Nanofluidic tools for bioanalysis : the large advantages of the nano-scale

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CHAPTER 6

Summary, Conclusions & Perspectives

6.1 Goal of the thesis

Metabolomics holds the promise to understand mechanisms of health and disease, by means of elucidating the role of metabolic processes involved. This understanding in turn can accelerate drug development and enable personalized medicine. The proper study of metabolomics however, requires cheaper and faster methods. Additionally, sometimes metabolomics will require the analysis of small to ultra-small samples, such as from single cells, which currently is not at all possible with conventional lab equipment. Ultimately, in order to understand the dynamic processes in the metabolic state of an entire human, an organ, tissue or a cell, time resolved studies will be required, taking samples of body fluids in a longitudinal manner or, when studying individual cells, at the level of individual cells.

A Lab-on-a-Chip platform in principle allows integration of complex separation methods in a miniaturized fashion and in that manner provides a standardized and automated analysis at reduced time and analysis costs. Such an efficient, standardized analysis platform could be also designed to allow the analysis of small sample volumes of complex samples such as a blood droplet to acquire information on the metabolome. Analyzing ultra-small samples volumes such as that of an individual cell, or an aliquot of an individual cell, then requires even further miniaturization.

Miniaturization towards the microscale will also allow sample volumes much smaller than possible with current conventional lab equipment, and it may enhance the resolving power of separations. However, this may require new ana-

lytical approaches and not only down-scaling of existing methods. Actually, microfluidics analysis systems are increasingly described in literature, and a few are commercially available, which handle volumes on the order of nanoliters (10^{-9} L).

Analysis on the sub-cellular scale, which is on the order of sub-picoliters ($<10^{-12}$ L) is rarely described in literature and not commercially available yet. For this purpose nanochannels, intrinsically small enough for sub-picoliters, hold great promise. Nanochannels are not just smaller channels; many of their properties are still unknown, making their study, nanofluidics, a fascinating field of research. Electrophoretic separation techniques provide a greater resolving power compared to LC separations, and become even more effective upon downscaling. Notably an electrophoretic separation technique with a very high separation potential is isotachophoresis.

The goal of this thesis is the exploration of nanofluidics for the analysis of minute metabolomic samples in miniaturized analysis devices. In this exploration we included the study of phenomena unique to nanofluidics. This understanding was used not only to bypass side effects while exploring the limit of miniaturization of isotachophoresis, but also to exploit them by the hyphenation of microfluidic ITP with nanofluidic phenomena.

6.2 Solution acidification in nanochannels, a surface charge model

In Chapter 2, the discovery of the unexpected dominant acidity of glass in nanochannels is described. This led to advanced modeling of the electrochemical double layer. The knowledge gained on nanochannels can be used to avoid unwanted titration effects in nanoscale analysis, or to possibly exploit them. The titration model can be used as a tool to study further fundamental nanochannel properties. For instance, in a follow-up of this work the effect of polymers on the surface of a nanochannel was studied by Anderson et al.[242] and recently used to study the influence of energetic surface heterogeneity on proton desorption during capillary filling of silica nanochannels by Piasecki et al.[243] Secondly, it offers the unique opportunity to study the titration of solutions without adding co-ions to the solution and resulting changes in ionic strength, e.g. from chlorine in HCl and sodium in NaOH, as the counter ion is a stationary group on the surface. This titration effect has since even been shown to allow actuation by an externally applied potential on the channel surface to actuate proton release and uptake, and titrate the filling solution[244].

This titration effect may be relevant for all applications in nanofluidic chips where changes in solvent composition occur. Particularly, it has implications for various separation methods when applied in nanochannels: for example in liquid chromatography often solvent gradients are used, and in electrokinetic methods such as field-enhanced sample stacking or isotachophoresis which are based on

discontinuities in solvent composition. On the other hand this model can support the understanding of large scale applications that utilize surface interface effects and interactions occurring at the nanoscale, including for instance open-tubular liquid chromatography in nanochannels.

