Chapter 1

General Introduction

GENERAL INTRODUCTION

Phenotyping based on profiling of small molecules

Phenotyping based on molecular profiling is a type of screening which investigates the phenotype of a biological system such as cells, tissues or whole organisms. The phenotype is a description of the total of physical characteristics of a biological system including its morphology, development, but also its biochemical properties. Profiling the phenotype is interesting since phenotypic effects can be induced by various (biological) perturbations such as a disease, drug interventions and genetic alterations. Phenotyping based on the profiling of small molecules such as metabolites can provide important insights into e.g. disease and the effect of drugs, as well as a systems understanding of the ability to adapt and cope with a perturbation[1].

For the profiling of small molecules there are different techniques available. There is often an inverse relation between assay comprehensiveness (analyte coverage) and the throughput of the assay (see Figure 1). Whereas a single analyte high-throughput screen (HTS), such as microscopy assays, enzyme-linked immunosorbent essays (ELISA) or fluorescence-activated cell sorting (FACS) can screen over a million compounds per week, comprehensive mass spectrometry (MS)-based assays which can potentially screen for hundreds of analytes, are generally much slower [2]. As a consequence, comprehensive profiling assays have the potential to provide more in-depth information about the phenotype of a specific biological system than a single read-out biochemical assay. Therefore, in choosing the appropriate profiling technology, the balance between assay throughput and analytical comprehensiveness should be considered carefully with probably in many cases preferably maximal depth at high sample throughput.

Many conventional HTS approaches rely on fluorescent- or radio-labeling since these labels can be rapidly detected with high sensitivity [3]. Label-free strategies, including MS, have gained wide interest, since they can be applied to a broader range of assays and target classes. Moreover, MS-based screening is a comprehensive, selective and sensitive analytical tool which is not susceptible to the limitations imposed by labelling and coupling enzymes[4]. As a consequence, profiling of small molecules by MS has the potential to be a very powerfull technique.

Mass Spectrometry

MS is a key analytical tool for detecting and identifying small (bio) molecules. Therefore in many bio-analytical applications MS has become a powerful quantitative and qualitative analytical technique. A MS comprises i) an ion source, where gas-phase ions (positively- or negatively charged) are formed, ii) a mass analyser for separation of ions based on their mass-to-charge ratio (m/z) and iii) a detector, where arriving ions are detected. A variety of different MS instruments are available, including high resolution (HR) MS such as fourier transform ion cyclotron resonance (FTICR), Orbitrap FT and time-of-flight (TOF) and low resolution MS such as ion traps and triple quadrupole systems. Among several available ionization techniques such as electron impact (EI), desorption electrospray ionization (DESI) and matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are predominantly used. MALDI and DESI techniques have recently attracted interest in the field of imaging MS (IMS). IMS involves comprehensive profiling of biological molecules by directly 'scanning' the analytes from a cell or tissue surface, without sample homogenization or extraction, thus maintaining the spatial

