Single-electrolyte isotachophoresis : on-chip analyte focusing and separation

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1 General Introduction

Challenges in metabolomics and the analysis of small samples

The field of metabolomics aims at the comprehensive analyses of classes of metabolites and the understanding how these small molecules interact within biological systems. Two major goals of metabolomics research are 1) the discovery of novel biomarkers which are indicative for health and disease states and 2) obtaining insights into disease mechanisms and identification of possible pharmacological interventions. It can be expected that as a result of the biomarker research, metabolite fingerprints will serve as diagnostic assays for diagnosis, prognosis and choice of pharmacological interventions The central paradigm of metabolomics in disease research is that certain metabolites or metabolite profiles are indicative for the progress of diseases like cancer. The discovery of such biomarkers and the development of clinically applicable assays would enable early diagnosis and treatment of such life-threatening diseases, significantly ameliorating the prospects for recovery.

Both for biomarker discovery and assay development, proper analytical methods are of crucial importance. Biomolecules and metabolites in particular have highly differing chemical structures, posing major challenges to analytical chemists. Especially when small sample volumes are used, conventional methods may have insufficient dynamic range, sensitivity, and/or separation power. Therefore new concepts in sample preparation and

separation are required. These issues appear to be most urgent in metabolomics because there are generally no amplification methods available (like PCR for genomics and transcriptomics research) nor common chemical structures (like peptide bonds, which are a common factor in all peptidomics and proteomics analyses). Nevertheless these -omics fields will also greatly benefit from novel analytical concepts as answer to these challenges.

The workhorses of bioanalytical laboratories usually include solid phase extraction (SPE), high performance liquid chromatography (HPLC) and gas chromatography (GC) instruments, often combined with mass spectrometry (MS). These techniques are extremely useful for the separation and detection of analytes in ultracomplex samples. However, they typically require microliters of sample, or a few hundred nanoliters at best. When dealing with small sample volumes, such as from a limited number of primary cells, small cells populations isolated from biopsies,only a single or a few analyses can be done with a single sample, forbidding repeated analysis and preventing the use of the sample for multiple tests. And in many cases the sensitivity will be not sufficient at all, as actually is currently for most methods for the analysis of individual cells.

A second disadvantage of these techniques is that they have only limited preconcentration capabilities. For example, in SPE preconcentration factors are in the order of 10-100, while biological concentrations of metabolites differ many orders of magnitude more. This limits the detectability of low-abundant compounds. It is very probable that currently many interesting biomarkers are going unnoticed through conventional analytical procedures, simply because detection limits may be orders of magnitude above biologically relevant concentrations and cannot be sufficiently improved.

The potential of electrokinetic methods

With electrokinetic methods, it is possible to obtain enormous improvements with regard to these two limitations. When it concerns small sample volumes, capillary zone electrophoresis (CZE) and related methods are well-known to be able to handle sub-nanoliter volumes, especially when using narrow-bore capillaries or microfluidic channels. An impressive example is the analysis of the contents of a single cell[1]. In such picoliter-sized samples low numbers of copies of molecules are present and usually only the most abundant species are detected. With nanochannel CZE, femtoliter volumes of samples can be analyzed[2, 3], though in practice such small volumes are only realistic if combined with inline sampling if one want to prevent significant; and maybe, the biggest problem one may encounter in such a case is adsorption at the surface of devices.

CZE is not as popular as HPLC, allegedly because it has inferior reproducibility and robustness. However, with proper expertise, CZE can be made as reproducible and robust as HPLC. Moreover, with CZE superior separation efficiency can be obtained, with plate numbers typically being around an order of magnitude higher than in HPLC.

An obvious limitation of CZE and other electrokinetic methods is that they can only separate charged compounds. Fortunately, the majority of metabolites and all peptides are or can be charged dependent on the pH, because acidic or basic groups are very common in biomolecules. For example, fatty acids, citric acid cycle products, molecules containing phosphate groups such as energy metabolism products and nucleic acids (including DNA and RNA), amino acids, peptides and proteins all include negatively charged moieties. Small ions also can be analyzed, as well as

positively charged molecules such as amines. A few classes of compounds remain which are not charged, including many sugars and sterols, but even for these neutral analytes there are interesting electrokinetic approaches, including micellar methods.

Concerning the detection of low-abundant compounds, CZE itself is not capable of preconcentration. Nevertheless there are numerous electrokinetic concentration methods which can easily be combined with CZE and which are able to achieve very impressive concentration factors: over thousandfold or even over millionfold[4-12]. Moreover, many of these methods can be used for selective trapping, which is extremely valuable when analysing low-abundant compounds in the presence of interfering molecules with much higher concentrations. Highly abundant matrix compounds, for example salts or albumin proteins can be discarded while concentrating the analytes of interest selectively. Such steps might be done inline, without the need for time- and sample-consuming pretreatment steps.

The approaches which result in up to or even over millionfold analyte trapping seem to fall within two classes: they are either isotachophoretic (ITP) or electric field gradient focusing (EFGF) techniques. This similarity is no coincidence according to the research presented in this thesis. In the subsequent chapters it will be argued and demonstrated that EFGF and ITP share many principles and phenomena.

Concepts of isotachophoresis (ITP), electric field gradient focusing (EFGF) and concentration polarisation (CP).

ITP and EFGF are two central concepts in this thesis. These concepts will be introduced extensively in chapter 2, but for the reader unfamiliar with these methods a brief overview will be given here.

