.

Secondly, in metabolomics there is a large variation in relative abundance of metabolites, and in addition these are found in different compartments and matrices¹². Metabolomics requires comprehensive analysis of metabolites in different matrices in the organism, from the whole body (e.g. blood), down to organs, tissues and ultimately individual cells. In drug research, an important example of the relevance of metabolomics on the smaller compartments tissues and single cells), is cancer, a disease which may include (highly) differentiated cells. This makes treatment difficult or a cure impossible, unless understanding of processes on the cellular level is included. The variation in matrices presents a huge analytical challenge. An indication of the sample sizes involved on the various levels from organism to sub-cellular level is illustrated in Figure 1.2. In comparison, in DNA analysis the challenge of low amounts has been eliminated by the polymerase chain reaction (PCR), now widely available, which can amplify DNA from even a single cell or one fragment, to detectable amounts. This has contributed to the fact that the entire human genome is now known. At present next generation sequencing has even become available. lowering the time and costs for the analysis of a whole genome significantly further.

Thirdly, to make optimal use of metabolomics, monitoring or time-resolved analysis of the (fast) metabolic processes is highly desirable. If one wants to take longitudinal samples of the same system, the amount of sample and the way in which it is obtained should not be of significant influence to the system. This would not be possible in single-cell genomics where it would simple mean removal of the cell's DNA, killing it.

Fourthly, the amount of information itself and the requirements on analysis and elucidation of the correlations places a high demand on data-analysis in terms of new techniques and computational power. These challenges fall beyond the scope of this thesis.

Note that the first three challenges mutually reinforce the requirements for low sample volume and corresponding small amount of analyte: particularly cells and tissues have a minute volume to begin with, the minimal disturbance needed for a longitudinal analysis constrains the volume that can be sampled and the need for multiple methods can necessitate splitting of this volume. Arguably, in a significant portion of future metabolomics applications, in drug development and personalized medicine, is not expected that a full metabolome analysis of single cells is required. For example, a blood sample may suffice, or a set of biomarkers as a group forms an adequate biomarker, and the technical challenge is correspondingly less. Regardless of the potentially large impact of single-cell metabolomics in the future, it is presently in its infancy with first results of whole cells being published in recent years ^{13–15} but developing very rapidly as reviewed elsewhere ¹⁶. In conclusion, to establish the potential of longitudinal single cell metabolomics requires integrated analysis methods that can work with minute amounts of analyte.



Figure 1.2 Overview of the sample volumes involved in bioanalysis of the various organism compartments. The sample volumes are indicative of the amount that can be extracted without disturbing homeostasis. Standard analysis methods in metabolomics currently comprise sample preparation, usually followed by separation, and detection/identification of the metabolites of interest, or of all metabolites detectable with a certain method. Usually, methods require 10,000 or more cells, or a few microliters of sample. However, some methods can detect metabolites in principle also in smaller samples, as is shown in Figure 1.4. Current methods for metabolomics are discussed below, particularly their compatibility with minute volume analysis and low absolute amounts of analyte, as needed for analysis down to single cells.

Conventional liquid chromatography (LC) is one of the most commonly used separation techniques in metabolomics, as it is applicable to a wide range of compounds and volumes (although volumes greater than of few tens of microliters are predominantly used in preparative and/or purification applications). The separation mechanism is based on the difference in distribution per analyte, between a stationary phase and a mobile phase¹⁷. Standardization and a high level of automation of this method is available, making the method relatively easy to implement. The extensive range of LC equipment commercially available includes LC columns, automated systems and standard interfacing with detection instruments and analysis software. Miniaturization of particle sizes in chromatography enables improved resolving power. In order to apply the same linear flow rate as for larger particles¹⁷ however, higher pressures are required, posing a challenge for interfacing and necessitating more powerful pumps. The limits for downsizing of particle sizes are currently about 1 um. Sample sizes on the order of tens of nanoliters can be analyzed with sufficient sensitivity by miniaturizing also the column inner diameter as demonstrated by nano-LC (Figure 1.3) which uses minute columns and commonly uses small particle sizes 17,18. Open tubular LC in nanochannels may provide higher separation power¹⁹⁻²¹, but experimental challenges limit this technique at the moment to a few specialized research labs.

Electrophoretic separation techniques are compatible with a wide range of sample volumes, and are predominantly utilized in the capillary format¹⁷. Their separation mechanisms are based on the difference in migration speed of ions in a matrix, e.g. a solvent or gel, under influence of an electric field. Since many metabolites are charged, electrophoretic separation can be applied to a wide range of compounds. Electrophoretic techniques, such as capillary zone electrophoresis (See Section 1.4), also provide significantly higher resolving power compared to most chromatography based techniques¹⁷. This is due to the absence of several factors that contribute to peak broadening in most LC approaches: 1) Capillary zone electrophoresis (CZE) takes place in an open capillary, whereas in conventional LC the liquid travels in many parallel paths which differ in velocity past the particles. 2) Bulk flow in LC is provided by pressure which has a parabolic flow profile, which contributes to peak broadening, whereas electro-osmotic flow in electrophoretic techniques has a flat flow profile. The downscaling of pressure

driven methods is challenging due the correspondingly higher pressures needed. This requires pumps that can supply the higher pressures precisely and robustly. and imposes higher demands on pressure resistance of connections and injection methods. Although commercially available, these systems are not standard and more expensive. Contrary to LC, implementation of miniaturized CE separations only requires a smaller cross-section while most other experimental conditions and equipment can remain the same. However, the consequences for detection (Figure 1.4) are not straightforward due to the decreasing analyte amounts and are considered separately in section 1.2.3. Furthermore, miniaturization has been shown to improve electrophoretic techniques, predominantly because it allows the use of higher field strengths. Downscaling causes a reduction in heat generation, where heat can cause bubble formation in the liquid and gradients of physicochemical properties in a capillary resulting in band broadening. Higher field strengths also enable faster separations with improved efficiency, as the dispersive effects from diffusion become smaller¹⁷. Electrophoretic techniques are therefore more suited for metabolomics of ultrasmall samples than LC as they provide the small volume compatibility and resolving power required.

