CE-MS for metabolomics: advancing performance and detection sensitivity
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Chapter 1

General Introduction and Scope

Introduction

Drug discovery and systems biology

Drug discovery is an interdisciplinary endeavor that involves iterative process of chemical, biochemical, and pharmacological assays [1-3]. Over the last five decades, the capitalized cost estimate for developing a new drug from concept has increased from millions to a few billion dollars [4], however, the success rate for drugs entering into the market is no higher than 24% [5,6]. Reducing the attrition rate of drug candidates in clinical development is the key challenge for pharmaceutical researches, and important for sustaining the viability of the entire industry [7,8]. Advancements in various technical aspects can help researchers avoid unnecessary costs. Preclinical characterization of pharmacokinetic and toxicological properties of chemical entities has already reduced downstream attrition [9]. On the contrast, the attrition rates in clinical trial stage are still unacceptably high, and one of the major issues is the lack of understanding about the nature of (new) drug targets, which increases the uncertainty about the desired therapeutic efficacy. Pharmaceutical industry has experienced a string of failed clinical trials in developing modifying therapies for Alzheimer's disease over last two decades. Sperling et al. proposed three assumptions in an attempt to explain those failures and emphasized the importance of the combination of the right target, the right drug, and the right time in order for the drug to exert its efficacy [10]. Selecting drug targets and employing discovered targets for early pharmacological evaluation are two key steps in drug discovery and aid in the reduction of high attrition rates in clinical trials [7].

Whether it is drug target identification or efficacy evaluation, systems biology appears to be a promising tool. Systems biology is a knowledge-based approach that attempts to understand biology at the system level and has the capability of integrating large quantities of complex data obtained by genomic, proteomic and metabolic analyses [11]. An important application of systems biology lies within the rapidly developing field of personalized medicine. Systems biology approach screens variations, mutations, gene variants, and metabolic profiles by analyzing human DNA, RNA and proteins, which reveals pathologically relevant information such as diagnosis, susceptibility and risk factors, and disease progression and therapy [12]. In this way, the current healthcare strategies can be optimized and therapeutic therapies personally customized.

Among the technologies applied for systems biology, genomics and proteomics first came to the spotlight and were expected to result in a plethora of validated new drug targets [6]. Genomics and proteomics only take into consideration the intermediate steps in the central dogma, while metabolomics explores the downstream products from genes and proteins [13]. It is the integration of metabolomics with genomics and proteomics that enables researchers to gain a holistic understanding about pathways and ultimately better assists with drug discovery and personalized medicine. However, it still remains to be a challenge to conduct multi-omics

integration because a lot of the analytical techniques and experimental designs lack the versatility to permit proper comparison or integration among different omics disciplines. Pinu et at. [14] pointed out that metabolomics is capable of providing a "common denominator" to the design and analysis of many other omics works due to its closeness to phenotypes, thus an increased awareness of metabolomics by fellow researchers conducting other omics could greatly benefit the quality of integrated-omics research.

Metabolomics

Metabolomics seeks to provide a comprehensive profile of all the (endogenous) metabolites present in a given sample at a specific point of time [15]. Metabolomic analysis of biological samples has found applications in pathology, diagnostics, and toxicology [16-20]. The field of metabolomics has developed significantly over the past decade and basically progressed from a fundamental research topic studied by a relatively small number of highly specialized research groups into a major field now used by hundreds of laboratories, core facilities and national centers.

The ultimate aim of metabolomics research is to effectively address a specific biological or clinical problem. For this purpose, two analytical strategies are generally employed, i.e. non-targeted and targeted metabolomics. Non-targeted metabolomics approaches are often used for explorative, discovery-based studies and involves the profiling of biological samples (often body fluids) without having *a priori* knowledge on the nature and identity of the measured metabolites. Such strategies may be referred to as metabolic profiling or fingerprinting. The second approach is focused on the quantitative analysis of pre-selected (target) metabolites in a biological sample. Both approaches can be used in a single study, where the first approach is used to discover putative biomarkers of disease, which are then validated and subsequently quantified by the second approach. The requirements on analytics for these two approaches are diverging, notably when it comes to sample pre-treatment, standardization, matrix effects, etc. The decision on which metabolomics approach to use is determined by the aim of the study.