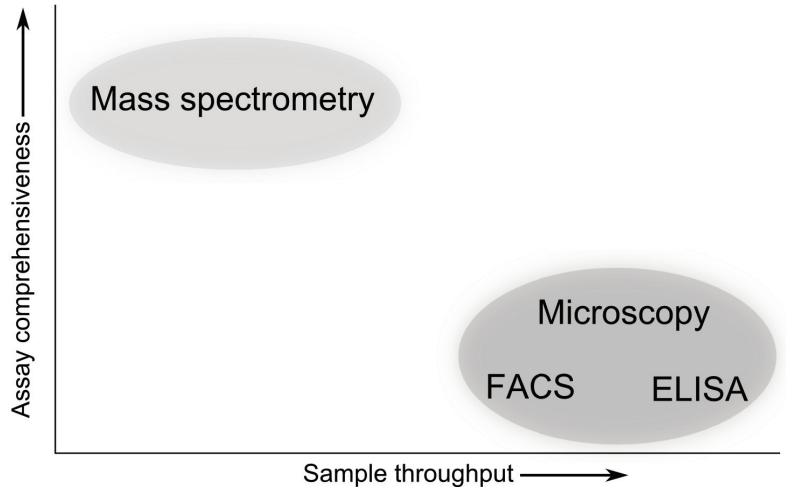

Figure 1: Schematic picture representing the comprehensiveness of the small molecule assay versus the sample throughput of the assay. Assays with potential hundreds of read-out variables such as MS are often much slower than other single variable read-out assays, such as microscopy assays, enzyme-linked immunosorbent assays (ELISA) or fluorescence-activated cell sorting (FACS).

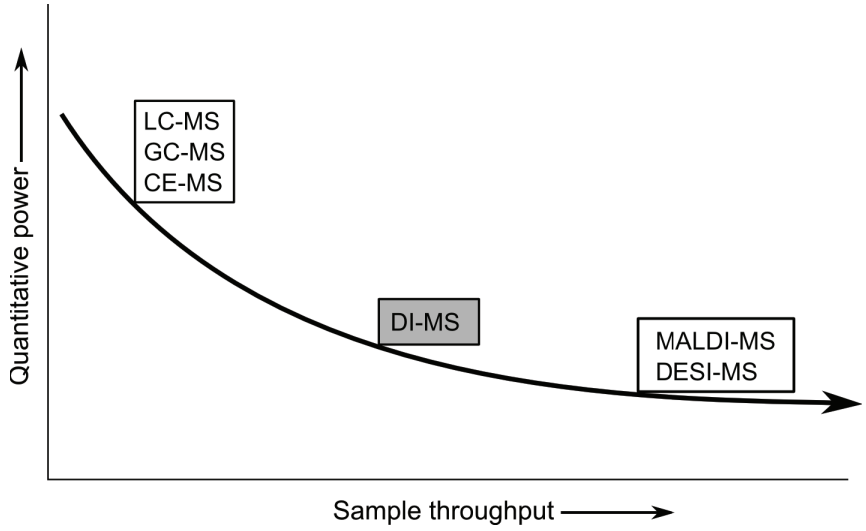

Figure 2: Schematic picture depicting the inherent trade-off between the quantitative power of a MS-based platform and its sample throughput.

distribution patterns of the analytes. IMS techniques can in principle be very fast, especially when only one spot per sample needs to be analyzed. Moreover, when employing e.g. DESI, limited or even no sample pretreatment is necessary. As a consequence, IMS has become a rapid and valuable tool across many fields, yet its major challenge is to acquire quantitative data regarding the surface concentrations of the analytes[5].

The developments in (HR)MS instruments have drastically increased ion selectivity for obtaining high resolution and moreover, enabled the detection of minute amounts of analyte (femtograms)[6]. However, one of the disadvantages of a MS is its limited dynamic range which is at best five orders of magnitude. More importantly, when using ESI, potential analyte competition during ionisation in the ion source, called ion suppression, imposes challenges in order to obtain quantitative results[7]. The same is true for most other ionization techniques. As a consequence, prior to MS detection, several separation techniques are often employed such as liquid chromatography (LC)-MS, gas chromatography (GC)-MS or capillary electrophoresis (CE)-MS in order to reduce the amount of potential co-eluting ions in the ion source and therefore reduce potential ion suppression effects. These hyphenated platforms can provide quantitative data, however at the expense of sample throughput (Figure 2).