Despite the advantages of electrophoretic techniques over LC, CZE as the most applied electrophoretic technique remains far less popular than LC due to several disadvantages, of which the following two are considered the most relevant: 1) in CZE, by nature of the separation mechanism, each analyte zone arrives at the detector at a different speed, affecting the observed length of the zone. The migration velocity is very sensitive to changes in temperature (\approx 1-2% per degree Celsius²²), making quantitative analysis and identification^a more challenging compared to LC, where the flow speed can be set constant. 2) CZE is performed in capillaries and small channels to prevent excessive heating. Although the compatibility with small volumes can be considered an advantage of CZE, this also proportionally reduces the loadability, when not hyphenated with preconcentration techniques, considered a drawback as it challenges detection for dilute analytes. Fortunately, the powerful electrophoretic technique of isotachophoresis suffers neither of these disadvantages, as detailed in section 1.4.2.

Figure 1.4 shows the most common detection techniques used for metabolomic analysis and an indication of their compatibility with smaller peak volumes, which are obtained when downscaling analytical methods. NMR is often used for profiling of the most abundant metabolites without prior separation, and for identification of (often isolated) compounds¹²; it is currently not suited as a fast online or inline detector for the profiling of low-concentration metabolites in a wider set of samples due to among others a lack of a sufficiently robust in-line interface. Mass spectrometry (MS) is the most generic detection technique, as MS can selectively detect a wide range of metabolites with good sensitivity. Furthermore, high resolution MS supports identification of the detected metabolites via determination of the elemental composition, if the mass resolution of a compound is high enough;

^aConventionally, e.g. in CE-UV, peak area is used in quantification and as slower zones are seen as broader peaks, experimental variations in speed likewise affect quantification accuracy²².



Figure 1.3 Typical separation techniques in metabolomics and the sample volume range in which they can be operated under optimal conditions. On the abscissa an estimate of the possible sample volume is given at which a method can be used with good performance whereas the height provides a tentative indication of how extensively the technique is presently used.

identification is further supported by controlled fragmentation of compounds and determining the elemental composition of these fragments. MS can be used as in-line detector with GC, LC, nano-LC and many electrophoretic techniques including capillary zone electrophoresis (CZE)¹². Not surprisingly therefore, mass spectrometry is the most used detection technique in metabolomics. Conductometry, measuring the local conductance of the eluate, is non-specific and provides relatively poor detection limits. Amperometric detection is very sensitive but only for a select few electroactive metabolites such as dopamine²³. Fluorescence detection is very specific and highly sensitive, most metabolites however requires labeling however, and when combined with laser illumination, detection limits down to single molecule can be provided for a selected few compounds²⁴.

1.2.3 Miniaturization of analysis methods & instruments: advantages and challenges for metabolomics

Halfway the 1970's, technology from the semiconductor industry made it possible to machine channels with dimensions on the order of micrometers. This enabled the downscaling of analysis platforms, with one of the first examples being a gas chromatograph scaled down to a single chip, composed of minute channels in 1979²⁵. It was recognized that integrated fluidic microchannel structures, so-called microfluidic chips, enabled a hitherto unprecedented control of minute volumes of liquids^{26,27} Microfluidics has since developed into a still expanding interdisciplinary research field characterized by the use of fluidic channels with dimensions on the order of micrometers. The growth of this research field was stimulated because the significant benefits towards bio-analysis were recognized early on. Obviously the intrinsic small size of the platform allows the



Figure 1.4 Detection methods in metabolomics and the optimal peak volume at the detector, i.e. the volume in which a peak introduced into the detector can be well measured. It should be noted that the peak volume is not the same as the sample volume, but can be smaller or larger. On the abscissa an estimate of volume compatibility is given down to the single molecule (SM) level, whereas the height provides a tentative indication of the applicability or current use of the technique for metabolomics.

manipulation of liquid volumes on the order of several microliters down to nanoliters, greatly reducing reagent use and sample sizes correspondingly. Even more important, networks of these channels in a so-called microfluidic chip allow the integration, automation and standardization of complex methods in a single fluidic chip. This so called Lab-on-a-chip concept can in principle improve speed, robustness and operator-friendliness of analytical methods. Using advanced microfabrication techniques, very complex devices can be made allowing a high degree of fluidic control^{28,29}. Lastly, in combination with an in-line detection method a complete miniaturized analysis platform can be realized, a so-called micro total analysis system (μ TAS).

