Profiling nucleotides in low numbers of mammalian cells by sheathless CE-MS in positive ion mode: Circumventing corona discharge

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Research Paper

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Negative ion mode nano-ESI–MS is often considered for the analysis of acidic compounds, including nucleotides. However, under high aqueous separation conditions, corona discharge is frequently observed at emitter tips, which may result in low ion abundances and reduced nano-ESI needle emitter lifetimes. In this work, we introduce a sheathless CE-MS method for the highly efficient and sensitive analysis of nucleotides employing ESI in positive ion mode, thereby fully circumventing corona discharge. By using a background electrolyte of 16 mM ammonium acetate (pH 9.7) a mixture of 12 nucleotides, composed of mono-, di-, and tri-phosphates, could be efficiently analyzed with plate numbers per meter above 220 000 and with LODs in the range from 0.06 to 1.3 nM, corresponding to 0.4 to 8.6 attomole, when using an injection volume of about 6.5 nL only. The utility of the method was demonstrated for the profiling of nucleotides in low numbers of mammalian cells using HepG2 cells as a model system. Endogenous nucleotides could be efficiently analyzed in extracts from 50 000 down to 500 HepG2 cells only. Moreover, apart from nucleotides, also some nicotinamide-adenine dinucleotides and amino acids could be analyzed under these conditions, thereby clearly illustrating the utility of this approach for metabolic profiling of low amounts of biological material.

Keywords:
corona discharge circumvention / HepG2 cells / mass spectrometry / nucleotides / sheathless interface
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Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

In metabolomics, LC hyphenated to high-resolution accurate mass TOF-MS is now routinely used for discovery studies. However, the highly efficient profiling of polar and charged metabolites remains a challenge with modern LC columns including HILIC and ion-exchange LC, especially for phosphorylated metabolites such as nucleotides [1], which play key roles in cell signaling and metabolism. The latter compounds have the tendency to interact with stainless steel in the LC–MS system and an additive to the mobile phase needs to be applied to deactivate the steel surfaces [2]. Moreover, method development for nucleotide profiling by ion-pair reversed-phase LC–MS, anion-exchange LC–MS, and HILIC–MS is often not straightforward. Another challenge concerns the analysis of nucleotides in low numbers of mammalian cells with the aim to get insight into how a small population of cells within a tumor or organoid responds to a drug as compared to another (adjacent) small population of cells. For this, a highly efficient microscale separation technique coupled to a high end MS instrument is required in order to enable the analysis of nucleotides in low amounts of biological material.

CZE is a highly efficient microscale separation technique and as compounds are separated on the basis of their charge-to-size ratio, CZE is especially suited for the profiling of polar and charged compounds under biocompatible conditions [3].

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Abbreviations: **ADP**, adenosine diphosphate; **AEC**, adenylate energy charge; **AMP**, adenosine monophosphate; **ATP**, adenosine triphosphate; **cAMP**, cyclic AMP; **DMEM F-12**, DMEM/Nutrient Mixture F-12 Ham; **ISVF**, ionspray voltage floating; **LLE**, liquid–liquid extraction; **RMT**, relative migration times; **t-ITP**, transient-isotachophoresis

Color online: See article online to view Figs. 1–4 in color.

Moreover, CZE–MS is well-suited for the sensitive analysis of compounds in material- or volume-limited samples as only nanoliter injection volumes are required from just a few microliters of sample or less in the injection vial [4–8]. A number of recent studies have clearly shown the utility of CZE–MS for metabolic profiling of large sample sets. For example, Harada et al. assessed the long-term performance of CE–MS for metabolic profiling of >8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52 month period [9]. The study provided an absolute quantification for 94 polar metabolites in plasma with a reproducibility comparable to other analytical platforms, that is, reversed-phase LC–MS and GC–MS, commonly employed for large-scale metabolomics studies.

The first CZE–MS methods for the global profiling of polar and charged metabolites were developed by Soga and co-workers [10,11]. For nucleotides and other anionic metabolites, a positively charged capillary coating was employed in combination with reversed CE separation polarity to allow their relatively fast analysis by CZE–MS [10]. However, adsorption of compounds carrying multiple negative charges, such as ATP (adenosine triphosphate) and ADP (adenosine diphosphate), to the positively charged capillary wall was an issue. Moreover, a conventional sheath-liquid interface was employed for coupling CZE to MS and it was found that under these conditions the stainless steel ESI spray needle showed oxidation and corrosion due to electrolysis [12]. The resulting precipitation of iron oxides plugged the capillary outlet and shortened capillary lifetime. In addition, many of the anionic metabolites appeared to form complexes with iron oxides and nickel ions from the steel needle. The metal–metabolite complex formation caused ionization suppression and significantly reduced detection sensitivity for anionic metabolites. In 2009, this issue was resolved by the use of a platinum ESI needle, however, the adsorption of some nucleotides and other acidic metabolites to the positively charged capillary wall remained an issue [12]. A platinum ESI needle is not needed when analyzing anionic metabolites in normal CE polarity mode at high pH separation conditions and negative ESI–MS mode. However, the CZE effluent is significantly diluted in CZE–MS employing a conventional sheath-liquid interface, resulting in LODs around the low µM-range for nucleotides which may not be sufficient for their reliable analysis in low numbers of mammalian cells.