Based on the insights obtained in Chapter 2, new approaches are also possible for the analytical method isoelectric focusing (IEF). Conventionally, this method separates amphoteric analytes, e.g. proteins, by an axial pH gradient. This gradient is imposed by adding a mixture of amphoteric background molecules. The constituents of this mixture are selected such that a continuous range of isoelectric points is present and that each of the compounds have a strong pH buffering capability. The in- and outlet of the channel or capillary are then placed in vials corresponding to the pH extremes of the ampholytes. When an electric field is applied over this mixture, the ampholytes migrate until they have reached a position where their net charge is zero, so that they are ultimately arranged on the order of their isoelectric point. This mixture is generally referred to as carrier ampholytes. Ampholitic analytes such as proteins, stop migrating at the position where the pH corresponds to their isoelectric point. This process simultaneously separates and concentrates these compounds. Unfortunately, the presence of carrier ampholytes can be unwanted or impractical, e.g. in combination with detection methods such as mass spectrometry. As a future prospect to exploit the nanochannel titration effect, the wall buffer capacity in a nanochannel can be used to perform isoelectric focusing (IEF). Nanochannels strongly buffer pH and do so inversely proportional to their channel depth. Potentially a nanochannel with slopes from deep to shallow may therefore induce a pH gradient, from low to high pH respectively, buffered by the wall. If so, such a nanochannel could enable IEF without any carrier ampholytes. The technology needed to make sloping channels with a continuous gradient is to our knowledge not available at this time. A stepped slope ($\approx$ 10 nm step size) however has been demonstrated[245], so selective trapping of compounds in batches may be attempted. A different approach could be the capping of the silanol groups in a standard non-tapered microfluidic or conventionally capillary channel, in a gradient from less to more capping to also achieve a buffer capacity gradient.

The model implicitly describes the charge asymmetry in solution (the number of protons released) in the nanochannel on the surface charge in charges per nm^2, as a function of pH and ionic strength. This in turn can allow prediction on the rate of filtration in membranes[246] and may be used to predict concentration polarization as used in depletion zone isotachophoresis (Chapter 5).

6.3 Isotachophoresis in nanochannels for sub-pL injection volumes

In Chapter 3 nanochannels were used for isotachophoretic separations of 0.2 and 0.4 pL samples to assess the limits of miniaturization. The developed setup demonstrated the feasibility of advanced separations and fluidic control on the nanoscale. The ITP separation was actually achieved for the smallest sample volume so far reported in the literature.

The feasibility of ITP in nanochannels is important for metabolomic analysis of minute sample amounts. ITP, being a focusing technique, can substantially improve detection limits, a crucial aspect for the challenging detection of very low abundant compounds. ITP can simultaneously be considered a sample pre-treatment technique, purifying analytes while separating them in their respective zones. Nanochannels in turn can maximize the resolving power of ITP. So in case of low sample amounts, perhaps counter-intuitively, the minute nanochannels can improve detection limits by improving resolution. Of course in nanochannels, detection techniques are challenging and some optical ones even impossible to use, but detection limits are imposed by the amount of analyte and not by the channel dimensions.

With the feasibility of nanochannel ITP established, in the future this technology can be developed and applied for the downscaling of several micro capillary and microfluidic analysis techniques. Possible other techniques of interest for further downscaling are hyphenated separation strategies such as ITP-CZE, transient ITP etc. It should be noted that with the demonstrated feasibility of nano-ITP, the application of the method is not limited to samples from single cells only. A targeted approach for one or a few compounds only is of value in many applications that involve rare or expensive compounds. The reported nano-ITP method can then contribute in two straightforward ways. Firstly, for a targeted analysis of one or a few compounds that are available in very low absolute amounts, using the advantage of enhanced separation that ITP in nanochannels offers. Secondly, for the analysis of intrinsically small volumes or small amounts which can be analyzed with nanochannel ITP after a first separation step, which can be achieved with zone electrophoresis, ITP or another separation technique.

At first glance, the minute size of nanochannels may not appear appealing for separations from the perspective of current standard sample volumes and world to chip interfacing challenges. In practice however, loadability of larger initial sample volumes in ITP is conventionally achieved by initiating the separation in a large cross-section while converging inline into a smaller one. This process takes longer but the final resolution is demonstrated to be independent of its history (at the same field strengths), and most importantly the resolution is independent of the original sample volume[60,247]. For the envisioned minute sample amounts ITP can then be started in a (large) microchannel (or capillary, or both, providing multiple steps as needed) converging to a final nanochannel cross-section.

This process will allow for potential sample dilution (from sample pretreatment often required for proper sample handling or hyphenated techniques) or simply for much larger sample sizes that contain a very low amount of the compound of interest. Effectively this means that the nanochannel ITP demonstrated here shows the potential for the analysis of 40 attomole of an analyte, regardless whether the original sample volume was 1 µL or 0.4 pL.