In general, miniaturization poses a challenge to the sensitivity of most detection techniques. A detection method is needed that is suitable for low amounts of molecules, and applicable in-line with micro- or even nanochannels. For the larger microfluidic systems, MS interfacing meets most detection needs¹², although it has to be mentioned that mass spectrometry as yet has not been miniaturized successfully to the footprint of the above mentioned liquid-manipulation and separation modules. Conductometric detection is also often encountered in microfluidics, as the required electrodes can be built into the chip, thereby fully integrating the detection³⁰. Laser-induced fluorescence (LIF) detection has so far been the method of choice for most microfluidic and nanofluidic systems because of its excellent detection limits²⁴, and its ease of use as an in-line detector in combination with transparent chips (e.g. glass). Unfortunately most metabolites require fluorescent labels, necessitating the integration of a successful labeling step. Also only a limited amount of different labels can be used, making LIF less suited for metabolomics profiling. Surface enhanced Raman spectroscopy (SERS) is an optical detection technique that provides specificity for unlabeled (bio)molecules in the form of vibrational signature spectra, and for a few compounds single molecule detection limits have been reached³¹ (see section 1.5). These advantages over other detection techniques such as LIF make SERS attractive as a detection technique for miniaturized analysis in e.g. metabolomics. Unfortunately, SERS is currently not compatible with in-line detection, due to irreversible binding of analytes to the SERS surfaces, a challenge addressed in Chapter 5.

Microfluidic separations have been applied to DNA fragments, proteins and metabolites^{32–36}. Notable commercially available bio-analytical platforms based on microfluidic chip devices include the Agilent® 2100 Bioanalyzer for the analysis of DNA, RNA and proteins. The company Fluidigm® provides a chip platform for single cell gene expression, targeted DNA sequencing, DNA polymerase chain reaction (PCR) and for protein crystallization²⁹. On the field of personalized health a lab-on-a-chip platform was developed by Medimate®, which enables the measurement of the pharmaceutical agent lithium in a drop of blood, in a few minutes, by patients themselves³⁰.

In section 1.2.1 the technological challenges for different types of metabolomics analyses were identified: the needs were stipulated for smaller volume analysis, integration of multiple methods, increased speed and/or cost reduction. In principle, miniaturization holds the promise to address all these aspects. Microfluidics provides minute sample compatibility, ease of use, automation and standardization. Particularly electrophoretic techniques were identified to be well suited for miniaturization, and in addition, downscaling may even improve the resolving power of electrophoretic techniques.

1.3 Nanofluidics

1.3.1 Nanochannels in separation science

The final frontier of miniaturization is the use of channels with at least one dimension smaller than a micron, so-called sub-micron channels or nanochannels. Nanochannels may ultimately approach the dimension of a water molecule (3 Ångström), and are studied in the field of nanofluidics^{37–42}. Nanochannels have been made possible by means of technology translated from the semiconductor industry⁴³, with an example given in Figure 1.5. Integration into a fluidic chip yields a promising analysis platform, capable of liquid manipulation on the picoliter to femtoliter scale. This provides a means to handle and manipulate minute biosamples as required for single-cell and sub-cellular analysis (Figure 1.2). So far, liquid chromatography^{44,45} and zone electrophoresis^{46,47} have been successfully demonstrated and studied in a nanofluidic environment. The advantages of downscaling electrophoretic separations in microchannels also apply to nanochannels⁴² as detailed in section 1.4.3.



Figure 1.5 Scanning electron microscopy image of a cross-section of a nanochannel. The channel is 40 μ m wide and 50 nm in depth ⁴³.

Nanochannels however, are not just smaller channels. Due to their high surface-to-volume ratio, surface effects are more pronounced or even dominant compared to the liquid^{37–41}, with the most relevant phenomenon being related to the electrochemical double layer (EDL)⁴⁸. This EDL is theoretically detailed in Chapter 2 of this thesis. The influence of surface effects on electrophoretic separations is discussed in the introduction of Chapter 3. The increased relevance of these surface effects differentiates nanofluidics from microfluidics, creating unique challenges and opportunities, making chemical analysis in nanochannels an exciting and fruitful field of research.

1.3.2 Nanofluidic concentration polarization

The concentration polarization effect, is a good example of a unique opportunity offered by the use of nanofluidics in Lab-on-a-Chip devices. It forms the basis of the novel separation technique depletion-zone isotachophoresis, described in chapter 4 of this thesis. Concentration polarization is known from the field of membrane physical chemistry and nanochannels function as a single membrane pore to cause concentration polarization. Since its first demonstration at a micro-nanochannel interface by Pu et al⁴⁹, concentration polarization has been studied by several groups^{39,50,51}. In Figure 1.6 the principle is schematically shown. In a nanochannel the countercharge from the walls in the double layer provides a significant contribution to the total amount of ions in the solution, shifting the ratio between co- and counter-ions. Under an applied electric field the asymmetric conduction by positive and negative ions induces the effect of concentration polarization. The charge asymmetry in the nanochannel as a function of ionic strength and pH, and hence the amount of ions depleted per unit current at its interfaces with a microchannel, is described by a model in Chapter 2.



