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Coupling miniaturized free-flow electrophoresis to mass spectrometry via a multi-emitter ESI interface†

Matthias Jender,[a‡] Stefan Höving,[a‡¶] Pedro Novo,[a] Erik Freier,[a] and Dirk Janasek*[a]

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We present a novel multi-emitter electrospray ionization interface for the coupling of microfluidic free-flow electrophoresis (μFFE) with mass spectrometry. 15 sample streams coming from 15 μFFE outlets were continuously analyzed in quick succession to monitor the electrophoretic separation in the microchip.

1 Introduction

Miniaturization of benchtop instruments in form of microfluidic devices offers a great opportunity for the handling, manipulation, preparation and analysis of minute, precious samples using these devices in *e.g.* point-of-care diagnostics[1], drug delivery[2], single cell analysis[3] or PCR tests[4]. Also the fractionation of analyte mixtures as up-stream or down-stream processing in complex workflows can be done in microfluidic devices. An exellent tool for conducting such preparative fractionations is free-flow electrophoresis (FFE). In FFE, the sample is continuously introduced into a separation bed. An electric field inducing the electrophoretic movement of charged species is applied perpendicular to the hydrodynamic flow through the separation bed. Thus, the charged species are affected by two orthogonally acting velocity vectors causing a deflection of the species from the hydrodynamic path, which in consequence leads to a geometrical two-dimensional separation of the sample mixture. At the end of the separation bed the fractionated analytes can be collected from several distinct outlets.[5] All separation modes known from capillary electrophoresis (CE) can be applied to FFE.[6–8] A miniaturized version of FFE (μFFE) has been reported for the first time by Raymond et al. in 1994.[9] They demonstrated that μFFE accomplishes all characteristics predicted by the scaling laws[10–12], and therefore could be used as a fast preparative and analytical instrument.[13–15] Since its first appearance, i) the design of μFFE has been optimized to improve the performance, ii) the known separation modes have been adapted to μFFE to widen the application field, and iii) several detection methods had been implemented to be more independent of analyte properties.[16–18] While optical detection of fluorescent species was the first choice due to its simplicity[9,11,19,20] other detection schemes like surface enhanced Raman scattering (SERS)[21] and hyphenation to mass spectrometry (MS) via electrospray ionization (ESI)[22,23] have been successfully demonstrated. Mass spectrometry is a well-known and well-established detection method for the sensitive and specific detection of a vast number of analytes in the (bio)chemical field. The hyphenation of FFE is not trivial since the 2D-separated samples have to be introduced successively into the mass spectrometer. Benz *et al.*[22] and Park *et al.*[23] used hydrodynamic flow rate alterations of the background electrolyte to steer electrophoretic bands sequentially to a single μFFE outlet connected to the MS. This method allowed the subsequent assessment of the electrophoretic bands' relative positions. However, the timely preparative collection of samples in the process was not feasible. Jender *et al.* demonstrated a setup where it was possible to analyze samples from multiple μFFE outlets via MS while maintaining the separation unaltered. Outlet channels were successively switched to the ESI emitter with the help of a multiposition valve. The disadvantage was the dead volume that needed to be replaced after each switching and thus slowed down the analysis[24].

An alternative to analyzing different samples with a single ESI emitter is the use of a multi-emitter. In literature, several multi-emitters, sometimes called multi-nozzle emitters or multi-sprayers, have been presented. The purpose of most studies was, however, an increase in sample throughput and an improved signal-to-noise ratio (SNR) by using multiple closely positioned emitters in parallel[25–27]. In other studies multi-emitters were used to analyze different samples by moving individual emitter tips in front of the MS inlet successively[28–30]. These setups either required finely tuned motorized components for the emitter positioning or the manual movement of the device. Other research groups presented multi-emitter systems where switching of the electrospray among two[31] or three[32] stationary emitters was possible. Via the targeted application of high-voltage, individual

[a] *Leibniz-Institut für Analytische Wissenschaften – ISAS – e.V., Bunsen-Kirchhoff-Str. 11, 44139 Dortmund, Germany. Tel: +49 231 1392-202; Fax: +49 231 1392-120; E-mail: dirk.janasek@isas.de*

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‡ These authors contributed equally to this work.