In EFGF, analytes are focused and separated along an electric field gradient. An electric field is expressed as a quantity E in volts per meter (V/m). The strength of an electric field determines how fast ions migrate through a medium. This velocity is also dependent on ion-specific factors like size and charge of a molecule and these factors are summarized in a value known as the ionic mobility. In principle, each kind of ion has a own ionic mobility. There are many EFGF methods, but commonly all have 1) a conductivity gradient at a predefined position, which gives rise to the electric field gradient and 2) a bulk flow in opposite direction of analyte migration. An ion migrating through an electric field gradient will accelerate or slow down. Analytes of interest encounter a point where the velocity of electromigration becomes equal to the counterflow velocity. At these points, the analytes will be focused. On shallow gradients, the focusing position will be dependent on the ionic mobility of the analyte concerned, resulting in simultaneous trapping and separation.

In ITP, two zones containing different electrolytes are used: a trailing electrolyte (TE) with low ionic mobility and a leading electrolyte (TE) with high ionic mobility. Analytes of interest should have intermediate mobility and are then focused between these two zones. Analytes present in sufficient quantities are concentrated until they reach a plateau concentration. A typical sign of a completed ITP separation is therefore a stair-like profile of

contiguous plateau zones, each analyte having its own plateau. These plateau zones move with equal velocities. This situation is different from CZE, where each zone travels with a different velocity, leading to resolved peaks. Moreover, analytes are in principle not being focused in CZE separations, while with ITP high concentration factors can be achieved.

A third important concept in this thesis is concentration polarisation (CP). CP occurs across conduits which exclude anions and permit cations, or vice versa. Such a conduit may be a nanochannel, where the close proximity of surface charges makes the pore selective, or a nanoporous membrane. As a result, upon application of an external voltage, an ion-enriched zone will form at one entrance of a conduit, and an ion-depleted zone at the other entrance. These zones give rise to conductivity gradients on which EFGF can be performed. Indeed, several EFGF approaches are based on CP.

In the research presented in this thesis, we demonstrate that CP-induced ion-depleted zones can replace the TE typically used in ITP separations. Therefore the LE is the only electrolyte needed in such experiments. Hence the titel of this thesis: single-electrolyte isotachophoresis, which might have seem a *contradictio in terminis* to someone familiar with isotachophoresis.

Scope of this thesis

The aim of this thesis was to develop novel, and to improve, electrokinetic methods for sample pretreatment and separation of charged biomolecules. The work in this thesis has been inspired by research on isotachophoresis (ITP) and other electrokinetic methods with excellent preconcentration capabilities, particularly electric field gradient focusing (EFGF). The ability to concentrate and separate analytes simultaneously and selectively makes ITP a

technique with much potential, but the fact that it requires multiple electrolytes makes it not easy to use.

Based on our discovery of the insight that ITP phenomena can be produced by EFGF methods, we have aimed at combining the best of two worlds. Methods have been developed that are based on isotachophoretic principles but that are simpler to use. The unique versatility of EFGF methods has been incorporated in these methods.

In addition, the aim was to create a conceptual framework for how to position novel methods developed in this thesis with already existing research and literature. Overall, with this thesis we aim to provide the bioanalysis and metabolomics communities, and the life sciences in general, with new insights and approaches for the analysis of small complex biological samples.

Thesis outline

ITP, EFGF and concentration polarization (CP) are complicated phenomena. Chapter 2 provides first an in-depth introduction to ITP and EFGF, as well as to CP devices. Next, evidence is presented for the argument that ITP and EFGF have identical properties; this argument is underpinned by theoretical and experimental evidence, both from previous literature and from the research presented in this thesis. The strengths of ITP and of the several EFGF methods, including the in Chapter 3 presented novel method, are compared and major challenges are identified. It is discussed that theoretical knowledge and practical tricks from ITP and EFGF may be combined and integrated.

In chapter 3 a novel electrokinetic separation and preconcentration method is presented which is called depletion zone isotachophoresis (dzITP). dzITP is performed in miniaturized EFGF devices based on CP. dzITP is investigated as

a single-electrolyte method which forms isotachophoretic zones at the border of a nanochannel-induced depletion zone. Actually, ITP is widely supposed to require a leading and a trailing electrolyte. Nevertheless, it is demonstrated that a single-electrolyte separation can have all main characteristics of ITP – the only condition is that an electric field gradient is present on which charged analytes can be focused. This fact is one of the most interesting consequences from the insight that EFGF has ITP nature. dzITP is developed based on glass chips containing a nanochannel, and concentration polarization leads to the formation of depletion zones, enabling dzITP. Discrete and continuous injections, long-term zone stability and voltage-actuated zone positioning is demonstrated, too.

In chapter 4, selective release of dzITP-separated compounds along a depletion zone is studied. dzITP is investigated as a tunable ionic mobility filter, which may be used in pulsed and in continuous mode. Selective enrichment of a low-concentration compound is demonstrated, as well as selective trapping of metabolites in diluted raw urine sample.

In chapter 5, the hypothesis is tested that an elastomeric microvalve in a PDMS chip forms a tunable reversible nanospace between the deflected valve membrane and the channel walls. The effect of valve pressure on valve electrical resistance is investigated, as well as the influence of different voltages and valve pressures on concentration polarization regimes. The PDMS valve was used to achieve efficient analyte preconcentration (over 1000-fold) before release of trapped analyte.

Finally, at the end of this thesis, an overview of the most important conclusions is given and the perspectives of the findings of this thesis are discussed.

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