



Utility of sheathless capillary electrophoresis-mass spectrometry for metabolic profiling of limited sample amounts

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ABSTRACT

Metabolomics studies using a small amount of cells may save time and money, while in some cases (e.g., profiling pathogenic cells in an early-stage tissue), only a small number of cells are accessible for analysis. The analysis of small amounts of biological samples challenges the analytical toolbox used in present-day metabolomics studies, and a significant number of crucial biological questions cannot be properly addressed. To allow metabolic profiling of limited sample amounts, the potential of capillary electrophoresis-mass spectrometry (CE-MS) using a sheathless porous tip interface has been assessed using HepG2 cells in starting amounts of 500 and 10,000 cells as a model system in this work. It is shown that highly efficient and information-rich metabolic profiles for cationic metabolites at low-pH separation conditions could be obtained by sheathless CE-MS using an injection volume of only circa 42 nL, which equals the content/aliquot of circa 0.25 and 5 HepG2 cells, respectively. With as little as the content of 0.25 cell injected, more than 24 cationic metabolites could be identified. A further improvement of sample preparation and/or the injection part is required in order to effectively analyze the compounds of interest in very low sample amounts by sheathless CE-MS. However, the results obtained so far clearly indicate the strong potential of the proposed method for metabolic profiling of limited sample amounts.

1. Introduction

The final aim of a metabolomics study is to find an answer to a given (well-defined) biological or clinical question [1]. For this purpose, advanced analytical separation techniques are used for targeted or non-targeted analysis of (endogenous) metabolites in biological samples to determine the influence of genetic variation or external stimuli [2]. If performed properly, the metabolomics study may reveal important insights into pathogenic factors or compromised metabolic pathways, which eventually may lead to an improved diagnosis and a personalized therapy [3–5].

Currently, the conventional analytical techniques can be used in a reliable way for metabolomics studies, however, these analytical tools are often not suited for the profiling of metabolites in small amounts of biological samples. There is a strong interest for analytical tools capable of providing highly sensitive metabolic profiles for microscale cell culture samples. For example, for researches focused on stem cells [6], circulating tumor cells in blood [7], cancer stem cells, and primary tumor cells in early-stage tissues [8,9], often only a small amount of

cells are available. Another type of biomass-limited samples come from the emerging microfluidic 3D cell models, which can simulate physiological tissues by arranging different cell types in a 3D environment within a proper micro-environment [10]. These microfluidic cell culture systems intrinsically deal with relatively low amount of cell numbers, i.e. typically in the range of hundreds to thousands of cells.

Therefore, highly sensitive microscale (or nanoscale) analytical tools are needed to enable metabolomics studies of limited sample amounts. CE-MS may be considered an attractive analytical tool for metabolic profiling of limited samples due to its nanoliter sample injection requirement from only a few microliters of samples in a vial [11].

Although, a conventional CE-MS method employing a co-axial sheath-liquid interface is suited for analyzing volume-restricted biological samples, the detection sensitivity obtained by such an approach is often not sufficient for the reliable and global screening of trace-level metabolites in small sample amounts. In this work, we have considered a sheathless CE-MS approach, which was originally developed by Moini [12], for metabolic profiling of limited amounts of cells, as this

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approach has provided very promising results for metabolic profiling of volume-limited samples, such as mouse urine and cerebrospinal fluid (CSF) [13]. For example, in case of metabolic profiling of CSF samples from mice after a simple 1:1 dilution with water, more than 300 molecular features could be observed by only employing an injection volume of circa 9 nL. Recently, we have developed a sheathless CE–MS method for the profiling of anionic and cationic metabolites employing a single bare fused-silica capillary emitter at low-pH separation conditions and applied it to the analysis of extracts from the glioblastoma cell line [14,15]. By using an injection volume of circa 20 nL, the developed CE–MS approach allowed the profiling of cationic metabolites in an amount corresponding to 400 glioblastoma cells. This encouraging result triggered us to assess the utility of sheathless CE–MS for highly sensitive profiling of cationic metabolites in limited amounts of mammalian cells. In order to do this, HepG2 cells were used as a model system and the cell pellet was lysed, diluted, processed and analyzed as microscale cell cultures. An on-line preconcentration strategy, transient isotachopheresis (t-ITP), was used in this study to further improve the detection sensitivity of the proposed sheathless CE–MS method.