¶ Present address: TU Dortmund University, Laboratory of Equipment Design, Emil-Figge-Str. 68, 44227 Dortmund, Germany.

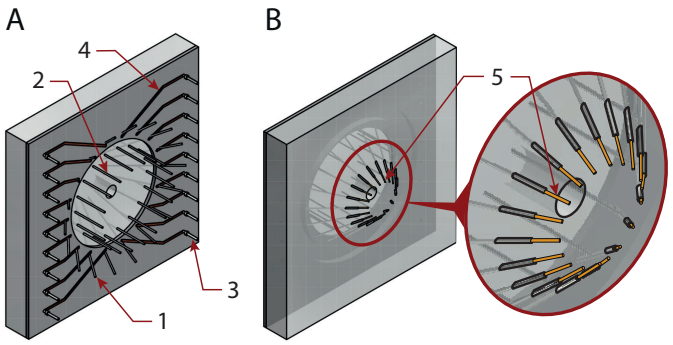

Fig. 1 Drawing of the multi-emitter interface from the (A) backside and (B) frontside. 1 – Fluidic ports for samples from μFFE outlets; 2 – Fluidic ports for make-up flow; 3 – Connector pins for high-voltage; 4 – Conductive traces for voltage feeding; 5 – FS capillary emitters fixed in steel capillaries.

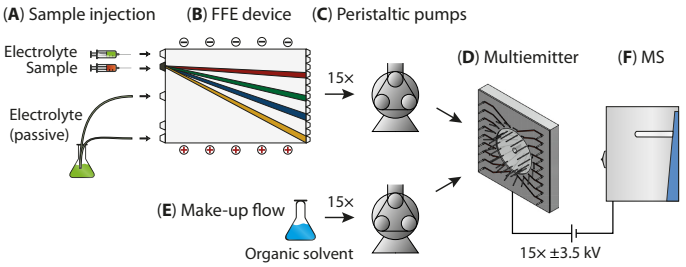

Fig. 2 Experimental setup of μFFE-MS with a multi-emitter.

emitters could electrospray different samples in quick succession, the switching speed being only limited by the scanning times of the mass spectrometer. In this work we adopted the principle of a stationary multi-emitter, but were able to increase the number of electrosprayed channels to 15 to perform a qualitative sample analysis of a directly coupled microfluidic FFE device. Thus, we were able to analyze a high-resolution μFFE separation within seconds by means of MS.

2 Experimental

2.1 Reagents

All solutions were prepared using ultrapure (type I) water (18.2 MΩ, Purelab Flex, ELGA LabWater, Lane End, UK). The μFFE background electrolyte solution contained 5 mM ammonium acetate and the electrode reservoir electrolyte solution 100 mM ammonium acetate, each at pH 7.7. Stock solutions of AMP disodium salt, ATP disodium salt hydrate, citric acid, UMP disodium salt (all purchased from Sigma-Aldrich, Steinheim, Germany) and CoA trilithium salt (purchased from Biomol, Hamburg, Germany) were prepared in water and stored at −80 °C. Sample solutions were prepared by diluting and mixing the stock solutions in water to their final concentrations. 2-propanol was ULC/MS grade and purchased from Biosolve BV (Valkenswaard, Netherlands).

2.2 Fabrication of multi-emitter interface and μFFE device

The inhouse fabricated multi-emitter interface, shown in Figure 1, provided 18 slots for the parallel connection of samples. Each slot contained a fluidic port for the sample (1), a fluidic port for make-up flow (2) and a connector for high-voltage supply (3). Attached to a circuit board on the multi-emitter backside, the connectors were electrically connected to the fluidic ports via conductive traces. The fluidic ports consisted of steel capillaries (0.41 mm ID, 0.71 mm OD, Vieweg, Germany) plugged into drilled channels of the multi-emitter's polycarbonate body. The two fluidic channels of a respective slot on the backside were merged into an emitter channel leading to the frontside of the interface (Figure 1 B). On this side, facing the MS inlet, steel capillaries were plugged into the 18 emitter channels, holding fused-silica (FS) capillaries (75 μm ID, 360 μm OD, Chromatographie Handel Müller, Fridolfing, GER) as ESI-emitters in place. The capillaries were arranged in a circle, tilted 45° towards its center and pointed towards the MS. In this arrangement, all emitter tips had the same distance and geometrical orientation toward the MS inlet. FS emitter capillaries were fixed in the steel capillaries via short pieces of Tygon tubing (250 μm ID).