In order to enable the analysis of nucleotides in limited amounts of cells or in even a single cell, CZE has been coupled to nano-ESI–MS using custom-built low-flow sheath-liquid interfaces and detection in negative ion mode. For example, Liu et al. developed a CZE–MS method for the profiling of nucleotides in extracts of single neuronal cells from *Aplysia californica* using MS in negative ionization mode [13]. A modified coaxial sheath-liquid nanospray interface was used that had a smaller diameter capillary outlet, that is 40 µm instead of 75 µm internal diameter, thereby allowing to use a sheath-liquid flow rate of 600 nL/min. These modifications reduced sample dilution and improved detection limits. The interface used in this study was constructed with a microtee assembly containing a platinum alloy emitter in order to prevent corrosion. The method provided a good separation for 16 mono-, di-, and triphosphate nucleosides with LODs ranging from 2 to 22 nM using an injection volume of only 10 nL. Though low nanomolar detection sensitivities could be obtained for nucleotides under these conditions, a serious concern is corona discharge when employing low-flow separations in combination with nano-ESI–MS in negative ion mode [14]. This effect can be attenuated by the addition of organic solvents, nitrogen gas, oxygen or other reagents acting as electron scavengers. For example, Portero et al. complemented a home-made low-flow sheath-liquid interface design for CZE–MS with a nitrogen gas filled chamber to minimize electrical discharges and to obtain a stable ESI spray in the negative ion mode [15]. In order to avoid corona discharge, Dodbiba et al. analyzed nucleotides in the positive ion mode by employing different cationic ion-pairing reagents that associate with nucleotides resulting in overall positively charged complexes [16]. Under these conditions, improved LODs were obtained for most of the studied nucleotides, however, for the nucleotide triphosphate compounds, such as for example ATP, low LODs were more difficult to obtain with the employed cationic ion-pairing agents.

In the present work, our aim was to develop a microscale analytical platform for the highly sensitive and efficient profiling of nucleotides in low numbers of mammalian cells under nano-ESI conditions and to fully circumvent corona discharge. For this purpose, CZE was coupled to nano-ESI–MS via a sheathless porous tip interface, originally developed by Moini [17] and now commercially available as CESI (Sciex), and the detection of nucleotides was performed in the positive ion mode, whereas they were electrophoretically separated at high-pH separation conditions. It is shown that apart from nucleotides also some nicotinamide-adenine dinucleotides and amino acids could be analyzed in low number of mammalian cells under these conditions, thereby clearly illustrating the utility of this approach for metabolic profiling of low amounts of biological samples.

2 Materials and methods

2.1 Chemicals and reagents

Ammonium acetate (99%), Dulbecco's PBS, and DMEM/Nutrient Mixture F-12 Ham (DMEM F-12) were purchased from Sigma–Aldrich (St. Louis, USA). Ammonium bicarbonate (99.5%) was provided by Fluka (Steinheim,Germany). Ammonium carbonate was obtained from Scharlau (Barcelona, Spain). Sodium hydroxide (analytical grade) was acquired from Merck (Darmstadt, Germany). Hydrochloric acid (37% solution in water) and ammonia (28–30% NH_3 in water) were supplied by Across Organics (Geel, Belgium). Methanol (ultra LC–MS grade) was purchased from ACTU-ALL chemicals (Oss, the Netherlands). HPLC grade chloroform and MS grade acetic acid were provided by Biosolve Chemicals (Valkensweerd, the Netherlands). Water

used in this work was produced by a Milli-Q® Advantage A10 Water Purification System from Millipore. Fetal calf serum HC-20C (Biowest) was obtained from VWR (Amsterdam, the Netherlands). Penicillin–streptomycin (Pen/Strep, 100 mg/mL each) was supplied by Duchefa (Haarlem, the Netherlands). Centrifugal filters with 3 kDa cutoff membrane were also provided by Merck. Standards of nucleotides, including NMP, NDP, and NTP (N = A, U, C, G), cyclic AMP (cAMP, used as reference when calculating relative apparent electrophoretic mobility), and isotope-labeled nucleotides (used as internal standards), UMP (15N2), ATP (15N5), and GMP (15N5), were all acquired from Sigma–Aldrich.

2.2 Preparation of solutions

Solutions of ammonium acetate, ammonium bicarbonate, and ammonium carbonate were prepared by dissolving proper amounts of powder in corresponding volumes of water. The pH of each solution was then adjusted with 28–30% ammonia to desired values, including 8.5, 9.0, and 9.7. The concentration of all tested buffers was 12.5 mM at the start. Stock solutions of all the nucleotides were prepared individually by dissolving dry powders in a mixture of 95% water and 5% methanol. A mix of standards was then generated by mixing aforementioned solutions in a volumetric flask and the final concentration was 100 µM for every compound. This mixture was then divided over separate Eppendorf tubes and stored at −80 °C. The same preparation procedure was conducted for labeled ATP and GMP, and 40 µL of mixed internal standard (ISTD) solution (50 µg/mL) was divided over aliquots in clean Eppendorf tubes for single uses later.

2.3 Sample preparation

Prior to sample preparation, chloroform saturated with water and methanol was prepared by vigorously mixing chloroform, methanol, and water at a 1:1:1 v/v/v ratio and removing the upper layer after centrifugation. A series of working solutions were diluted from a 100 µM stock solution with water, covering the range of 3.9 nM to 2 µM for calibration curves. ISTD mixture was diluted with methanol to a final concentration of 400 ng/mL for each compound. Into clean Eppendorf tubes, 120 µL methanol, 30 µL ISTD solution, 100 µL water, 50 µL working solution (substituted with water for blanks), and 125 µL saturated chloroform were added. The final ratio of methanol/water/chloroform was 1:1:0.83 v/v/v in sample mixture. The mixtures were then vortexed for 1 min followed by centrifugation using 20 817 × *g* at 4 °C for 10 min. A total of 220 µL supernatant was then transferred to 500 µL Eppendorf tubes and placed in a Savant SC210A SpeedVac Concentrator (Thermo Scientific) for solvent evaporation at room temperature. Dried residues were reconstituted with 30 µL of ice-cold MeOH/H_2O (1:1, v/v) mixture, followed by vigorous vortex and centrifugation using 20 817 × *g* at 4°C for 15 min prior to analysis.