Figure 1.6 Principle of concentration polarization. Depicted is a nanochannel that connects two microchannels. The surface charge is in this system is negative. A) The system is filled with a solution of a monovalent salt. In the narrower nanochannel the counter ions of the surface charge significantly contribute to the total amount of ions in the solution, yielding a positive to negative ion concentration ratio of 3:1 in the solution. In the microchannel the contribution of the surface charge contribution is negligible and the ratio is 1:1. B) Under application of an electric field a current of 4 elementary charges per unit time is carried by negative and positive ions equally in the microchannel. In the nanochannel the asymmetric distribution of ions results in a correspondingly asymmetric charge transport. Summation of the ions transported in region I yields a net result of 1 salt ion pair being depleted per time unit. In zone II the net result is the enrichment by one salt ion pair per time unit. Over time the net effect of this charge asymmetry in the nanochannel leads to expanding depletion and enrichment zones

1.4 Electrophoretic separation techniques

1.4.1 Principles of electrophoretic techniques

Electrophoretic separation is based on the migration of ions in liquids effectuated by an applied electric field, a principle established by Friedrich Kohlrausch in 1897⁵². Each ionic species i has a different velocity, v_i , in an electric field, E, according to its electrophoretic mobility μ_i :

$$v_i = qE/6\eta\pi R = \mu_i E \tag{1.1}$$

with q the charge of the ion and R its effective hydrodynamic radius in a liquid of viscosity η . The most straightforward separation technique that exploits the difference in μ_i of each ion is zone electrophoresis (ZE)¹⁷. In capillary-ZE (CZE), a sample is injected at the inlet of a capillary, commonly by pressure; next, under influence of a subsequently applied electric field each ionic species migrates at a velocity according to its mobility. Detection of the separated ions can be performed with optical methods along the capillary or at the capillary end with for example mass spectrometry or electrochemical detection¹⁷.

Electrophoretic techniques are particularly suited to miniaturization^{32–35} as they benefit amongst others from improved heat dispersion as already described

above. Electrophoretic separation techniques that have been successfully applied in microchannels include zone electrophoresis, iso-electric focusing and iso-tachophoresis (ITP)^{32–35}. With the advent of nanochannel fabrication technologies⁴³ the nanofluidic domain is receiving increasing attention. So far, zone electrophoresis^{46,47} and isoelectric focusing have been successfully demonstrated in a nanofluidic environment^{46,47}. Despite the benefits the use of nanochannels is less popular than the use of microchannels, although the advancement of fabrication techniques has mostly closed the gap with microchannels. Arguably this is due in part to detection limits becoming a limiting issue, while also for many applications downscaling further than microfluidics is not necessary. An expansion towards nanofluidic technology in future applications is expected for the analysis of metabolites or other biomolecules in individual cells or even smaller, in organelles.

1.4.2 Isotachophoresis

Isotachophoresis is an electrophoretic separation technique that also provides the advantage of focusing $^{53-56}$. Isotachophoresis is effectuated by placing the sample (consisting of diluted analyte ions) next to a zone of fast ions of high concentration (the leading electrolyte, LE) and applying a voltage difference. As a consequence the sample ions undergo isotachophoretic focusing and separation as detailed in Figure 1.7. After some time an equilibrium is achieved when all ions move at the same velocity (Figure 1.7B), hence the name iso-(same)-tacho-(speed)-phoresis (migration). At equilibrium the analytes will have reached a concentration C_{i} , the so-called plateau concentration, related by a factor k to the LE concentration, C_{LE} :

$$C_i = kC_{LE} \tag{1.2}$$

The factor k depends among others on the mobility of the analyte and the coand counterion mobilities in the leading electrolyte.

Isotachophoresis concentrates a sample zone containing a mixture of analytes at low concentration into a sequence of concentrated zones of these ions. In conventional ITP, a second electrolyte is added behind the sample, chosen to have ions with a lower mobility than those in the sample, a so-called trailing electrolyte (TE), as detailed in Figure 1.8. The LE is chosen to have a concentration as high. and the trailing as low as practical, for instance as reported by Jung et al⁵⁷. These authors used 1 mol/L NaCI as LE and 5 mmol/L HEPES, pH 5.3 as TE, demonstrating a concentration increase by a factor of 2 million. The added benefit of a high concentration LE, besides causing a very high final plateau concentration for analytes, is that the voltage will drop predominantly over the sample and trailing zone, and the correspondingly high local field will maximize the focusing speed.

When available in sufficient amounts, a maximum concentration is reached and analytes are separated in neighboring zones, in so-called plateau mode ITP.



Figure 1.7 Principle of isotachophoresis. A) Sample ions (triangles), are positioned next to a high concentration of co-ions (ions with the same charge sign) of the leading electrolyte (LE), selected for having a higher electrophoretic mobility (counter-ion contributions are neglected). The electric fields in each zone induces migration velocities (Eq 1.1) as schematically depicted by the arrows. The sample ions move faster in the sample zone than the leading ions in the leading zone, as the concentration of leading ions is higher, and therefore the local conductivity higher and the field strength lower. Consequentially the sample ions increase in concentration at the interface which sharpens. B) In equilibrium (after migrating further along the channel) the ion velocities have normalized, with the sample ions having reached their plateau concentration, and a corresponding electric field is reached (Eq 1.2).