The μFFE chip used for the separation of samples was designed and fabricated as described in our recent publication[24]. In short, the device was made from medical grade double-sided adhesive tape (9965, 3M, Saint Paul, USA) and poly(methyl methacrylate) (PMMA) plates by lamination. The microfluidic structures were cut into the adhesive tape and afterwards sandwiched between the PMMA plates. The adhesive tape's thickness of 90 μm defined the microstructures' height. Electrodes were embedded in reservoirs and separated from the separation bed by laminated polycarbonate membranes (0.1 μm pore size) to prevent the entry of bubbles generated at the electrodes.

2.3 Experimental setup

The experimental setup was based on our previous publication, where the μFFE-MS coupling (based on a multiposition valve and a single ESI emitter) and analytical details are described in more detail[24]. The illustration in Figure 2 gives an overview of how the devices were arranged for the experiments herein. Sample and part of the electrolyte were injected with a syringe pump (neMESYS B002-01, Cetoni, GER) (A), the remaining electrolyte was passively drawn into the μFFE device (B) by the negative pressure the peristaltic pump (Ismatec ISM939D, Cole-Parmer, USA) (C) created at the μFFE outlets. The syringe pumps ensured a precise control of the flow, while the peristaltic pump guaranteed equal flow rates at all 15 outlets and thus a stable separation. In the multi-emitter device (D) sample was combined with a make-up flow (E) to ensure a stable electrospray. The multi-emitter was positioned in front of a linear ion trap MS (LTQ XL, Thermo Fisher Scientific, USA) (F) with the help of a custom-made 3D-printed mount, the emitter tips being about 5 to 10 mm apart from the MS inlet.

An inhouse fabricated 15-position high-voltage generator capable of delivering 3.5 kV (negative or positive) was used to apply potential to the individual emitters for 0–999 ms, followed by an arbitrary down time of 0–999 ms before automatically switching to the next emitter. With cycle times in the millisecond range, all samples coming from the 15 μFFE outlets could be analyzed by MS within seconds.

2.4 FFE separation and MS detection

With the experimental setup described above, we performed an exemplary separation of an analyte mixture with online MS detection. The compounds for the separation were AMP, ATP, Coenzyme A (CoA) and citric acid, important players of cellular respiration. A sample solution containing 100 µM of each analyte was injected into the µFFE with a volume flow rate of 2 $\mu L\,min^{-1}$. The peristaltic pump, set at 3 $\mu L\,min^{-1}$ per channel, created an overall flow rate of 45 $\mu L\,min^{-1}$. The background electrolyte, suitable for the FFE separation and for ESI-MS, was 5 µM ammonium acetate. 100 µM ammonium acetate was used to continuously flush the electrode reservoirs. At the multi-emitter the sample solution coming from the µFFE device was complemented by a make-up flow (3.5 $\mu L\,min^{-1}$ per channel) consisting of 60 % 2-propanol to improve the electrospray. 100 µM uridine monophosphate (UMP) was added to the make-up flow for one of the channels as a reference mark. We arbitrarily chose µFFE outlet channel #2. The reference sprayed from emitter #2 helped to allocate the continuously acquired mass spectra to the respective emitters. High-voltage of 3.5 kV was applied to individual channels for 210 ms each, followed by a downtime of 210 ms. During an uptime 2–3 mass spectra were acquired. The downtime was added to prevent two channels from being analyzed within one scan. Also, the blank spectra acquired during downtimes helped to identify the switching to the next channel during data evaluation. The time to analyze all 15 channels amounted to 6.3 s $(15 \times (210\ ms + 210\ ms))$.