Direct infusion-MS (DI-MS) is a fast and comprehensive analytical method in which a sample is introduced into the MS without prior separation, possibly after sample pretreatment. In DI-MS, samples can be introduced into the MS in several ways: (i) manually, by means of a syringe pump, or (ii) automated. Flow injection analysis-MS is a commonly used automated DI-MS approach which involves the injection of an analyte plug (typically a few microliters) into a flowing solvent stream by employing e.g. a LC autosampler and pump, omitting a chromatographic column[8]. Additionally, automated nanoESI robots are available which can directly introduce a sample into the MS through a small nanoESI microchip-nozzle (typically 5 μm), in a 'carryover-free' manner[9]. However, although DI-MS can be fast and enables high-throughput analysis, it is often prone to ion suppression due to co-eluting ions such as (endogenous) salts and it is often not possible to differentiate between isomers. By employing nanoelectrospray (nanoESI) instead of conventional ESI, ionization efficiency can be increased and ion suppression can be reduced [10]. As a consequence, effective sample pretreatment is a key step prior to DI-MS analysis in order to obtain quantitative data[6]. When samples are effectively purified and the extracted analyte is within the dynamic range of the MS, analytical performance of DI-MS should be comparable with hyphenated MS platforms. As discussed, MALDI and DESI-MS are in principle faster per sample point than DI-MS as they can faster acquire MS spectra per sample. When e.g. using chip-based nanoESI-DI-MS, after every sample the nozzle has to be changed in order to prevent carry over, which will usually take a few seconds.

Sample pretreatment

A typical MS-based analytical workflow comprises sampling, sample pretreatment and MS analysis. Many MS-based methods require proper sample pretreatment procedures in order to overcome the mismatch of the sample with the analytical method. The sample pretreatment procedure should allow the reproducible enrichment/extraction of the analytes so that the concentrations are within the linear range of the MS. Moreover, depending on the analytical MS-platform, interfering molecules such as proteins and salts should be removed in order to maintain the integrity of the system and to reduce ion suppression and poor detection sensitivity. Furthermore, sample pretreatment is required to release the analytes of interest from the sample matrix and transfer the analytes into a medium or form compatible for the

subsequent analysis with the analytical instrument. However, MS-based methods have the drawback that they often require tedious protocols for sample pretreatment that decrease the throughput of the assay. Therefore, to achieve high-throughput automated and integrated analytical systems have to be developed which include the sample pretreatment procedure. Although these goals can partly be met by traditional methods, new concepts are required which simplify sample pretreatment and maintain robustness and a high-throughput capacity. Additionally, when sample volume is limited, miniaturization of the sampling as well as the sample pretreatment procedure is needed which can also potentially lead to the concept of massive parallelization.

Metabolomics

The metabolome is the whole set of small molecules (typically <1000 Da) in a given biological sample. It can provide a functional readout of cellular biochemistry and therefore has the strongest correlation with the phenotype[1]. Therefore, metabolomics offers a promising phenotyping platform which has the potential to e.g. discover biomarkers for the diagnosis and prognosis of diseases and the prediction of the efficacy of drug interventions[11] [12]. Metabolites are a highly diverse range of molecules with vast differences in chemical structure, physicochemical properties and dynamic range (up to nine decades[13][14]). As a consequence, metabolomics may be the most challenging phenotyping omics science. Whereas genomics and transcriptomics can amplify DNA sequences using polymerase chain reaction (PCR), in metabolomics there is no effective method which can 'amplify' all low-abundant metabolites in an unbiased manner.

The metabolome can be considered as to the largest part a regulated dynamic phenotype in which changes in metabolite levels can e.g. reflect a transition from a healthy to a diseased state or the resilience of a system, in the context of systems homeostasis and allostasis. For the discovery of metabolic profiles to predict disease progression or the effect of interventions at an individual level, performing longitudinal studies is key in monitoring individuals which may result in personalized biology and eventually personalized medicine. For this strategy, following individuals over years will require high-throughput metabolomics approaches[1]. In addition, due to the vast biological variability of individuals, many individual samples may be analysed in a metabolomics study in order to ensure statistically meaningful results. As a consequence, such metabolomics studies should be performed preferably in a HT manner. In addition, each study should not only be carried out to analyse more samples within a period of time, but also to quantify as many relevant metabolites as possible in a single analysis, for which MS is in principle very suitable[15].