The plateau concentration depends amongst others on the concentration of the leading electrolyte (LE), (see Eq 1.2) and the mobility of the analyte^{53–56}. In conventional separations the resolution between analytes is expressed as the distance between the, assumed Gaussian distributed, peaks relative to a measure of their width. In ITP however zones always partially overlap, in the form of a diffused zone, and the ability to distinguish trace analytes in their individual zones depends on the length of a zone, relative to their interfaces. Therefore, resolving power in ITP can be expressed in terms of the length of the plateau zone normalized by the characteristic length of diffused zone^{58–60}. Since the length of a plateau zone is proportional to the absolute amount of trace analyte, the separation efficiency of ITP in equilibrium is also proportional to the absolute amount of analyte. Note that this makes the resolving power in ITP independent of the initial analyte zone width or the sample volume, as opposed to for instance zone electrophoresis.

If not enough absolute amount of analyte is present, the plateau concentration is not reached, and analytes focus as peaks (so-called peak mode ITP) which may partially or even completely overlap with neighboring peaks or zones⁵⁸.

1.4.3 Benefits of the miniaturization of isotachophoresis

ITP particularly benefits from miniaturization, as the length of the ITP plateau zones, which are a measure of resolving power, increase proportionally with decreasing channel cross-section, if the amount of analyte is kept constant, as described extensively by Bagha et al⁶⁰. This inherent downscaling benefit originates



Figure 1.8 ITP focusing over time for two analytes. Assumed is a minimal EOF (not shown), so that displacement (towards the right) is due to net migration. Assuming ground at the origin, this figure is equally applicable to describing ITP for positive analytes and a negative potential at x or the reverse. The E field is inversely proportional to the concentration (not shown here, but for reference see Figure 1.7). A) Initial state. The sample analytes, assumed dissolved in trailing electrolyte, are interposed between leading electrolyte (LE) and trailing electrolyte (TE) B) After application of an electric field along the axis of the separation channel, focusing is ongoing. Part of the sample ions S₁ and S₂, have reached their plateau concentration (Eq 1.2), but full separation is not complete. C) ITP in equilibrium, with the analytes fully focused and resolved.

from the fact that ITP is a concentration-driven focusing technique that focuses up to a plateau (Eq 1.2); in smaller cross-sections therefore a smaller amount of analyte is needed for the same concentration per length. Or phrased differently: the same amount of analyte forms a longer zone illustrated in Figure 1.9. Optimal use of this advantage is made in case of low abundant analytes and if the detector is concentration sensitive. In case of detectors sensitive to the absolute amount per cross-section, better detection limits are achieved with larger cross-sections (e.g. a longer path length in optical detection), but the poorer separation requires that the detector is capable of distinguishing simultaneously arriving compounds.

An important proof-of-principle of ITP downscaling has been given by Walker et al.⁶¹, who were the first to demonstrate ITP in a microfluidic device. Many applications of ITP in the microfluidic chip-platform have been reported since, as extensively reviewed elsewhere^{33,62–64}. Jung et al. reported concentration factors of up to a million times, achievable due to downscaling to microfluidic dimensions⁵⁷. Isotachophoresis in nanochannels is assessed for the first time in Chapter 3 of this thesis, for the purpose of ultra-small sample analysis such as that from a single cell.



Figure 1.9 Theoretical schematic of the effect of downscaling the channel cross-section on the resolution of ITP of two analytes of constant absolute amount. LE and TE concentration (not shown) and field strengths are assumed constant; all subfigures are for ITP in equilibrium. The two analytes and their constant amounts are indicated by the 3 circles and triangles each. Upon downscaling of the cross-section from A to B to C, correspondingly less ions are needed to achieve the plateau concentration per length. A & B correspond to peak mode equilibrium, with no separation, C Equilibrium in plateau mode with the two compounds each in its own zone corresponding to an improved separation vs. B.

1.4.4 Depletion zone isotachophoresis (dzITP)

A synergy between ITP and the nanofluidic effect of concentration polarization can provide a new hyphenated separation and focusing technique, dzITP. An important simplification compared to conventional ITP is that now only a single background electrolyte is needed, that doubles as the leading electrolyte. The zone depleted by the nanochannel provides a function similar to the terminating electrolyte, from whereupon the rest of the process in the channel can be described in analogy to ITP principles. Several of dzITP's potential applications and benefits are described in Chapter 5 of this thesis.

The working principle of dzITP can be described as a combination between ITP and concentration polarization in a microchannel perpendicular to a CP-induced by a tangential nanochannel. In a classical nanofluidic pre-concentration device, ions are focused due to a local gradient in the electric field induced by a depletion zone ⁶⁵ (For details on the depletion zone see section 1.3.2 & Figure 1.6). In such a device however, a form of ITP can be induced: dzITP. The principle of dzITP is illustrated in Figure 1.10. In short, the depletion zone locally increases the electric field along the microchannel. While bulk flow, in this case by EOF, is constant along the microchannel, locally the individual migration velocity of ions is not, as a result of this difference in E-field; In the depletion zone migration is dominant over EOF while elsewhere the EOF is dominant. This creates a focusing condition at the interface between the leading electrolyte containing sample ions and the depletion zone, at the location where electromigration is exactly balanced by the EOF. This location is very stable and can be positioned by the applied potentials.

The depletion rate is a function of the charge asymmetry in the nanochannel per unit current (see section 1.3.2). The excess op positive ions, responsible for the charge asymmetry in the nanochannel, may be predicted based on the results reported on nanochannel deprotonation in Chapter 2, which give the amount of charge per surface area as a function of pH and ionic strength.