In this thesis ITP was successfully performed in a nanochannel in the presence of a biomatrix. A next step in the application of metabolite profiling can be the analysis of an individual cell. Although the injected volume was 400 fL, the injection was carried out from a larger sample volume because of the dead volume still present in the set-up. Therefore, integration of the sampling procedure and handling on the chip prior to analysis is crucial when analyzing ultra-small samples of less than 1 pL as mentioned in the previous paragraph. The proof of concept of nanoscale ITP was achieved for two labeled amino acids and fluorescent detection. For the analysis of a larger set of analytes in a complex sample however, such as needed for metabolomics of a single cell, the separation has to be coupled to a suitable detection technique. The addition of both very small-scale sampling and detection techniques will determine also the way how to set up the ITP separation. Fluorescence is probably the most suited detection technique at this scale; another candidate, investigated in this thesis is surface enhanced Raman spectroscopy (See Chapter 1).

A limit on miniaturization of ITP, in terms of maximum potential was unexpectedly imposed by a newly discovered nanofluidic phenomenon: electrocavitation[120]. It was discovered that a water column can be broken in a controlled manner in a nanochannel, with negative pressures developing on the order of -2000 bar. The feasibility to use nanochannels as a platform for controlled cavitation studies is currently underway. Positive pressures have also been realized, by straightforwardly reversing the polarity of the voltage, to the limit of breaking the bonding between bottom and top substrate in the chip, delaminating them. Potentially counter intuitively, micro and nanochannels may handle very high pressures: $>$ 1400 bar[248]. This is because pressure is force per area, so that with the very small surface area the force is relatively low. The realization that huge pressures, potentially exceeding 1400 bar, are possible in nanochannels without any pumps is particularly interesting for ultra-high pressure liquid chromatography (UHPLC) applications. Pumps based on the related principle of electro-osmotic flow are actually commercially available from several sources e.g. Dolomite Microfluidics, UK. Normally the application of UHPLC is limited by availability and cost of pumps and interfacing, with currently pressures of up to 1200 bar available, whereas the aforementioned large pressures are developed inside the nanochannels and require in principle no pressure interfacing to the outside world at all. In addition nanochannels, due to their high surface-to-volume ratio are an ideal open-tubular platform for LC[19–21]. This hyphenation of very high pressures and nanochannel open-tubular LC could therefore yield ultra-high pressure open-tubular/nanochannel liquid chromatography.

6.4 Depletion zone Isotachophoresis

In Chapter 4 the advantages of hyphenating microfluidics and nanofluidics for isotachophoretic separations was investigated, which led to the discovery of a new powerful separation method: depletion zone isotachophoresis (dzITP). This microfluidic separation method utilizes a nanochannel/microchannel interface to generate concentration polarization, forming an ion depletion zone in the microfluidic channel. This depletion zone acts similar to a trailing electrolyte allowing the performance of isotachophoresis with a single electrolyte.

dzITP is of high value in metabolomic sample analysis as it inherits from ITP the double function of being both a sample pretreatment and/or purification technique as well as a focusing and separation technique. In addition, its gradient focusing aspect grants a significant greater control of the analyte zone position, while the depletion zone removes the need for a trailing electrolyte. The need for only a single electrolyte adds a very important degree of freedom and experimental simplification to ITP, increasing its applicability.

The focusing abilities of dzITP, on one hand depend on the leading electrolyte, a feature which has been extensively modeled and described for conventional ITP[60,247]. On the other hand it depends on the formation of a depletion zone at the interface of a single nanochannel with a microchannel as has also been investigated and modeled[50,157]. The latter study shows that the rate of depletion zone growth is linear in time at constant current, $\Delta L_{depletion} \approx t$, or as time squared for constant voltage, $\Delta L_{depletion} \approx t^2$. This means that the nanochannel pumps ions at a constant rate over time, and hence that the charge asymmetry over time does not change in the nanochannel, so that the pumping property of the nanochannel can be described with a single value. As already mentioned, an estimate of this value can be based on the model provided in Chapter 2, which allows the charge asymmetry in solution in the nanochannel to be calculated, in charges per nm^2, as a function of pH and ionic strength. This knowledge would for instance allow tuning the EOF in the microchannel to a maximum value while just maintaining the depletion zone, to maximize the accumulation of analytes in, and the sharpness of the focused zones. Still, despite these available pieces of the puzzle and its potential benefits, the combined fundamental principle of dzITP remains to be studied and modeled in more detail.