Aim and outline of the thesis

The aim of this thesis is to develop new methods for the mass spectrometry (MS)-based high-throughput profiling of small molecules. Direct infusion (DI)-MS can potentially provide high-content and high-throughput analyses, but effective and rapid sample pretreatment procedures are needed in order to obtain enough quantitative performance, i.e. precision and accuracy, in the mass spectrometric analysis while maintaining assay throughput. Moreover, when the analysis of small sample volumes is required, new miniaturized concepts in sampling

as well as sample pretreatment are needed. In addition, miniaturization of the experimental setup can play an important aspect with regards to the potential of massive parallelization and, consequently, can tremendously increase sample throughput.

In **Chapter 2** an overview of recent developments in sample pretreatment procedures for MS-based metabolomics is given. Deproteinization, removal of interfering molecules, liquid-liquid extraction (LLE), solid-phase extraction (SPE), electromigration-based extraction methods and possibly emerging sample pretreatment techniques for metabolomics are described and discussed. Advantages and limitations of these techniques for metabolomics are given, and aspects such as potential for automation and high-throughput analysis are evaluated

In **Chapter 3** the potential of nanoESI-DI-High Resolution-MS for the metabolic phenotyping of early zebrafish embryogenesis is investigated. It is studied whether efficient but limited sample pretreatment is sufficient to obtain metabolic profiles to distinguish early developmental stages during zebrafish embryos embryogenesis. Reproducible and high-quality MS data is generated by implementing an automated data-processing tool, which includes data clean-up steps and a dedicated normalization-optimization algorithm. Principle component analysis reveals that periods of 1 hour time shifts post fertilization can be differentiated from each other.

In **Chapter 4**, a new and fast and selective electromigration-based sample enrichment and purification technique called 3-phase electroextraction (3-phase EE) is presented and coupled to nanoESI-DI-MS. The electromigration of analytes from an aqueous sample through an immiscible organic filter phase into a small-volume aqueous acceptor phase is demonstrated. It is shown that selectivity of 3-phase EE can be tuned by changing the composition of the organic filter phase which also prevents proteins from migrating into the acceptor phase. Proof of principle towards online 3-phase EE-nanoESI-DI-MS is demonstrated and thus the compatibility of 3-phase EE with HTS is shown.

In **Chapter 5** a proof of principle of a new miniaturized LLE technique in a 384-well plate is presented, based on gas pressure mixing followed by passive phase separation. This fully-automated approach is integrated online into a nanoESI-DI-MS robot. It is shown that this high-throughput platform is suitable for screening drugs from human plasma with similar or better analytical performance compared to a conventional LLE procedure. Finally, the micro LLE method applied to dried blood spots is demonstrated.

In **Chapter 6**, a miniaturized sampling technique based on microneedle sampling is developed in order to monitor drug uptake in the (small-volume) yolk of zebrafish larvae using MS analysis. It is demonstrated that conventional whole zebrafish embryo lysis is not suitable for measuring drug uptake, due to potential adherence of drugs to the skin. The *in vivo* uptake MS-data is correlated to the results of *in vitro* and *in vivo* high-throughput drug screening platforms and it was shown that this new approach can give relevant information for interpretation of drug efficacy data.

REFERENCES

[1] R. Ramautar, R. Berger, J. van der Greef, and T. Hankemeier, "Human metabolomics: strategies to understand biology.," Curr. Opin. Chem. Biol., vol. 17, no. 5, pp. 841–6, Oct. 2013.

[2] Y. Feng, T. J. Mitchison, A. Bender, D. W. Young, and J. A. Tallarico, "Multi-parameter phenotypic profiling: using cellular effects to characterize small-molecule compounds.," Nat. Rev. Drug Discov., vol. 8, no. 7, pp. 567–78, Jul. 2009.

[3] Z. A. Gurard-Levin, M. D. Scholle, A. H. Eisenberg, and M. Mrksich, "High-throughput

screening of small molecule libraries using SAMDI mass spectrometry.," ACS Comb. Sci., vol. 13, no. 4, pp. 347–50, Jul. 2011.