2. Materials and methods

The reagents, background electrolyte (BGE), sheathless CE equipment (CESI8000) and mass spectrometer are as described in our previous work [15]. HepG2 cells were cultured, harvested and rinsed with PBS (37 °C) before being split into Eppendorf tubes (circa 2,000,000 cells per tube); the amount of rinsing solvent was minimized. Cell pellets were kept at –80 °C prior to quenching with cold methanol/water mixture (v/v, 80/20). The quenched cell lysate mixture was further diluted to 10,000 and 500 cells per 50 µL using the same methanol/water mixture. For cationic metabolic profiling, the intracellular metabolites were extracted in a similar manner as reported in [15]. 50 µL of the aforementioned samples were used for sample preparation, where 50 µL of cell-free isotope-labeled amino acids solution (0.2 µg/mL) was added as internal standards (ISTD). Pre-chilled water, methanol and chloroform were then added to the mix resulting in a final ratio of 1:1:0.5 (v/v/v). After vigorous vortex shaking and centrifugation, the supernatant was transferred to centrifugal filters with a 5 kDa cutoff filter to further remove interfering residues. Prior to analysis, dried metabolite extracts were reconstituted in 50 µL of 250 mM ammonium acetate (pH 7.0) solution in order to allow the use of t-ITP. Circa 42 nL (corresponding to circa 6.6% of the total capillary volume) was hydrodynamically injected (i.e., by using a pressure of 6 psi for 60 s) into the sheathless CE–MS system, which in case for the HepG2 cell samples corresponded to an aliquot/content of circa 5 and 0.25 HepG2 cells, respectively. The calculation of the number of cells per injection was based on the following procedure: a mixture of water, methanol and chloroform was used for liquid-liquid extraction, using in total 600 µL of water and methanol. The highly polar and charged compounds will be in the water-methanol layer. After ultrafiltration, 360 µL of this fraction was evaporated and the dried extract was reconstituted in 50 µL of ammonium acetate solution, which contains the content of $10,000 \times (360/600)$ cells if we start with 10,000 HepG2 cells. For CE–MS analyses, an injection of volume of 42 nL is used, which corresponds to the content of circa 5 cells based on $42 \text{ nL} / 50,000 \text{ nL} \times 10,000 \times (360/600)$.

Sheathless CE–MS experiments were performed employing a 30 µm i.d. \times 91 cm bare fused-silica capillary, which was thermostated using recirculating liquid coolant regulated at 25 °C, and coupled to a Sciex TripleTOF 5600+ MS system using the NanoSpray III source. ESI was performed in positive ionization mode by setting the ionspray voltage at 1100 V while the values for gas 1, gas 2, and interface heating temperature were set at 0, 0 and 50 respectively. MS data were recorded in the m/z range of 65 to 1000. Declustering potential was set at 50, which favors the detection of compounds in the low mass region. Electrophoretic separation was conducted in normal polarity mode by

applying 30 kV to the CE inlet electrode. Prior to CE–MS, the MS instrument was optimized using an ESI tuning mix for positive ion mode. 10% acetic acid (pH = 2.2) was used as BGE. The OptiMS cartridge was pre-conditioned and rinsed as described in [14]. Plate numbers of a few representative compounds were calculated using their migration time and the peak width at half height. Limits of detection (LODs) for the metabolites in the test mixture were determined as the concentration yielding a S/N-ratio of 3 via extrapolation of the S/N-ratio produced by the lowest concentration used for the design of calibration curves (extracted ion electropherograms were used for this purpose). The identification of the peaks detected in HepG2 cell extracts by sheathless CE–MS was based on a comparison of the recorded m/z values and migration times with that obtained for the metabolites from the standard mixture.