Current techniques used in metabolomics mainly include two categories: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)-based techniques. NMR is a non-destructive strategy that does not require complicated and laborious sample preparation [21], which sometimes, in turn, increases the difficulty of analyzing NMR spectra derived from complex mixtures. In terms of analysis time and cost per sample, NMR is more suitable for high-throughput studies than MS [22]. The major drawback of NMR in metabolomics applications is the relatively low sensitivity, thus its acquired metabolic coverage isn't comparable to MS.

Mass spectrometry (MS)-based techniques such as GC-MS and LC-MS are the most commonly used analytical platforms for metabolomics studies. GC-MS is the method of choice for volatile and thermally stable metabolites, and it involves complex and laborious sample derivatization procedures. LC-MS benefits from its widespread availability and continuous instrumentation

development and has been applied in a plethora of metabolomics explorations. The profiling of endogenous metabolites is predominantly performed with *state-of-the-art* reverse phase LC-MS (RPLC-MS) methods. However, RPLC-MS is highly suitable for analyzing compounds of medium or low polarity, but often problematic for highly polar metabolites. The analysis of polar compounds could then be alternatively analyzed by employing hydrophilic interaction liquid chromatography (HILIC) columns. HILIC utilizes modified LC columns that possess strong retention capability for highly polar compounds, which renders HILIC a complementary technique to RPLC and has been applied in various research fields as described in the literature [23-25]. The drawbacks of HILIC usually include poor retention time reproducibility and analytical drift after analyzing multiple samples [26].

CE-MS for metabolomics

Within the metabolomics field, another useful analytical technique for the profiling of (highly) polar and charged metabolites is capillary electrophoresis (CE) hyphenated to MS (CE-MS). In CE, compounds are separated on the basis of differences in their intrinsic electrophoretic mobility, which is dependent on the charge and size of the analyte. Therefore, CE is especially suited for the analysis of polar and charged metabolites. Compared to chromatographic-based separation methods, the separation efficiency of CE is very high due to the flat flow profile of the electro-osmotic flow and there is no mass transfer between phases and as only longitudinal diffusion contributes to band broadening under proper experimental conditions. The intrinsically high separation efficiency of CE is very advantageous for the high resolution separation of structurally similar metabolites in complex samples. Moreover, as the separation mechanism of CE is fundamentally different from chromatographic-based separation techniques, a complementary view on the composition of metabolites present in a given biological sample is provided [27-29]. HILIC and CE are both suited for the analysis of polar metabolome, and the extent of overlapping in their metabolic coverage triggered some interesting studies. Kok et al. employed both CE-MS and HILIC-MS for anionic profiling of rat urine and discovered that these methods exhibited a high degree of orthogonality [30]. Although the proposed HILIC method obtained more features and high intensities (mainly due to the much larger injection volume), CE-MS detected over 200 unique features.

CE coupling to MS is accomplished via electrospray ionization (ESI) with different interfacing designs. The development of interfacing technologies has helped expand the applications of CE-MS. So far the most commonly used interface is coaxial sheath liquid interface, and sheath liquid CE-MS has found usefulness in profiling various biological samples [31-33]. Due to the limited volume of injected samples and the dilution effect caused by the sheath liquid, the compromised concentration sensitivity has always been an issue with this setup. To overcome the sample injection limitation, various online sample preconcentration techniques have been investigated, including dynamic pH injunction, transient isotachophoresis (tITP), field amplification sample

stacking (FASS), field amplification sample injection (FASI), and sweeping [34-37]. Moreover, efforts have also been devoted to developing more sensitive interfaces by minimizing or circumventing the direct mixing of sheath liquid with CE effluent [38-42], thus improving detection sensitivity. To date, the most successful alternative interface has been the sheathless nanospray interface with a porous tip emitter developed by Moini [40], which has greatly benefited the detection sensitivity and metabolome coverage, as showcased in the literature [43-46].