2.4 Cell lysate preparation

A total of 500 mL DMEM F12 was supplemented with 45 mL FCS and 1 mL Pen/Strep and used as the culture medium in this work. HepG2 cells were cultured and harvested in house. Harvested cells were counted with a TC10 Automated Cell Counter (Bio-Rad Laboratories) and live cell density arrived at 7.4 × 10^6 cells/mL. Five milliliter of prewarmed (37°C) culture medium was first added onto Petri dishes (60 mm, $n = 3$), and 135 µL of the obtained cell mixture (containing about 10^6 live cells) was gently pipetted into the medium right after the cell mixture was properly dispersed. The Petri dishes were then gently shaken to help distribute the cells evenly before incubation at 37°C in 95% air/5% CO_2. The Petri dishes were taken out of the incubator after all the cells had adhered to the bottom of the dishes (after roughly 7.5 h). The medium was then aspirated and 6 mL prewarmed (37°C) PBS was carefully added into each dish to wash away residual culture medium. The PBS was then removed and 1 mL ice-cold methanol/H_2O (80:20, v/v) mixture was added into every Petri dish to quench intracellular enzymatic reactions. The dishes were then moved onto ice and scraping was employed to get all the cells off the surface. Cell lysates were transferred into separate Eppendorf tubes and centrifuged using 14 000 RPM at 4°C for 10 min. The supernatant from each tube was then filtered with centrifugal filters with 3 kDa cutoffs and centrifuged using 10 000 × *g* at 4°C for 1.5 h. Filtered cell lysates were then transferred and stored at −80°C prior to further sample preparation. The cell lysate samples were processed in a similar manner as previously described [18]. The cell lysate obtained corresponded to a cell density of 50 000 cells/50 µL and was further diluted to 10 000, 5000, 2500, 1000, and 500 cells/50 µL with ice-cold methanol/water (80:20, v/v). Fifty microliter of every (diluted) cell lysate was transferred to clean Eppendorf tubes, followed by addition of methanol, water, ISTD solution, and saturated chloroform to reach the same final ratio as aforementioned. The rest was conducted exactly as previously stated.

2.5 CZE–MS analysis

Sheathless CZE–MS experiments were conducted employing a 30 µm i.d. × 91 cm bare fused-silica capillary, regulated at 25°C with recirculating liquid coolant, and coupled to a Sciex TripleTOF 6600 MS system via NanoSpray III source equipped with an XYZ stage. The porous tip of the sheathless capillary was positioned roughly 3 mm away from the MS inlet. ESI was performed in positive ionization mode and manual tuning was conducted after installation of the capillary to determine the optimal IonSpray Voltage Floating (ISVF) and curtain gas values. Different ISVF values ranging from 1600 to 1850 V were selected for different background electrolytes (BGEs) tested and curtain gas values varied between 10 and 15 psi. During the method validation process and cell lysate analysis, ISVF values were set between 1920 to 1960 V and curtain gas values between 12 and 14 psi. The values for gas

1, gas 2, interface heating temperature, and declustering potential were set at 0, 0, 50°C, and 50 V, respectively. An accumulation time of 0.25 s was used and the collision cell was set at 10.0 eV. MS data were recorded in the m/z range of 65–1000. The OptiMS cartridge was pre-conditioned and rinsed as described in our previous work. Sample tray was kept at 8°C. Samples were hydrodynamically injected into the capillary at 2 psi for 30 s, which corresponds to about 6.5 nL (i.e., 1% of the total capillary volume). A push plug of BGE injected at 5 psi for 60 s was introduced right after sample injection. Electrophoretic separation was performed in normal polarity mode by applying 30 kV to the CE inlet electrode and 1 psi forward pressure was applied during the separation. The selection of an optimal BGE was done by testing different BGEs in combination with nucleotides dissolved in water. The selection of optimal BGE was based on the separation resolution, peak shapes, intensities, and S/N ratios obtained for NTPs.

2.6 Determination of analytical performance characteristics

Calibration curves were generated by plotting peak area ratios (i.e., peak area of nucleotide divided by peak area of internal standard) against their corresponding concentrations employing 5, 10, 25, 50, 100, 250, 500, and 1000 nM as concentration for each nucleotide. Labeled UMP was used as internal standard at a fixed concentration of 200 nM. Values for the slope, intercept, and correlation coefficient were obtained by linear-regression analysis of the calibration curves. LODs were calculated as the concentrations providing a signal intensity equivalent to S/N of 3 based on an injected concentration of 5 or 10 nM (extracted ion electropherograms were used for this purpose). As LLE is required for the analysis of nucleotides in HepG2 cells, calibration curves were also constructed for nucleotide standard solutions in the concentration range as listed in Table 2 using LLE in combination with sheathless CZE–MS.

Accuracy was determined by comparing calculated concentrations of nucleotides with established calibration curves with the nominal concentrations using a concentration of 250 nM. Intra- and interday variation of relative migration time and peak area were determined by analyzing four replicates of a 250 nM nucleotide mixture, which were processed by LLE, within a day and on 3 consecutive days. Relative migration time (RMT) ratios were calculated using the migration times of corresponding internal standards. For determination of cellular energy status, adenylate energy charge (AEC) was calculated using obtained concentrations of AMP (adenosine monophosphate), ADP, and ATP, with the formula expressed as $\mathrm{AEC} = ([\mathrm{ATP}] + 0.5 \times [\mathrm{ADP}])/([\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}])$ [19].

In order to evaluate the repeatability of this method for profiling nucleotides in cell lysates, cell samples were prepared in triplicates for the cell lysate obtained from one Petri dish at 1000 and 2500 cells/50 µL, respectively, and cell lysates from the other two dishes were also diluted to the same cell densities and analyzed, which could reveal information regarding intra- and inter-dish variability.