We conducted µFFE-MS experiments at different separation conditions to prove the functionality of both the electrophoretic separation and the multi-emitter device. We started by measuring (Xcalibur, Thermo Scientific) all channels without a separation voltage applied. After a few seconds, 100 V were applied to the µFFE device while continuing to acquire mass spectra. In additional experiments we measured the separation at 150 V and 200 V. During measurements the extracted ion chromatogramms (EICs) of the analytes of interest were generated (Xcalibur Qual Browser, Thermo Scientific). From these data, we created heatmaps displaying signal intensities of the respective analyte in the 15 channels over time.

3 Results and Discussion

The EIC from the first experiments is shown in Figure 3. In the beginning the peaks appeared regularly all 6.3 s, which corresponds to the time required to cycle through all channels. The presence of all analytes together in one channel led to ion suppression and thus smaller signals for AMP, CoA and ATP. Around minute 2 the applied separation voltage came into effect causing peaks to shift. After a transition time the separation became stable, as the regularly occurring peaks indicate. The increase in peak frequency indicates that analytes were spread to multiple outlets. For further evaluation the data points from the EIC were allocated to their respective µFFE outlet channels using spreadsheet software (Microsoft Excel). The results are displayed in heatmaps in Figure 4. The heatmaps of the 100 V separation show the distribution of the analytes over the 15 channels during the experiment. Before being affected by the electric field AMP, CoA, ATP and citric acid

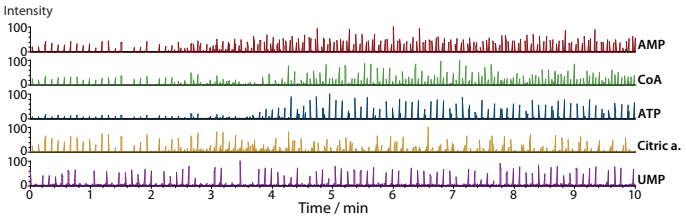

Fig. 3 EIC of the substances acquired with the multi-emitter during the 100 V separation in µFFE. The intensities of the analytes AMP (red), CoA (green), ATP (blue), citric acid (yellow) and the reference marker UMP (purple) are plotted over 10 minutes. Noticeable is the appearance of regular peak patterns which are the result of the cyclic switching of the multi-emitter. Absent of an electric field, analytes are not separated initially and thus come through the same outlet channel in beginning with a peak every 6.3 s. The signal for UMP also appears every 6.3 s, but with an offset relative to the other signals since it is in another channel. After 30 s the separation voltage of 100 V is applied. With the electric field active, AMP, CoA, ATP and citric acid start to migrate to different outlet channels between minutes 2-4, which is indicated by additional irregularly appearing peaks. Around minute 4 the peak pattern becomes stable again. The increase in the number of peaks shows that an analyte is spread to multiple outlets in the electric field. Peak counts for AMP and CoA, for example, are double.

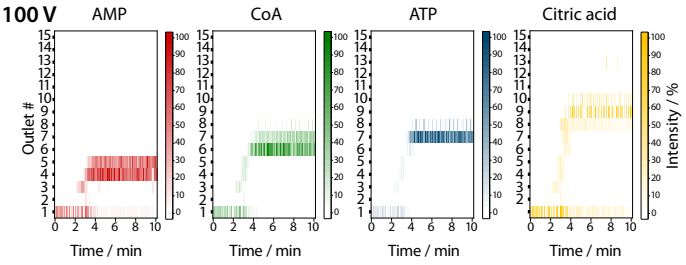

Fig. 4 Heatmaps of the analytes separated in µFFE at 100 V. Each heatmap shows the distribution of an analyte over the 15 µFFE outlet channels during the course of the measurement. Absent of an eletric field, all analytes are in outlet channel #1 in the first 2 minutes. Then the electrophoretic migration starts and the signals shift to other channels. The separation is stable after roughly 4 minutes. AMP (red) is shifted to outlets #4 and #5, CoA (green) to #6 and #7, ATP (blue) mostly to #7 and citric acid (yellow) to #8, #9 and #10. Outlet channel #1 shows extended signals after the migration of the analytes, which is probably caused by diffusion from dead volumes at the multi-emitter oder the µFFE outlets.