The electric field in the depletion zone vs. the EOF determines its function similar to a trailing electrolyte, and can be considered therefore to be a very relevant subject for study. In practical applications, if the electric field is known, the mobility cut-off value of the depletion zone can be described in analogy to the trailing ion and assigned a value in terms of a ”virtual” electrophoretic mobility. Also, the field strength in the depletion zone not only depends on fixed parameters such as the channel geometry and chosen parameters such as pH and ionic strength, but also on parameters which can be adjusted such as the applied potentials. Particularly relevant is the field along the microchannel, which determines the EOF, vs. the field over the nanochannel towards ground, which

determines the depletion rate per unit time. That means that the value of the depletion zone's "virtual" electrophoretic mobility may be modulated (and in fact changed under constant potential actuation) by setting the voltages and monitoring the current. More preferably the current is actuated directly as it translates directly into the ionic fluxes. Compared to conventional ITP, where the trailing ion is selected, dzITP would have the added advantage of what is defined here as virtual mobility actuation. Once the current and voltages have been calibrated, the need for visual confirmation or monitoring is not any more needed. This will be relevant for when optical access is impractical, e.g. when the channels are not accessible or compounds do not fluoresce, and particularly for integration with non optical detection techniques such as mass spectrometry.

A more direct mode of selective release for dzITP, effectuated by locally and/or temporarily collapsing the depletion zone, has been demonstrated in our group[249], by leveraging the voltages so that the bulk flow elutes past the depletion zone at a length of focused zone per unit time. This process can be pulsed so that a select length of analyte zone may be released, or controlled so that a specific length per unit time is released. The instability of this process was overcome and tunable release was achieved by means of visual monitoring. Interestingly when elution has been matched to the slowest compound (the compound that focuses nearest the depletion zone), it is no longer focused itself (its flux along the depletion zone is now the same as along the whole channel) and it has taken over the role from the depletion zone as the trailing ion.

Although dzITP reported here uses bulk flow from EOF alone, which is practical as pumps are not needed, this feature is not required, and the combination of EOF with pressure flow would enable all field strengths to be applied over the nanochannel, resulting in a simplified system with a potentially faster analyte focusing speed. Likewise, dzITP is not limited to a depletion zone made at a nanochannel/microchannel interface[68] but its principles should also hold for a depletion zone made by other means, adding to the versatility of the technique, which remains to be fully explored. For instance, with bipolar electrode focusing, where the depletion zone is locally induced by electrodes in the channel[69], also plateau concentrations as function of the background electrolyte concentration were reported, but apparently not considered associated with ITP by the authors. Another example by which a depletion zone focusing was induced is by means of a Nafion membrane, replacing the nanochannel[71], or placed locally on the bottom of a microchannel[72]. In a functionally analogous system, the depletion zone function may also be fulfilled by an organic phase as in electroextraction[250]. Although the EOF is absent in such a system, when the electrolyte into which the ions are extracted is chosen to be a leading electrolyte vs. the desired analytes, the lower mobility ions will focus at the interface, and ions faster than the leading ion will merely stack and travel onwards, creating a cutoff filter for high-mobility ions.

In experiments using just a single nanochannel (during our studies on electrocavitation) a focused analyte plug was maintained at constant position in a long

nanochannel under influence of an applied field. It is tentatively hypothesized that this is a fundamental form of dzITP. The EOF in the single nanochannel, like in dzITP provides a net transport of negative analytes towards the cathode down the nanochannel, but is met from the other end by a depletion zone forming at the anode from nanochannel concentration polarization, with focusing at the interface: single nanochannel dzITP (K.G.H. Janssen et al, manuscript in preparation).

6.5 Surface enhanced Raman spectroscopy

In Chapter 5 the first results of a surface enhanced Raman spectroscopy sensor (SERSOR) are reported. In contrast to conventional SERS, dynamic analyte detection can be performed as a coating protects the surface from irreversible binding and/or fouling, while it is thin enough to allow SERS. This strategy is unconventional as it creates a distance between analyte and surface and forgoes the standard drying of the sample on the substrate. Since the measurement is performed in solution, its detection limits are much higher than reported in conventional SERS for dyes. We believe this disadvantage to be more than offset however by the ability to perform Raman spectroscopy at an improved detection limit and to perform dynamic measurements of biological compounds in solution.