3 Results and discussion

As outlined in Section 1, the profiling of anionic (i.e., acidic) metabolites by CZE–MS has only been considered by a few groups as it is a challenging endeavor to develop robust CZE–MS methods when employing ESI in negative ion mode in combination with high aqueous separation conditions. In this context, we have previously developed a CZE–MS method utilizing a sheathless porous tip interface, which was first developed by Moini [17], for the profiling of anionic metabolites at low-pH separation conditions in combination with ESI in negative ion mode [20]. Under these conditions we observed that corona discharge was minimal, while at high-pH separation conditions employing ammonium acetate with a low percentage of isopropanol as BGE corona discharge rapidly decreased the lifetime of a single porous tip emitter. Recently, Sarver et al. also reported on the detrimental effect of corona discharge when using borosilicate emitters in an electrokinetic sheath-liquid interface for coupling CE to MS in negative ion mode [21]. Though, corona discharge could be attenuated in our previously developed CE-MS method for anionic metabolic profiling, the employed low-pH separation conditions were not optimal for profiling acidic compounds, especially for the analysis of nucleoside triphosphates, such as ATP, GTP, and UTP, which were not detected. The chemical stability of these compounds at low-pH separation conditions may be limited and it was recently proposed by Siegel et al. that these compounds should be preferably analyzed at high pH (separation) conditions [2]. Given this context and our interest to profile nucleotides in low numbers of mammalian cells, the aim of this work was to develop a highly sensitive sheathless CE-MS method for nucleotide profiling at high-pH separation conditions using ESI-TOF-MS in the positive ion mode, thereby fully circumventing corona discharge.

3.1 CZE–MS method optimization for nucleotide analysis

Prior to assessing the performance of the sheathless CZE–MS method for the profiling of nucleotides, three types of volatile BGEs (selected on the basis of the examination of the literature), including ammonium acetate, ammonium carbonate, and ammonium bicarbonate, were tested first for the analysis of nucleotides by CZE–MS. As a starting point, 12.5 mM of each BGE using different pH values in the range from 8.5 to 10.5 was considered, followed by further optimization of the BGE concentration. All the nucleotides studied were negatively charged during the electrophoretic separation performed in normal polarity mode (i.e., anode at inlet side) with the different BGE conditions. Their detection by TOF-MS was performed in positive ion mode, thereby

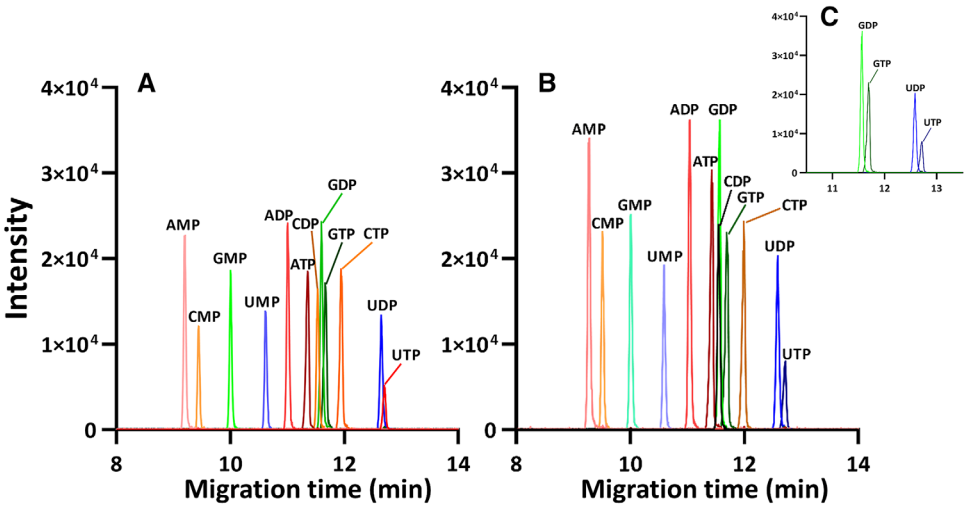

Figure 1. Multiple extracted ion electropherograms obtained for the analysis of a standard nucleotide mixture (100 nM) by sheathless CE-MS in positive ion mode. Separation conditions: BGE, 16 mM ammonium acetate (pH 9.7); Separation voltage: +30 kV; sample injection: 2.0 psi for 30 s. (A) Nucleotides dissolved in water; (B) Nucleotides dissolved in methanol:water (1:1, v/v); (C) Zoom-in of Figure 1B showing the (partial) separation for GDP/GTP and UDP/UTP.

fully circumventing corona discharge, by focusing on the signal intensity obtained for the protonated compounds. Optimization of the BGE conditions revealed that 16 mM ammonium acetate with a pH of 9.7 provided most optimal signal intensities, peak shapes, and resolution for the nucleotides with acceptable CE currents (~4.1 µA). A representative electropherogram for a 100 nM nucleotide standard mixture is shown in Fig. 1A, which clearly indicates that very narrow peaks were obtained by sheathless CZE–MS under the employed separation conditions. The most intense signal intensities were attained for protonated nucleotides ($[M+H]^+$), whereas very low signal intensities were observed for ammonium and/or sodium adducts.

By dissolving the nucleotides in methanol/water (1:1, v/v) instead of water only, and keeping the injection volume (about 6.5 nL) constant, slightly enhanced peak intensities and improved S/N ratios were obtained as a result of field-amplified sample stacking (Fig. 1B). Under the final optimal conditions, plate numbers obtained for the nucleotides ranged from 200 000 to 250 000. These values clearly indicate the high separation efficiency of the proposed sheathless CZE–MS method.