Figure 1.10 Principle of depletion zone isotachophoresis (dzITP) described by means of two negative analytes during the focusing and depletion process. The lower part of the graph shows part of a chip with an H-shaped channel pattern, comprising a nanochannel connecting two microchannels with reservoirs for fluidic and electrical connections at the ends (not shown). Indicated are the potentials applied, with ground on the nanochannel side and two different positive voltages at the microchannel ends. The gradient along the microchannel induces an EOF along the microchannel in the negative direction (right to left). In the top part of the figure indications for the electric fields per region are shown. The depletion zone created by the nanochannel (see section 1.3.2 & Figure 1.6, for details) has a large E-field (in practice several tens of times that of the LE zone). Indicated for two analyte ions (the LE ions are not shown) are the velocity contributions per zone. In the LE zone the EOF is dominant and the net velocity of analytes is negative transporting them towards the nanochannel. In the depletion zone the field is high, and migration of sample ions is positive. In the intermediate region the velocity of EOF matches that of the individual migration, and focusing occurs in order of mobility, as in ITP.

Depletion zone isotachophoresis belongs to the family of counter-flow gradient focusing techniques (CFGF)⁶⁶, more specifically to the electric field gradient focusing branch to which also other gradient focusing techniques such as gradient elution isotachophoresis (GEITP)⁶⁷, belong. These methods are characterized by a gradient in electric field strength along a microchannel in the presence of an

opposing bulk flow, and each analyte is focused at a unique location based on its electrophoretic mobility. What sets dzITP apart from other gradient-focusing techniques such as gradient elution isotachophoresis (GEITP)⁶⁷, is that the depletion zone takes on a role similar to that of the trailing electrolyte so that only one electrolyte (a leading electrolyte) is needed in dzITP vs. e.g. GEITP. For negative analytes furthermore only EOF is needed to generate bulk flow, with the very high field in the depletion zone ensuring that there is a stable focusing position for any analyte. Technically the depletion zone is not a trailing electrolyte, as the mobility of its co-ion is not lower than that of the analytes, but explicitly the opposite, as it needs to function as leading ion.

The combination of depletion zone and focusing has been reported in literature before, in different configurations, as reviewed elsewhere⁶⁸. For example in bipolar electrode focusing, where the depletion zone is locally induced by electrodes in the channel, also plateau concentrations as function of background electrolyte were reported⁶⁹. Another example by which a depletion zone can be induced is by means of a Nafion membrane, either replacing the nanochannel in the configuration by means of a junction in a capillary⁷⁰, or in a chip⁷¹, or placed locally on the bottom of a microchannel (filling only part of the height)⁷². What sets dzITP apart from other depletion zone focusing techniques is the crucial realization, that from the depletion zone onwards the interfaces between subsequent (higher mobility) analytes and interfaces the principles of ITP apply (See Figure 1.8) and as a consequence the toolbox of depletion zone focusing can be used in combination with that of ITP, as examples given in Chapter 5 of this thesis will show. This realization has apparently not occurred in other groups, most likely as they used only one analyte.

1.5 Surface-enhanced Raman spectroscopy

Surface enhanced Raman spectroscopy (SERS) is a special form of Raman spectroscopy, a technique that can detect very low amounts of molecules. Since it also is a form of vibrational spectroscopy it provides fingerprint spectra. Furthermore, particularly relevant for downscaling is that in SERS the signal originates from the sample volume up to some tens of nanometers from a surface. This makes SERS by its nature a detection principle suitable for nano-scale detection. These qualities make SERS a strong candidate for the integration in an analysis platform for minute metabolomic samples. Disadvantages include that a SERS surface needs to be integrated in the system and that currently irreversible binding of analytes and/or matrix compounds readily fouls the surface. This fouling makes it not compatible with inline use although off-line applications have been abundantly reported for some decades⁷³

The use and principles of Raman spectroscopy and SERS are detailed below. To make SERS suited as a dynamic biodetector for multiple analytes, fouling as mentioned above should be prevented. This is the motivation for the evaluation of a thin (to allow SERS), anti-fouling coating, to turn a SERS substrate into a sensor responsive to concentration as well as concentration changes in time. This SERS sensor concept we have called SERSOR, and it is the subject of Chapter 5 of this thesis.

1.5.1 Raman & surface-enhanced Raman spectroscopy

In Raman spectroscopy a sample is irradiated using a laser of well-defined wavelength, and the energy of the scattered photons measured. Most of these photons will scatter away without a change in energy, so-called Rayleigh scattering, but some, approximately 1 in 10⁶ photons will have exchanged energy with the vibrational levels of the samples molecules, so-called Raman scattering. The principle of Raman scattering is theoretically described below.

In practice, Rayleigh scattering is filtered out and the residual Raman spectrum resolved by diffraction and imaged on a CCD. The vibrational information in the spectrum is directly related to the functional groups of a molecule and can provide a fingerprint. In addition Raman spectroscopy is quantitative as the amount of scattering observed correlates with the molecular concentration. Last but not least Raman spectroscopy, unlike infrared-absorption spectroscopy, which is another vibrational technique, is not hindered by measuring in water. Acquiring fingerprint spectra and the possibility of measuring in solution make Raman spectroscopy highly attractive in bioanalysis. An example of a Raman measurement of some metabolites is shown in Figure 1.11.