A SERSOR has significant potential in miniaturized metabolomic analysis, or bioanalysis in general. Firstly, as it provides vibrational spectra, a SERSOR also supports identification of unlabeled biomolecules while it is more sensitive than Raman alone. Secondly, by its nature as a surface detection technique it detects analytes within only tens of nanometers (Eq 1.9) from its surface, and by its concentration sensitivity (the coating ensuring that the signal predominantly originates from solution), it is intrinsically compatible with minute volume and/or low abundant compound detection.

Although of interest for inline detection or process monitoring in general, including detection in microchannels, the potential advantages of a SERSOR may stand out most as an inline detector for miniaturized separation techniques, particularly for concentration-driven techniques such as ITP or dzITP. Before the SERSOR can fully prove its potential, two aspects need to be improved. First of all, the coating stability needs to be improved beyond the first results reported in Chapter 5, preferably with the new generation of improved colloid coated substrates that has already been achieved (section 5.7). For this purpose a coating based on amine binding to the silver should be evaluated, because the coating inspired by gold coating chemistry, which was based on a thiol group, has likely reacted with the silver upon irradiation, damaging both surface and coating and contributing to the poor initial robustness observed. Secondly the SERSOR needs to be implemented in a micro- or nanochannel. Fortunately, a promising process that may integrate a SERS surface in small channels was already demonstrated, in a nanochannel, in the form of silver mirrors for a Fabry-Perot interferometer manufactured into the side walls of the nanochannel[251].

6.6 Roadmap for nanofluidic tools in metabolomics and bioanalysis

Within bioanalysis in general and metabolomics in particular, the need for miniaturized sample analysis challenges analytical methods and instrumentation. In answer, nanofluidic tools can address these challenges.

Developing a method for metabolomic analysis for samples down to those from a single cell, has proven to be a tremendous multidisciplinary challenge. Ultrasmall volumes have to be handled, which requires working in nanochannels. However, analytical approaches in nanochannels require full understanding of processes originating from the significant surface/volume ratio, for example theoretical modeling of silicon oxide surface chemistry. For the analysis of small volumes, isotachophoresis is a promising analytical method. Designing a nanofluidic chip allowing isotachophoresis of 400 fL injection volumes has proven to be a challenge which can ultimately be solved. It was found that development of a fluidic setup to provide the macro- to nano-interfacing of the fluidic, optical and electrical connections is required to realize such a device. Such a development is actually often neglected. Also, the development of an automated and standardized protocol and the design and manufacturing of devices to allow this is crucial, and requires at the nanofluidic level much attention.

In the described research nanofluidics has been established as a platform capable of supporting the handling of the low sample amounts required for single-cell analysis and for the achievement of separations. Still, single-cell metabolomics can only be realized if the envisioned nanofluidic platform allows integration of sampling, separation and detection steps. As a consequence the following next research steps are: 1) Developing of non-invasive cell-sample extraction on-nanochip or at least compatible with introducing the sub-cellular sample volume into a nanochannel. A device with these properties has been demonstrated by J. Emmelkamp et al.[252]; 2) Interfacing the analysis device with a label-free detection method of metabolites such as mass spectrometry or SERS. If the detection method provides sufficient detection limit, but requires fully resolved compounds, the techniques of transient ITP or ITP-CZE can be considered. Other than these aspects, the required know-how of nanofluidics, interface engineering and separation methods is now available.

We expect that the successful performance of metabolomics down to the single cell level, will grant insight not only into the cell on its own but also its workings as smallest functional component of an organism. The understanding of individual cells is fundamental in processes such as developmental biology from stem cell to tissue or even embryo to organism. A single cell metabolomics method will not only be useful for those cells that are rare or unique but it will for the first time allow the validation of studies that relied so far on the averaging of many cells. The search for mechanisms and pathways relevant in apoptosis, cancer, diabetes, and neuronal diseases could be significantly accelerated if these processes can

be investigated in a time resolved manner at the individual cell level. Nanofluidics can greatly contribute to providing the tools for applications like these in metabolomics, not just by the use of smaller channels with lower volume, but since knowledge of fundamental nanofluidic phenomena can help in designing novel analytical approaches, whether at the nanoscale or larger, as was demonstrated in this thesis.