Recently, Yamamoto et al. have demonstrated that the use of alkaline aqueous ammonia solutions (with a pH above 9.0) as BGE leads to chemical degradation of the outer polyimide capillary coating, causing incidental capillary fractures [22]. In our approach, this is not an issue as the outer part of the capillary, which is the porous tip emitter, does not contain an outer polyimide layer.

3.2 Analytical performance evaluation

The performance of the sheathless CZE–MS method was evaluated by establishing calibration curves, LODs, migration time, and peak area precision. Eight-point response curves (based on extracted-ion peak area versus concentration) were established using working solutions with individual nucleotide concentrations in the range from 5 to 1000 nM. For all nucleotides, apart from UTP, a good linearity was observed with R^2 values above 0.994 (Supporting Information Table S1). For all analyzed nucleotides, LODs were calculated as the concentrations providing a signal intensity equivalent to S/N of 3 based on an injected concentration of 5 (or 10) nM. The LODs ranged from 0.06 to 1.33 nM, thereby clearly indicating that the proposed sheathless CZE–MS method provides very high sensitivity for nucleotides, allowing detection down to 0.4 to 8.6 amol of injected amount. To our knowledge, these are the lowest LOD values reported for nucleotides by CZE–MS so far. When compared to the state-of-the-art ion-pair nanoscale RP-LC–MS method developed for profiling nucleotides and other anionic metabolites [23], which provided absolute LOD values of 100, 250, and 750 amol for AMP, ADP, and ATP, respectively, our method showed an improvement in LOD of 19-, 104-, and 326-fold, respectively, for these compounds. More recently developed ion-pair reversed-phase LC–MS methods yielded LODs in the range from 1 to 10 nM for AMP, ADP, and ATP using multiple reaction monitoring and an injection volume of 5 µL [24, 25]. HILIC–MS-based approaches recently developed for nucleotide profiling in various biological samples provided LODs typically in the range from 2 to 100 nM using MS/MS mode and employing an injection volume of 5 µL [26–28]. In this context, the proposed sheathless CZE–MS method (used in full scan MS mode only) provided concentration LODs for the studied nucleotides that are comparable to or better than LODs obtained by LC–MS-based approaches employing multiple reaction monitoring. It is anticipated that lower LODs can be obtained by sheathless CZE–MS by also using MS/MS and/or by injecting more using in-capillary preconcentration techniques. When considering the obtained LODs in terms of absolute amount injected, it is obvious that the proposed sheathless CZE–MS method has very promising characteristics for the sensitive profiling of nucleotides in biomass-limited samples.

For the analysis of nucleotides in HepG2 cells by sheathless CZE–MS, an LLE step is required to selectively remove

Table 1. Analytical performance characteristics of the optimized sheathless CZE–MS method including LLE for the analysis of nucleotides

Nucleotides	Linear range (nM)	Slope ($n = 3$) (Mean ± SD%)	R^2 ($n = 3$) (Mean ± SD%)	LOD (nM)	LOQ (nM)	Accuracy ($n = 4$) (Mean ± SD%)	Matrix effect ($n = 4$) (Mean ± SD%)
ADP	3.9 - 2000	1.804 ± 0.055	0.9970 ± 0.0033	0.2	0.5	95.2 ± 2.4	93.6 ± 6.4
AMP	3.9 - 2000	3.424 ± 0.203	0.9943 ± 0.0021	0.5	1.8	100.8 ± 2.9	92.0 ± 8.1
ATP	3.9 - 2000	1.897 ± 0.022	0.9994 ± 0.0006	0.3	1.1	98.4 ± 1.5	93.9 ± 6.2
CDP	3.9 - 2000	1.224 ± 0.016	0.9972 ± 0.0025	0.7	2.4	97.9 ± 2.0	96.4 ± 7.8
CMP	15.6 - 2000	2.064 ± 0.259	0.9952 ± 0.0026	0.5	1.6	102.0 ± 2.9	90.9 ± 8.6
CTP	3.9 - 2000	1.503 ± 0.201	0.9978 ± 0.0007	0.1	0.5	102.4± 4.3	94.7 ± 9.8
GDP	3.9 - 2000	1.565 ± 0.234	0.9960 ± 0.0034	0.4	1.4	96.1 ± 2.3	91.9 ± 10.0
GMP	3.9 - 2000	2.503 ± 0.221	0.9995 ± 0.0005	0.4	1.2	94.1 ± 1.2	92.2 ± 6.9
GTP	3.9 - 2000	1.416 ± 0.115	0.9966 ± 0.0029	0.7	2.4	90.7 ± 0.8	94.9 ± 6.3
UDP	7.8 - 2000	1.026 ± 0.035	0.9977 ± 0.0011	0.5	1.6	99.5 ± 1.1	92.9 ± 5.2
UMP	3.9 - 2000	2.156 ± 0.170	0.9976 ± 0.0019	0.9	3.0	99.1 ± 3.5	96.3 ± 8.0
UTP	7.8 - 2000	0.472 ± 0.011	0.9988 ± 0.0009	0.2	0.5	99.1 ± 7.2	95.1 ± 4.7
ATP 15N5	NA	NA	NA	NA	NA	NA	NA
GMP 15N5	NA	NA	NA	NA	NA	NA	NA

Table 2. Precision data (RSD, %) obtained for migration time and peak area of a 250 nM nucleotide mixture by sheathless CZE–MS in positive ion mode