Notably, a strong advantage of CE-MS over HILIC-MS is that it is especially useful for analyzing volume-restricted samples. Volume restricted samples are intrinsically small and often low in metabolite concentrations, such as mouse cerebrospinal fluid (CSF), samples from microfluidic organ-on-a-chip models, and circulating tumor cells in bloodstream, but they can be information-rich and could be the key to unravelling certain biomedical mysteries. The heterogeneous tumor cell populations that reside in the tumor ecosystem represent a considerable challenge for tumor therapy [47, 48]. Insights into the metabolic patterns of these small tumor cell populations can contribute to the understanding about tumor cell heterogeneity. Better understanding about the cell heterogeneity in tumors will lead to a better chance of eradicating all different cell subpopulations and smother any possibility of inducing drug resistance. Sheathless CE-MS has proved to be a useful technique in the metabolic profiling mouse CSF samples [45] and low numbers of cancer cells [49].

Challenges in CE-MS for metabolomics

Until now, CE-MS has only been used by a limited number of research groups for metabolomics studies. The coupling of CE to MS is often perceived as technically challenging by the scientific community. Moreover, there is a lack of standard operating procedures, which are critical for performing (long-term and inter-laboratory) reproducibility studies. The major drawback of CE is the inadequate robustness. However, it is important to note that a number of recent studies clearly exemplify the usefulness of CE-MS for metabolic profiling of large sample sets [50, 51]. For example, the group of Soga and co-workers, the group that introduced the first CE-MS methods for metabolomics in 2003 [52], has assessed the long-term performance of CE-MS for metabolic profiling of more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period [50]. The study provided an absolute quantification for 94 polar metabolites in plasma with a similar reproducibility when compared to other analytical platforms, i.e. reversed-phase LC-MS and GC-MS, employed for large-scale metabolomics studies.

Nonetheless, there is still a lack of reliable CE-MS methods for the efficient profiling of highly polar and charged metabolites in the literature, especially in volume-limited samples. In this thesis, a key aim was to develop viable CE-MS approaches for the highly efficient and sensitive profiling of polar and charged metabolites. For achieving this purpose, both the conventional and the sheathless porous tip interface have been considered for coupling CE to MS. The repeatability of CE-MS methods for peak areas and migration times of metabolites was closely monitored. Special

attention has been devoted to highlight relevant methodological aspects for metabolomics studies in protocol papers and to share experimental procedures via peer-reviewed (video) articles. Conveying the work in this way is urgently needed to convince the scientific community about the unique and complementary capabilities of CE-MS for metabolomics.

Scope and outline of the thesis

At the start of this work, some efforts had already been devoted to downscaling analytical techniques and/or workflows for the analysis of (endogenous) metabolites in minute sample amounts. These analytical technologies, though very promising, were not suited for the highly sensitive and efficient profiling of a large number of metabolites in small-volume biological samples in a robust way [53-55]. Therefore, in this thesis, CE-MS strategies employing both a sheath-liquid and sheathless porous tip interface were developed and evaluated for the global profiling of metabolites in low amounts of sample material. These methods covered a wide range of cationic and anionic metabolites, including compounds like amino acids, amines, organic acids and nucleotides, etc. This evaluation was conducted by considering aspects such as migration-time and peak area repeatability, separation efficiency, linearity and detection limits. The applicability of the developed CE-MS methods has been tested by analyzing clinically relevant volume/material limited samples. Special attention has been devoted to highlighting relevant methodological aspects in order to expand the (complementary) role of CE-MS in metabolomics.