3. Results and discussion

The aim of this study was to develop a sheathless CE–MS method for the profiling of metabolites in limited amount of cells, using HepG2 cells as a model system for this purpose. First, we have evaluated the performance of sheathless CE–MS for the analysis of a home-made cationic metabolite mixture and subsequently applied this approach to the profiling of intracellular metabolites in extracts from HepG2 cells. An injection volume of circa 4.7 nL (which corresponds to 0.73% of the total capillary volume) of a 5 µM cationic metabolite mixture resulted in an acceptable detector response for most test compounds. The LOD values (S/N = 3) obtained for the test compounds, by extrapolating the S/N-ratio obtained for the injection of a 5 µM metabolite mixture, ranged from 1.4 to 92 nM (except for aspartic acid, 417 nM), which is a significant improvement as compared to the LOD values (0.1 to 10 µM) typically found for these compounds when employing conventional sheath-liquid CE–MS systems [16,17], and also with comparison to CE–MS using a flow-through microvial interface in which LODs from 0.1 to 12 µM were obtained for a cationic metabolite mixture [18]. It should be noted, however, that these are rough comparisons as different MS systems have been used in the other works. Recently, Hirayama et al. developed a new sheathless interface for coupling CE to MS [19]. The interface was designed by creating a small crack approximately 2 cm from the end of the capillary, which was covered with an electro dialysis membrane (cellulose acetate, molecular weight cut-off of 100 Da) to minimize the migration of small metabolites across the crack. This approach provided LODs for cationic metabolites in the range from 30 nM to 1.7 µM (when using an injection volume of 1.4 nL), which were rather comparable with results obtained by the sheathless CE–MS method proposed here when considering the difference in injection volume. However, the new CE–MS approach of Hirayama and co-workers is not suited for the profiling of compounds with a molecular weight below 100 Da, therefore, many relevant metabolites may not be detected by this approach.

Though, a significant improvement in LODs was provided by the sheathless CE–MS method using a porous tip interface, these values may not be sufficient for the highly sensitive profiling of metabolites in small sample amounts. Therefore, t-ITP was applied to further increase the injection volume to circa 42 nL. The cationic metabolite mixture with different physico-chemical properties was first analyzed using this method. Fig. 1 shows a typical result obtained by sheathless CE–MS for the analysis of a 1 µM cationic metabolite mixture employing t-ITP for preconcentration. Compared with the results from injection without t-ITP, the plate numbers for a few selected compounds like phenylalanine, tyrosine and lysine increased from 76,659, 89,493 and 273,631 to 444,761, 397,870 and 637,912, respectively, thereby indicating a significant decrease of peak widths at half height and as a result higher peak heights. The LOD values obtained with t-ITP injections ranged from 0.06 to 8.41 nM, with nearly one-third of the compounds achieving sub-nanomolar LOD values. It should be noted that these values were extrapolated from S/N-ratios obtained for the injection of a

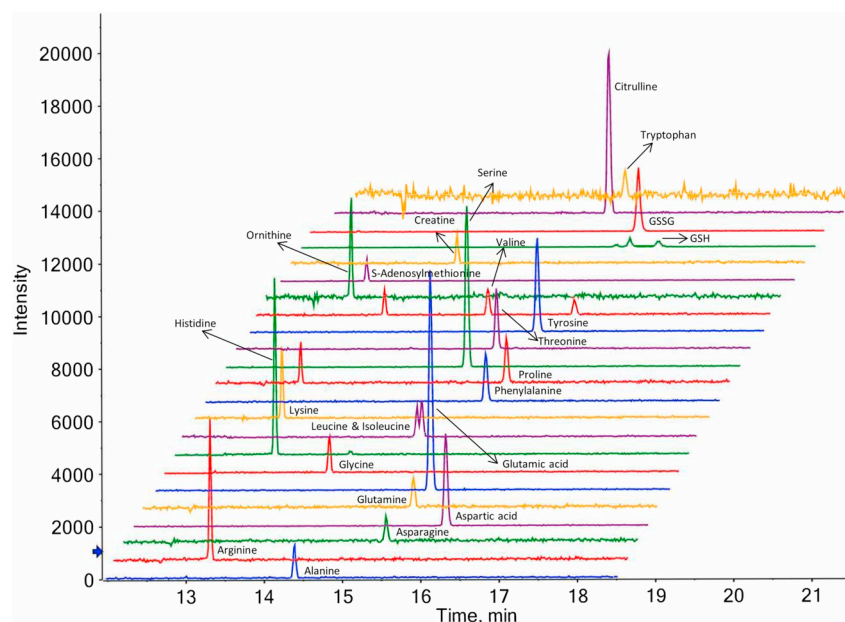


Fig. 3. Multiple extracted ion electropherograms for a selected number of metabolite peaks detected in an extract of 500 HepG2 cells by sheathless CE–MS in positive mode using a porous tip emitter. Separation conditions: BGE, 10% acetic acid (pH 2.2); Separation voltage: 30 kV; sample injection: 6.0 psi for 60 s.