Nucleotides	Intra-day MT (RSD,%) ($n = 4$)	Intra-day peak area (RSD, %) ($n = 4$)	Inter-day MT (RSD, %) ($n = 12$)	RMT (mean) ($n = 12$)	Inter-day RMT ratio (RSD,%) ($n = 12$)	Inter-day peak area (RSD,%) ($n = 12$)	Inter-day peak area ratio (RSD,%) ($n = 12$)
ADP	1.78	7.04	5.82	0.91	0.68	8.45	5.96
AMP*	1.50	5.25	4.77	0.99	0.53	12.80	4.37
ATP	1.91	7.22	6.43	1.00	0.00	6.98	3.46
CDP	1.87	8.40	6.10	0.96	0.46	8.36	5.24
CMP*	1.51	5.10	4.90	1.02	0.65	12.16	5.76
CTP	1.99	7.05	6.75	1.06	0.32	8.14	5.38
GDP	1.59	8.05	5.32	0.90	1.10	9.65	6.72
GMP*	1.28	4.87	4.23	1.00	0.01	14.64	2.59
GTP	1.78	7.91	6.01	0.98	0.41	8.23	1.66
UDP	1.72	7.20	5.70	0.99	0.74	7.28	3.74
UMP*	1.34	6.16	4.39	1.06	0.16	10.42	7.41
UTP	1.89	6.84	6.48	1.09	0.15	9.05	9.44
ATP 15N5	1.91	7.67	6.42	NA	NA	8.64	NA
GMP 15N5	1.29	4.57	4.24	NA	NA	16.51	NA

*Peak areas and migration times of these nucleotides were normalized with isotope-labeled GMP 15N5, whereas the other compounds were normalized with isotope-labeled ATP 15N5 for determining the inter-day RMT and peak area ratio.

proteins and apolar compounds from the highly polar and charged metabolites in the cell lysate. As sample preparation is often the most crucial step in the entire analytical workflow, we have determined the analytical performance of the sheathless CZE–MS method with LLE included. Table 1 gives an overview of the analytical performance characteristics showing that excellent linearity was observed for all nucleotides with coefficients of determination (R^2) between 0.9943 and 0.9993 over the tested linear ranges. The LODs and LOQs ranged, respectively, from 0.1 to 0.9 nM and from 0.5 to 3.0 nM (Table 1), indicating the strong potential of the method to profile nucleotides in low numbers of mammalian cells, but also to potentially profile these compounds in human plasma samples [26].

Precision of the sheathless CZE–MS method for nucleotide analysis was assessed based on the repeated analyses of samples prepared at the intermediate concentration level (250 nM). Intraday RSD values ($n = 4$) for peak areas and migration times of all the analytes were better than 8.40 and 1.99%, respectively, while interday RSDs ($n = 12$) were below 14.64 and 6.75%, respectively (Table 2). By using RMT ratios instead of migration times, interday RSD values for RMTs were below 1.10%. Peak area ratios were calculated for all nucleotides with interday RSD values lower than 9.5%

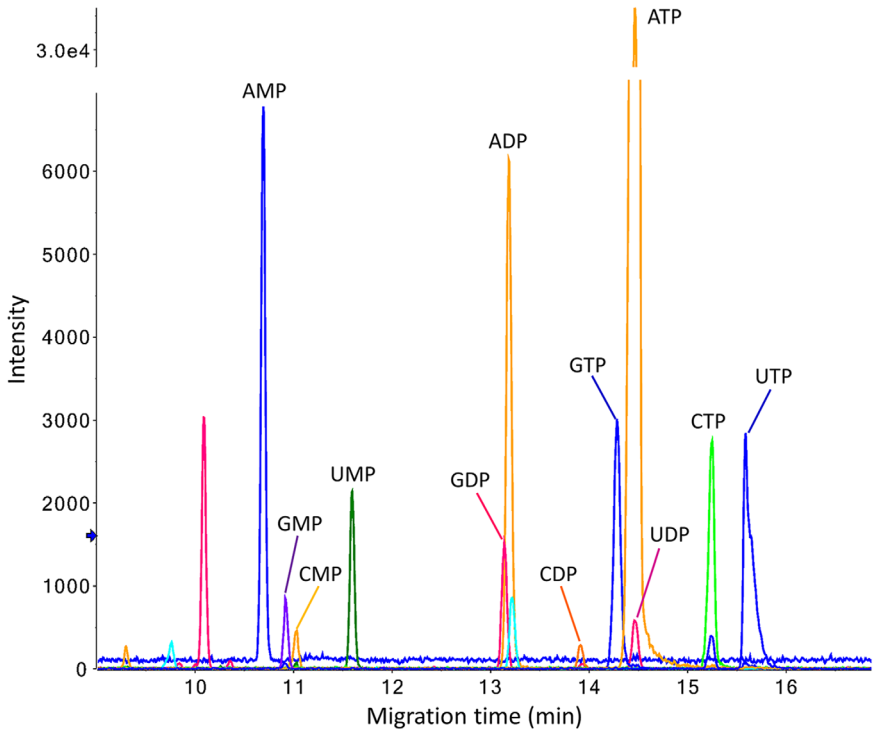

Figure 2. Reconstructed ion electropherograms obtained for the analysis of nucleotides in an extract from 10 000 HepG2 cells as starting material by sheathless CZE–MS in positive ion mode using a porous tip emitter. Separation conditions: BGE, 16 mM ammonium acetate (pH 9.7); Separation voltage: 30 kV; sample injection: 2.0 psi for 30 s.

(Table 2). Even without internal standard correction, acceptable RSD values (i.e., below 14.64%) for interday peak areas were obtained by the sheathless CZE–MS method. Next, accuracy was investigated for the proposed method by back calculating the concentration of nucleotides spiked to the cell extract with established calibration curves and comparing the calculated and nominal concentrations (250 nM). The accuracy for every nucleotide included was shown to be between 90.7 and 102.4% (Table 1). In order to study the matrix effect, a HepG2 sample matrix extracted from 5000 cells ($n = 4$) was prepared in which subsequently the responses (peak areas) obtained for post-extraction spiked nucleotides (and corrected for endogenous nucleotides) were compared with the responses obtained for standards. As shown in Table 1, a marginal to negligible matrix effect was observed for all the nucleotides, which is of crucial importance when analyzing nucleotides present at trace levels in biological samples.