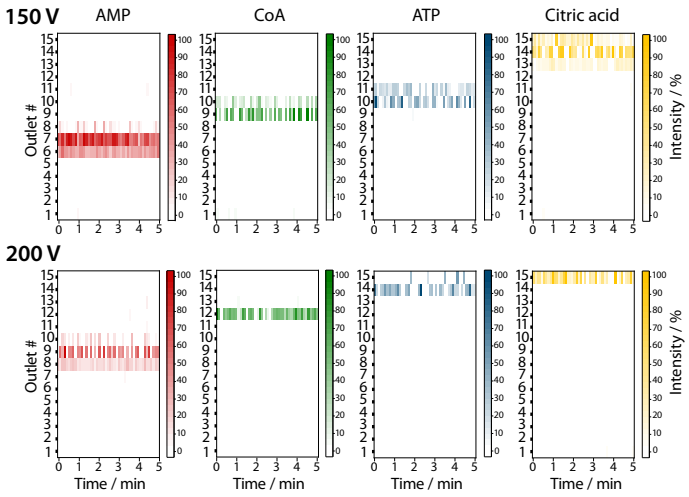

Fig. 5 Heatmaps of the analytes separated in μFFE at 150 V and 200 V. Each heatmap shows the distribution of an analyte over the 15 μFFE outlet channels during the course of the measurement.

were detected at outlet #1. In the electric field the analytes were deflected and spread out between outlets #4 and #10 as a result. However, diminishing signals of AMP and citric were still present for some minutes at outlet #1. This was most likely caused by diffusion from dead volumes at the μFFE outlets or the multi-emitter. By increasing the separation voltage to 150 V and 200 V an increased electrophoretic migration was expected. In contrast to the 100 V-experiment, the measurements were only started with the separation being already in a stable state. The results are shown in heatmaps in Figure 5. All analytes migrated further due to the increase in field strength. At 200 V, with citric acid being already at the lateral end of the separation bed, all analytes were separated from each other without significant overlap.

In all measurements, gaps were observed in the mass spectra (missing peaks) and the resulting heatmaps (white spaces). The gaps can be attributed to electrospray interruptions due to droplets accumulating at the emitter tips during the 6.3 s cycle time. These droplets did not always fly off when high-voltage was applied and thus kept growing until they got into contact with each other forming short circuits and preventing the electrospray from ignition. Faster cycle times, lower flow rates or higher voltages could solve this problem in the future.

4 Conclusions

The integration of a μFFE device with multi-emitter-MS was shown for the first time. The multi-emitter proved to be a useful tool for the qualitative measurements of multiple parallel channels. With cycle times of a few seconds the whole set of 15 samples was analyzed continuously, which enabled the monitoring of varying sample compositions over time. Compared to previously presented μFFE-MS-setups, a faster and higher resolved separation was possible.

For further study some measures can be taken to improve the presented setup. (i) With the setup described here, it is possible to analyze the fractions in quick succession without flow rate alterations. However, in order to collect the analytes preparatively, the integration of an actuator into the μFFE chip would allow the fast switching between preparative collection and detection. ii) An alternative to the aforementioned measures to solve the issue of merging droplets at the emitter tips could be the employment of dielectric barrier electrospray ionization (DB-ESI) where the amount of charges transferred to the liquid is an exponential process with the highest amount transferred in the first milliseconds.[33] (iii) The architectural imperfections of the μFFE and the multi-emitter device currently causing dead volumes can be prevented by high-precision 3D-printing. (iv) Data evaluation and representation in heatmaps, as performed in this study, can be automated using scripts to enable a comprehensive online-monitoring of the separation.

In conclusion, the multi-emitter presented herein is well suited for the monitoring of a μFFE separation and also has great potential for other areas of application in the field of microfluidics, where multiple samples need to be analyzed in quick succession.

Conflicts of interest

There are no conflicts to declare.

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