[4] T. P. Roddy, C. R. Horvath, S. J. Stout, K. L. Kenney, P.-I. Ho, J.-H. Zhang, C. Vickers, V. Kaushik, B. Hubbard, and Y. K. Wang, "Mass spectrometric techniques for label-free high-throughput screening in drug discovery.," Anal. Chem., vol. 79, no. 21, pp. 8207–13, Nov. 2007.

[5] S. R. Ellis, A. L. Bruinen, and R. M. A. Heeren, "A critical evaluation of the current state-of-the-art in quantitative imaging mass spectrometry.," Anal. Bioanal. Chem., vol. 406, no. 5, pp. 1275–89, Feb. 2014.

[6] A. Namera and T. Saito, "Recent advances in unique sample preparation techniques for bioanalysis.," Bioanalysis, vol. 5, no. 8, pp. 915–32, Apr. 2013.

[7] A. Furey, M. Moriarty, V. Bane, B. Kinsella, and M. Lehane, "Ion suppression; a critical review on causes, evaluation, prevention and applications.," Talanta, vol. 115, pp. 104–22, Oct. 2013.

[8] T. Fuhrer, D. Heer, B. Begemann, and N. Zamboni, "High-throughput, accurate mass metabolome profiling of cellular extracts by flow injection-time-of-flight mass spectrometry.," Anal. Chem., vol. 83, no. 18, pp. 7074–80, Sep. 2011.

[9] R.-J. Raterink, F. M. Kloet, J. Li, N. A. Wattel, M. J. M. Schaaf, H. P. Spaink, R. Berger, R. J. Vreeken, and T. Hankemeier, "Rapid metabolic screening of early zebrafish embryogenesis based on direct infusion-nanoESI-FTMS," Metabolomics, vol. 9, no. 4, pp. 864–873, Jan. 2013.

[10] A. Schmidt, M. Karas, and T. Dülcks, "Effect of different solution flow rates on analyte ion signals in nano-ESI MS, or: when does ESI turn into nano-ESI?," J. Am. Soc. Mass Spectrom., vol. 14, no. 5, pp. 492–500, May 2003.

[11] R. Kaddurah-Daouk, M. B. Bogdanov, W. R. Wikoff, H. Zhu, S. H. Boyle, E. Churchill, Z. Wang, A. J. Rush, R. R. Krishnan, E. Pickering, M. Delnomdedieu, and O. Fiehn, "Pharmacometabolomic mapping of early biochemical changes induced by sertraline and placebo.," Transl. Psychiatry, vol. 3, p. e223, Jan. 2013.

[12] M. Mamas, W. B. Dunn, L. Neyses, and R. Goodacre, "The role of metabolites and metabolo mics in clinically applicable biomarkers of disease.," Arch. Toxicol., vol. 85, no. 1, pp. 5–17, Jan. 2011.

[13] N. Psychogios, D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, and D. S. Wishart, "The human serum metabolome.," PLoS One, vol. 6, no. 2, p. e16957, Jan. 2011.

[14] S. Krug, G. Kastenmüller, F. Stückler, M. J. Rist, T. Skurk, M. Sailer, J. Raffler, W. Römisch-Margl, J. Adamski, C. Prehn, T. Frank, K.-H. Engel, T. Hofmann, B. Luy, R. Zimmermann, F. Moritz, P. Schmitt-Kopplin, J. Krumsiek, W. Kremer, F. Huber, U. Oeh, F. J. Theis, W. Szymczak, H. Hauner, K. Suhre, and H. Daniel, "The dynamic range of the human metabolome revealed by challenges.," FASEB J., vol. 26, no. 6, pp. 2607–19, Jun. 2012.

[15] J. Han, R. Datla, S. Chan, and C. H. Borchers, "Mass spectrometry-based technologies for high-throughput metabolomics," Bioanalysis, vol. 1, no. 9, pp. 1665–1684, 2009.