3.3 Profiling nucleotides in low numbers of mammalian cells

The applicability of this method for profiling nucleotides in low numbers of mammalian cells was evaluated using HepG2 cells as a model system. For this, a serial dilution of cell lysate with a methanol/water (8:2, v/v) mixture was performed, yielding a sample range from 50 000 to 500 HepG2 cells per 50 µL, in which then the nucleotides were analyzed by the proposed sheathless CZE–MS method. Figure 2 shows a representative electropherogram obtained by sheathless CZE–MS for the analysis of nucleotides in an extract from 10 000 HepG2 cells.

Figure 3A shows reconstructed electropherograms for seven nucleotides that could be observed in 500 HepG2 cells only under these conditions. Figure 3B shows that a linear detector response was obtained for endogenous ATP concentrations when going from 500 to 50 000 HepG2 cells as starting amount. Throughout the analysis of cell lysates at different cell densities, no sign of degradation or in-source fragmentation was observed for compounds like ATP and ADP. This is important for quantitative studies, as ATP/ADP ratios are used for the determination of the adenylate energy charge (AEC), which is a measure of chemical energy available for metabolic processes. AEC is a common key feature to all cellular organisms and maintains a value between 0.7 and 0.95 in most cell types grown under optimal conditions. The calculated AEC values were between 0.72 and 0.85 for all different cell content concentrations in our work, indicating that the proposed method can be a useful tool in assessing AEC values in biomedical/clinical studies intrinsically dealing with low amounts of mammalian cells. Actually, we would like to propose AEC as an additional metric for evaluating the reliability of the sampling and sample preparation process when working with low amounts of cells.

The repeatability of the method for profiling nucleotides in biological samples was demonstrated by analyzing triplicates from the same cell lysate and comparing cell lysate aliquots from different culture dishes using 1000 and 2500 cells as starting amounts in the analytical procedure. Table 3 clearly indicates that good repeatability could be obtained using both 1000 and 2500 HepG2 cells with intra-dish CV

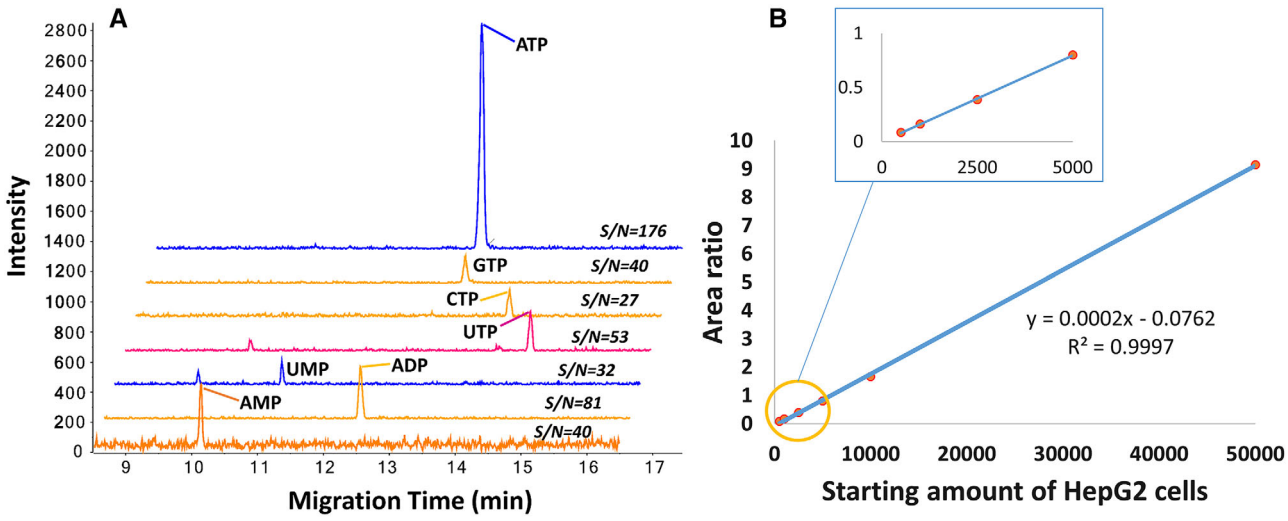

Figure 3. (A) Multiple extracted ion electropherograms obtained for nucleotides in an extract from 500 HepG2 cells by sheathless CZE–MS in positive ion mode. Separation conditions: BGE, 16 mM ammonium acetate (pH 9.7); Separation voltage: 30 kV; sample injection: 2.0 psi for 30 s. (B) Scatter plot generated by plotting the area ratios of endogenous ATP to labeled ATP (ISTD) against corresponding starting numbers of HepG2 cells.