An overview of CE-MS advancements in metabolic profiling is presented in **Chapter 2**. This chapter discusses aspects such as interfacing designs and provides an overview of strategies on how to improve metabolic coverage, and sample throughput. The applicability of CE-MS for metabolic profiling of especially limited sample amounts is demonstrated by the analysis of cell culture extracts. The advancements in CE-MS interfaces, such as the sheathless interface with a porous tip emitter, have shown great promise in enhancing the coverage of the polar metabolome. However, the general application of CE-MS in metabolomics research is still limited as this approach is considered as technically challenging and lacking reproducibility.

In order to expand the utility of CE-MS in metabolomics research, standardized protocols are urgently needed. **Chapter 3** includes two technical notes, introducing both the basic operations and biological applications of CE-MS. The first part introduces a protocol on how the CE system is coupled to MS via a sheathless porous tip interface, including a description of an extensive conditioning procedure for the first use of the capillary. This section also showcased the versatility of CE-MS for the global profiling of anionic and cationic metabolites using a single capillary and buffer composition. The second part in this chapter described an analytical workflow based on sheathless CE-MS for metabolic profiling of low amounts of HepG2 cells, with special attention on the sample preparation step.

In **Chapter 4**, the utility of CE-MS employing a sheathless porous tip interface is demonstrated for metabolic profiling of limited amounts of HepG2 cells, using the sample preparation procedure described in the previous chapter. By using sheathless CE-MS in combination with transient isotachophoresis (tITP), this chapter illustrated the feasibility of CE-MS for highly sensitive profiling of metabolites in material-limited samples. It is shown that the proposed approach allows to obtain metabolomics data from an injection volume corresponding to the content of less than a single HepG2 cell.

In **Chapter 5** a sheathless CE-MS method is proposed for the profiling of nucleotides at high pH separation conditions. In this work, nucleotides were separated while negatively charged and detected by MS in the positive ion mode, thereby fully circumventing the commonly observed *corona discharge* when coupling high pH nanoscale separations to negative ESI-MS mode. The proposed sheathless CE-MS method was validated in aspects of linearity, precision, accuracy and matrix effect. Overall, the developed approach allows highly efficient and sensitive profiling of nucleotides in HepG2 cells, down to the injection content equivalent to less than a single cell.

CE-MS approaches employing a conventional sheath-liquid interface have been widely used for metabolic profiling studies, however, the robustness and usefulness of this technique has not been assessed in detail for biomarker discovery studies yet. Therefore, **chapter 6** outlines an innovative validation strategy to illustrate the utility of CE-MS for biomarker discovery studies using metabolomics. In essence, the approach was based on the determination of simulated biomarkers in spiked human plasma. Different strategies were adopted in creating differences among sample groups, and those artificial "biomarkers" could be accurately identified with the use of CE-MS analysis combined with multivariate data analysis.

Chapter 7 demonstrates our work using conventional CE-MS in biomarker discovery using volume-limited plasma samples from a mouse model for epilepsy. The mouse model was created by direct electric shock on mouse corneal and blood samples were collected from both the control group and model group By using our previously developed and validated CE-MS method for biomarker discovery. The study clearly revealed the advantage of CE-MS in profiling volume-restricted samples and revealed metabolic markers potentially indicative of epilepsy onsets in mice.

Chapter 8 reports the first quantitative assessment of polar and charged metabolites in the nanomaterial corona using a CE-MS-based metabolomics approach. This study revealed that polar ionogenic metabolites adsorb to nanomaterials and that nanomaterial properties have a significant impact upon the qualitative and quantitative composition of the metabolite corona. Furthermore, formation of metabolite corona was quantitatively assessed using protein-free and complete human plasma samples, which revealed that the presence of proteins in the sample is vital to characterizing a biologically relevant metabolite corona as differences between protein-free and intact plasma are significant and lead to different corona formation.

Finally, **Chapter 9** offers a general conclusion of the studies described in this thesis. Perspectives and recommendations on further improvement and applications of the proposed CE-MS methods are also discussed.

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