Detection limits of Raman spectroscopy depend strongly on the molecular species, or more specifically its type of functional groups as well as wavelength and power of the laser used, but typically they are in the sub-millimole per liter range for biomolecules. In case of lower concentrations or corresponding very low abundance as in miniaturized systems, the detection limit of Raman spectroscopy is therefore challenged. In 1974 however, an enhancement of the Raman signal was observed on roughened silver or gold metal electrodes⁷⁴. This significant enhancement is maximized when the metal is rough on the order of some tens of nanometers, preferably from the group of the coinage metals, and greatly decays with distance from the surface, d, on the order of d^{-12} .

The technique, called surface-enhanced Raman spectroscopy which makes use of this principle has greatly improved detection limits compared to normal Raman spectroscopy while still providing fingerprint spectra. These detection limits can equal the detection limits of LIF, reaching down to a single molecule for a select few compounds and generally providing nmol/L-µmol/L detection limits for many endogenous (unlabeled) biomolecules. An example of a SERS measurement of the biomolecule adenine is given in Figure 1.12 using silver metal colloids.

Importantly, this interaction requires the proximity of the molecule near the metal surface, at a distance on the order of tens of nanometers. For this reason SERS is a nanoscale detection method by nature (as detailed below in section



Figure 1.11 Raman scattering measurement of 20 mmol/L of ATP and ADP. On the x-axis the energy lost between emitted and scattered photons to the vibrations of the molecule is given, a Stokes shift, in so-called Stokes-Raman spectroscopy, Despite the similarity between ATP and ADP the difference in the spectrum, related to the phosphate groups is readily apparent upon straightforward substraction. Measured using a solid-state laser emitting at 785 nm, 10 s exposure time.

1.5.1). In addition, the SERS-active metals gold and silver adsorb analytes on their surface, which is even more beneficial for the detection limit. Unfortunately, this corresponds to fouling when dynamic measurements are required or surface competition when multiple analytes are to be detected, preventing calibration and quantitative measurements.

Principles of Raman spectroscopy

Consider a molecule in an incident oscillating electric field E (e.g. a laser light source), at frequency ω_I . This field induces a dipole moment P:

$$\overrightarrow{P} = [\alpha] \overrightarrow{E}$$
(1.3)

with:

$$\vec{E} = E_0 \cos\left(\omega_I t\right) \tag{1.4}$$



Figure 1.12 Surface-enhanced Raman measurement of adenine 50 nmol/L. The signal from adenine (blue particle) is enhanced by the proximity to silver particles (orange spheres) of 30-100 nm in size.

with α the polarizability (a 3x3 tensor), a characteristic describing the properties of the molecule's electron cloud. It indicates how much the electron cloud will shift in an electric field and affects the strength of the induced dipole.

If energy is absorbed in a vibrational level that changes the dipole moment P (with α constant), this corresponds to a net shift of the electron cloud, for instance in the linear molecule CO₂: O-C-O excited to the asymmetrical vibration from O—C-O to O-C—O. This process corresponds to those vibrations observed in infrared absorption spectroscopy. A change in corresponds to a symmetric perturbation of the electron cloud, so there is no net change in the polarization. This corresponds to Raman scattering. For instance in CO₂ the molecule can be excited to the symmetrical vibration O-C-O to O—C—O. More complex molecules can have a simultaneous change of both P and α , and can be observed in both IR and Raman scattering.

Raman scattering can both lose and absorb vibrational energy from the molecule, so-called Stokes and Anti-Stokes Raman respectively. In this work exclusively Stokes Raman is used. When a volume containing N molecules is probed, the total Stokes Raman signal I_{SR} is proportional to the Raman cross section σ^R and the incident laser intensity I_0 . In formula:

$$I_{SR} = N I_0 \sigma^R \tag{1.5}$$

Note that this relation illustrates why Raman is quantitative, since the signal scales directly with the amount of molecules N.

Principles of surface-enhanced Raman spectroscopy (SERS)

The principle of the enhancement is attributed to the metal, which has a much larger chance of interaction with the photon, mediating the energy exchange between photons and molecule^{75,76}. An incoming photon induces a dipole oscillation in a metal, with roughness on the order of tens of nanometers, carried by displacement of surface electrons, a so-called surface plasmon. This surface plasmon in turn produces an electric field. A molecule near the surface will feel a total field from both the laser and the induced dipole,

$$\vec{E}_T = \vec{E}_0 + \vec{E}_{dip} \tag{1.6}$$

The enhancement is defined as the ratio between the total field with and without the metal present:

$$A = \frac{\overrightarrow{E}_T}{\overrightarrow{E}_0} \tag{1.7}$$

This enhancement factor must be taken into account not only for the enhancement of the field of the laser $A(\omega_L)$, from laser to metal particle and metal particle to laser: $A(\omega_L)A(\omega_L)$, but also for the enhancement of the Raman scattered field $A(\omega_R)A(\omega_R)$, from molecule to particle and from particle to photon. Equation 1.5 then becomes⁷⁷:

$$I_{SR} = NI_0 \sigma^R \left| A\left(\omega_R \right) \right|^2 \left| A\left(\omega_L \right) \right|^2$$
(1.8)

Where ISR is the total Stokes Raman signal, N the molecules probed, σ^{R} the Raman cross section and the incident laser intensity I_0 . This energy exchange is by far the most efficient through dipole interactions. As metal structures with roughness on the order of tens of nanometers support these exclusively (exact size varies per metal), this is therefore the optimal size range.