Table 3. Concentrations determined for some nucleotides in extracts from 1000 and 2500 HepG2 cells by sheathless CZE–MS. Intra-dish refers to analyzing nucleotide concentrations from the same cell lysate extract, whereas inter-dish refers to analyzing concentrations from three different culture dishes

	1000 HepG2 cells				2500 HepG2 cells			
	Intra-dish ($n = 3$)		Inter-dish ($n = 3$)		Intra-dish ($n = 3$)		Inter-dish ($n = 3$)	
Nucleotides	Concentration (nM)	Precision (RSD, %)	Concentration (nM)	Precision (RSD, %)	Concentration (nM)	Precision (RSD, %)	Concentration (nM)	Precision (RSD, %)
AMP	13.9	11.4	15.9	10.6	32.5	11.6	34.4	6.9
ADP	13.8	1.8	15.7	11.8	37.2	9.8	41.4	13.4
ATP	87.5	0.6	85.5	3.0	228.1	0.2	219.1	3.9
CTP	9.2	7.5	8.5	6.6	22.5	3.4	21.9	3.4
GTP	14.9	4.5	13.9	3.5	29.4	10.3	29.6	19.9
UMP	4.5	10.7	3.5	21.2	10.6	9.1	9.5	20.0
UTP	41.2	5.1	39.9	8.2	105.6	3.9	101.3	8.3

*Only seven of the 12 nucleotides that were used for method evaluation could be detected in extracts from 1000 and 2500 HepG2 cells

values of no more than 11.4 and 11.6%, respectively. However, for the comparison between different dishes cultured at the same time, overall greater variation was observed for the evaluated metabolites, notably for low abundant metabolites (Table 3).

The detection sensitivity of the proposed sheathless CZE–MS method can be further improved by using in-capillary preconcentration techniques, such as for example transient-isotachophoresis (t-ITP). When using 2 M acetic acid as a leading electrolyte and an injection volume of about 39 nL, improved peak intensities and S/N ratios of nucleotides in an extract from 500 HepG2 cells, as shown for AMP, ADP, and ATP, were obtained (Fig. 4B compared to Fig. 4A). The increase in S/N ratios obtained for AMP, ADP, and ATP was 5.75-, 3.71-, and 6.21-folds, respectively, as compared to the results obtained for these compounds using standard injection. Therefore, the implementation of t-ITP in sheathless CZE–MS may be considered for the detection of nucleotides present at trace levels in very low amounts of biological material.

To assess whether the proposed method could be used for the analysis of other compounds beyond nucleotides, more features were extracted and provisionally annotated with standards or theoretical *m/z* values using the data obtained for 10 000 HepG2 cells as starting material (Supporting Information Fig. S1). It was noticed that amino acids, such as proline, glutamic acid, and aspartic acid, showed satisfactory peak shapes with good intensities. Other key metabolites, such as NAD+, NADH, and FAD, were also detected though with a relatively low detector response. Further optimization of the sheathless CZE–MS method is needed in order to improve the detection sensitivity for the latter compounds.

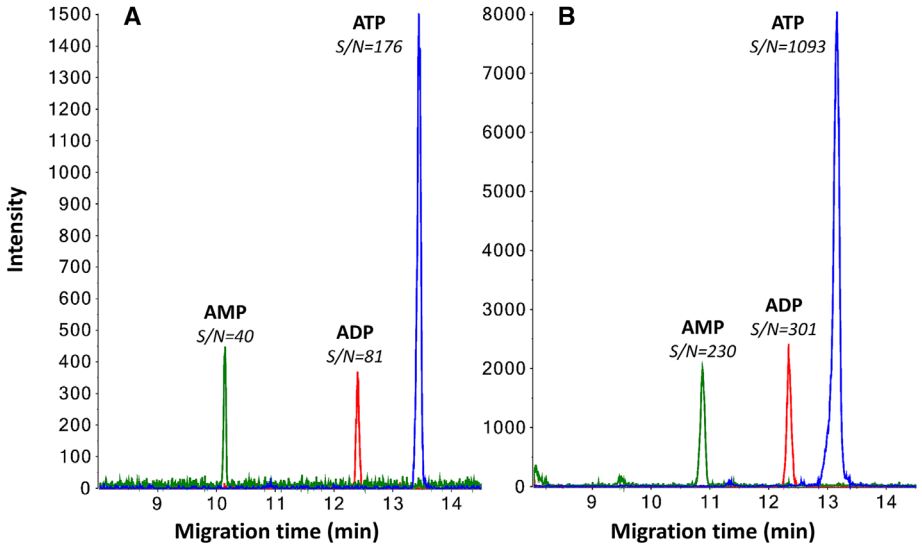

Figure 4. Peaks for AMP, ADP, and ATP obtained from the analysis of 500 HepG2 cells as starting material by sheathless CZE–MS in positive ion mode using a porous tip emitter. Separation conditions: BGE, 16 mM ammonium acetate (pH 9.7); Separation voltage: 30 kV; (A) Sample injection: 2.0 psi for 30 s. (B) 2 M acetic acid as the leading electrolyte. Sample injection: 6.0 psi for 60 s using t-ITP for in-capillary preconcentration.

4 Concluding remarks

In this work, we have developed a sheathless CZE–MS method for the highly sensitive and efficient profiling of nucleotides in low numbers of mammalian cells. To fully circumvent corona discharge, which is typically observed in nano-ESI employing negative ion mode detection for acidic compounds, nucleotides were analyzed in the positive ion mode. Sub-nanomolar detection limits were obtained for the nucleotides by sheathless CZE–MS by using an injection volume of only about 6.5 nL, corresponding to low attomoles injected into the capillary. Compared to other state-of-the-art methods developed for nucleotide profiling, the proposed method provided the lowest LOD values in terms of absolute amounts and clearly shows the value of the approach for analyzing these compounds in low amounts of biological material, as exemplified here for nucleotide profiling in extracts from 50 000 to 500 HepG2 cells only. The detection sensitivity of the method for nucleotide profiling in extracts from HepG2 cells can be further improved by reconstituting the dried extract in 5 µL instead of 30 µL using microvials. In order to apply the proposed method for comparative metabolic profiling studies, a more thorough validation is needed. An aspect not studied in detail in the present work is the injection technique for material-limited samples, that is, how to get the relevant fraction/compounds of the sample effectively into the CE system. In this context, miniaturization and optimization of injection techniques and sample preparation will be crucial for material-limited metabolomics studies.

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The authors have declared no conflict of interest.

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