Table 1

List of compounds detected in an extract of HepG2 cells with sheathless CE–MS in positive ion mode using a porous tip emitter by comparing the migration time and m/z ratio with those obtained for in-house metabolite standards. Separation conditions: BGE, 10% acetic acid (pH 2.2); separation voltage: +30 kV; sample injection: 6.0 psi for 60 s.

Metabolites determined			
Detected m/z	Metabolites	Detected m/z	Metabolites
76.0393	Glycine	138.0913	Tyramine
90.0550	Alanine	147.0764	Glutamine
106.0499	Serine	147.1128	Lysine
116.0706	Proline	148.0604	Glutamic acid
118.0863	Valine	150.0583	Methionine
120.0655	Threonine	156.0768	Histidine
132.0655	Hydroxyproline	166.0863	Phenylalanine
132.0768	Creatine	175.1190	Arginine
132.1019	Isoleucine	182.0812	Tyrosine
132.1019	Leucine	205.0972	Tryptophan
133.0608	Asparagine	307.0833	GSSG
134.0448	Aspartic acid	308.0911	GSH ^a
137.0458	Hypoxanthine		

Metabolites to be determined			
Detected m/z	Detected m/z	Detected m/z	Detected m/z
86.0972	130.0499	152.0562	188.1281
88.0402	130.0859	160.0965	190.1071
90.0918	133.0972	163.0179	212.2008
100.1120	134.0809	164.0917	220.1544
102.0550	136.0634	165.0543	221.1544
102.0550	136.0754	168.0761	223.0740
104.0701	138.0520	170.1181	241.0313
106.0866	139.0495	174.1123	258.2065
116.0703	139.0499	176.1030	284.0989
118.0863	146.1173	178.1071	399.1445
128.1067	150.1120		

^a Though detected, GSH is not stable under the employed conditions as it is prone to oxidation.

profiling of biomass-limited samples. Though, primarily introduced here as a screening method, we have also compared the detector response (using peak area as read-out) for a few selected endogenous

metabolites as a function of the starting amount of HepG2 cells. This analysis revealed a linear detector response when going from 10,000 to 500 HepG2 cells (see Supplementary Fig. 1), which corresponds to an injection content from 5 cells to 0.25 cell, indicating the potential of the sheathless CE–MS method for quantitative metabolomics studies of limited sample amounts.

In the present work, the primary focus was on developing an assay for profiling cationic metabolites in limited amounts of cells. Follow-up work will focus on the profiling of anionic metabolites, including compounds like nucleotides and sugar phosphates, in order to further expand the metabolic coverage of this method. In this context, (further) optimization of BGE composition and the in-capillary preconcentration procedure is needed, notably for anionic metabolites. Another important aspect to consider is the sample preparation strategy used for a given material-limited cell line based metabolomics study, as we anticipate that the chosen sample preparation strategy, instead of the type of cell, will have a major influence on the performance of the analytical method. In the work outlined here, the final dried cell extract was reconstituted in 50 μ L of solvent, however, the reconstitution volume may be dramatically reduced by the use of nanovials and, as such, we expect that we can further improve the detection sensitivity of our method for material-limited metabolomics studies. The sample throughput of the current approach is limited, however, the group of Britz-McKibbin recently developed a multi-segment injection strategy which significantly improved the sample throughput for targeted metabolomics studies [20]. We will also explore the possibility of multi-segment injection for improving analysis times in sheathless CE–MS-based metabolomics studies. Overall, the aim is to use the proposed sheathless CE–MS method for metabolic profiling of cell culture samples from 3D microfluidic organ-on-a-chip systems developed in our laboratory [21].

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2018.12.004>.

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