The strength of the induced field, E_T , scales strongly with the amplitude of the plasmon oscillation, which in turn depends on the relative freedom of the electrons in the metal which corresponds to the conductance. The coinage metals, silver, gold and copper have the highest conductance of all metals. They are in the same row of the periodic table, and share the property of having a full d shell while the s shell has only one electron. In physics this comprises the definition of a noble metal. This s electron grants the high conductance: in ascending order copper gold and finally silver provide the strongest enhancement.

An approximation of the enhancement factors in Equation 1.8 can be given for nanorough surfaces and as a model for the colloids used in Chapter 5, by means of describing the enhancement factor for a small spherical metal particle⁷⁷, equation 1.7 then becomes:

$$A(r,\omega) = \frac{\overrightarrow{E_T}}{\overrightarrow{E_0}} \approx 1 + \left(\frac{\varepsilon(\omega, R) - \varepsilon_m}{\varepsilon(\omega, R) + 2\varepsilon_m}\right) \left(\frac{R}{R+r}\right)^3$$
(1.9)

with R the radius of the particle, r the distance between molecule and metal, $\varepsilon(\omega, R)$ the dielectric function of the metal depending on ω and the radius of the particle R (which explains why ε_m the dielectric constant of the metal, The approximation of eq 1.9 illustrates the experimentally observed strong decay of SERS signal with distance from the surface. Namely, the enhancement $A(r, \omega)$ drops with increasing distance d as $\approx r^{-12}$. At approximately 100 nm, equation 1.9 is reduced to 1, corresponding to normal Raman.

1.6 Goal & Scope of this thesis

Life sciences research, and especially metabolomics, will benefit greatly through the development of improved bioanalytical separation techniques. New approaches enabling the analysis of minute volumes of complex composition are therefore urgently required. Small-volume analysis can be achieved by miniaturization in fluidic chips, not in the least as the resolving power of both chromatographic and electrophoretic techniques can improve upon downscaling. Analysis of complex samples is possible by making use of the potential provided by Lab-on-a-Chip platforms, which can in principle provide standardized, automated and also faster analyses and integrated detection. Electrophoretic separation techniques provide the greatest resolving power and can become more effective upon downscaling; this is particularly true for isotachophoresis and trace analytes as explained in the previous subsection. This makes the smallest practical channels available, nanochannels, of great interest to explore for this purpose.

The goal of this thesis is to make analysis devices smaller, more efficient, and simpler to use for the analysis of small biosamples, specifically in metabolomics. For this purpose, nanochannels were utilized for electrokinetic separations and especially isotachophoresis and the new fundamental nanofluidic phenomena encountered were studied and reported. Nanochannels are not just smaller channels; many of their properties are still unknown, belonging to the field of nanofluidics with phenomena relevant whenever a dimension approaches the nanoscale including e.g. nanopores, biological ion channels, nanocavities etc. This makes the study of nanochannels, from the perspective of a platform to study nanofluidics, an interesting field of research. Possible limits of downscaling by nanochannels are also explored in this thesis.

In Chapter 2, a fundamental property of silicon oxide nanochannels, relevant when miniaturizing separations, is experimentally investigated for the first time and theoretically modeled. Namely, in nanochannels the surface-to-volume ratio is so large that the acidic glass is able to titrate solutions contained in them. To illustrate the scale and relevance of this effect: a 50 nm deep channel easily titrates 1 mol/L of buffer. This effect is relevant for the understanding of the electrochemical double layer on silicon oxide surfaces in general, including capillaries and (micro)channels. When downscaling devices to perform separations in nanochannels, this effect can be a dominant factor. A model to predict this

behavior was established, and used in the further chapters to assist in the design of miniaturized isotachophoresis.

In Chapter 3 the limit in miniaturization of isotachophoresis (ITP) is explored, by assessing the performance of ITP in sub-micron channels and nanochannels for sub-picoliter metabolomic samples. Successful focusing and separation of a biosample representing approximately 5% (0.4 pL) of the volume of a typical human cell was demonstrated. This represents the smallest application of ITP to date. Furthermore, a hitherto unknown fundamental effect was discovered that provided a practical, fundamental limitation on the further downscaling of ITP: electrocavitation. Electrocavitation is also considered relevant on its own as a new phenomenon for the study of cavitation of liquids.

Chapter 4 presents the new technique of depletion zone isotachophoresis (dz-ITP), a successful synergy of nanofluidic phenomena and a microfluidic analysis method. The effect of concentration polarization induced by a nanochannel was used to create a depletion zone which in turn acts as the trailing electrolyte for ITP in a perpendicular microchannel. This technique enabled ITP with a single electrolyte, an important simplification of ITP, whose complexity until now hampered the widespread application of this powerful separation technique. In addition, dzITP provides a far greater control of the separation process compared to conventional ITP. This significant improvement of ITP resulting in dzITP provides a new separation technique that is very attractive for the sample preparation or analysis of complex samples such as in metabolomics.

In Chapter 5 the first steps in the development of a SERS based sensor are reported. Protecting the surface with a coating of polyethylene glycol gives the SERS surface the capability of being re-usable. This SERSOR concept was developed particularly with the purpose of inline detection of unlabeled biomolecules. A proof of concept is provided but coating robustness remains to be improved.

In Chapter 6 the results of the research described in this thesis are summarized and general conclusions and perspectives are provided.