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Short communication

Interfacing microchip isoelectric focusing with on-chip electrospray ionization mass spectrometry

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ABSTRACT

In this work, we demonstrate the interfacing of microchip capillary isoelectric focusing (cIEF) with online mass spectrometric (MS) detection *via* a fully integrated, on-chip sheath flow electrospray ionization (ESI) emitter. Thanks to the pH-dependent surface charge of the SU-8 polymer cIEF can be successfully run in native SU-8 microchannels without need for surface pretreatment prior to analysis. On the other hand, the inherent electroosmotic flow (EOF) taking place in SU-8 microchannels at high pH can be exploited to electrokinetic mobilization of the focused pH gradient toward the MS and no external pumps are required. In addition to direct coupling of a cIEF separation channel to an ESI emitter, we developed a two-dimensional separation chip for two-step, multiplex cIEF-transient-isotachophoretic (tITP) separation. In this case, cIEF is performed in the first dimension (effective $L = 20$ mm) and tITP in the second dimension ($L = 35$ mm) followed by ESI/MS. As a result, the migration order is affected by both the p*I* values (cIEF) and the intrinsic electrophoretic mobilities (tITP) of the sample components. The selectivity of the separation system was shown to be different from pure cIEF or pure ITP, which allows at best for baseline separation of two compounds with nearly identical p*I* values. The repeatabilities of the migration times of the two-step cIEF-tITP separation were 3.1–6.8% RSD ($n = 3$). Thanks to the short separation channel, relatively short focusing times of 60–270 s (depending on the applied focusing potential) were sufficient for establishment of the pH gradient and cIEF separation of the sample components, yielding total analysis times (including loading, focusing, and mobilization) well below 10 min.

1. Introduction

Microchip capillary electrophoresis (MCE) in combination with fluorescence (FL) detection is the standard for microfluidic separations thanks to its high speed of analysis and good feasibility for proteomics research [1,2]. In addition to free zone electrophoresis, capillary isoelectric focusing (cIEF) is another established technique for the separation of zwitterionic biomolecules (*e.g.*, peptides and proteins) based on their isoelectric points (p*I*), often followed by electrokinetic or hydrodynamic mobilization toward a detector [3]. Microchip-based cIEF is typically combined with direct optical imaging of the focused sample components (through the chip cover layer), thus avoiding the need for mobilization [4–6]. However, a major limitation of optical detection (UV or FL) in proteomic applications is the fact that the ampholytes (required for the establishment of the pH gradient) strongly absorb UV light below 280 nm. Thus, only tryptophan and tyrosine containing peptides and proteins can efficiently be detected. Coupling of cIEF to mass spectrometry (MS) is therefore of great interest to many proteomics applications, where more specific identification is needed. However, the ampholytes also tend to suppress the ionization efficiency of the target compounds [7,8], and thus, the potential of microchip cIEF-MS in proteomics research is often overlooked.

In this study, we demonstrate efficient coupling of microchip cIEF separation to on-chip electrospray ionization (ESI)/MS *via* a dead-volume-free sheath flow interface that allows for the reduction of the ampholytes' suppression effect. Specifically, we exploit the parallel microfabrication technology (photolithography and adhesive bonding) for the implementation of fully integrated multidimensional separation systems [9–15]. Zero-dead-volume valving and transfer of samples from one dimension to another is easily achieved through electroosmotic flow (EOF) supported by SU-8

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[16]. In addition to direct coupling of microchip cIEF to ESI/MS, we also demonstrate a multiplex cIEF-transient isotachophoretic (tITP) chip design where peptides are first separated based on cIEF (pH 3–10) and then isotachophoretically during EOF-driven mobilization through a subsequent separation channel before ESI/MS. This approach allows for additional selectivity, as the migration order of the peptides is dissimilar from pure cIEF or ITP.

2. Materials and methods

Two types of microchip designs were fabricated (see Supplementary data and Fig. 1) as described earlier [10,12,13,17,18]. One design was used for direct coupling of cIEF to ESI/MS (Fig. 1A and C) and another one for multiplex-cIEF-tITP-ESI/MS (Fig. 1B and C). Before measurements, the SU-8 microchips were placed on a manually controlled *xyz* alignment stage that was mounted in front of the MS instrument to replace the conventional ion source. In addition, thin sheets of hydrophobic poly(dimethyl siloxane) (PDMS) were attached on top of each microchannel inlet to avoid sample spreading on a relatively hydrophilic SU-8 surface.

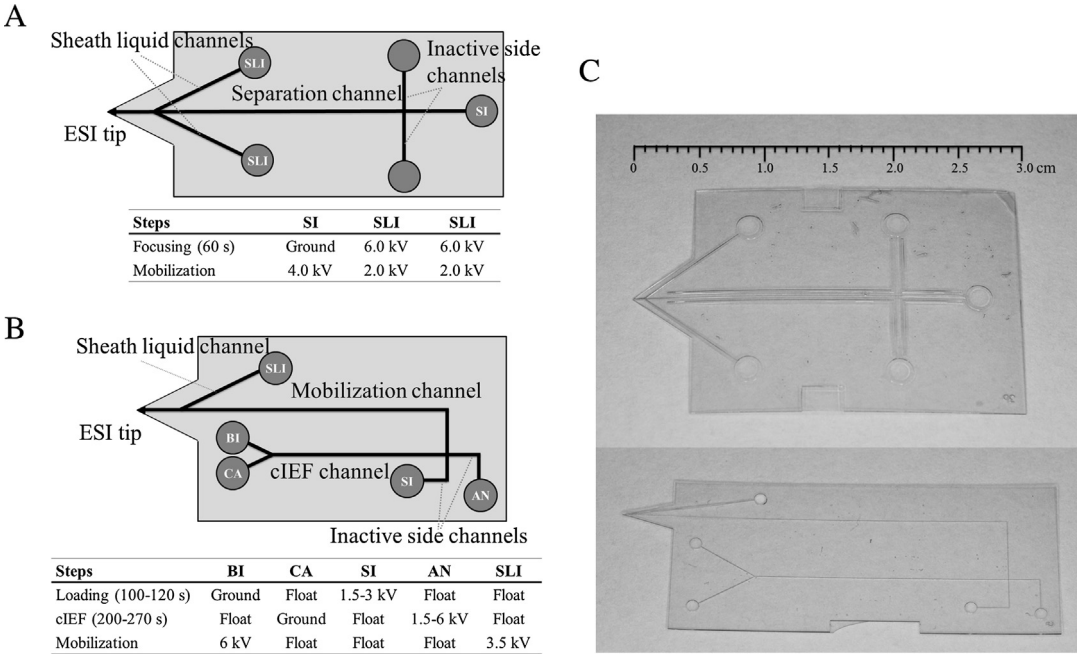
For direct coupling of microchip cIEF to ESI/MS the separation and sheath liquid channels (Fig. 1A) were filled with sample solution (peptides dissolved in 2% ampholyte solution (pH 3–10) including 25% methanol) and sheath liquid (methanol:water 80:20 incorporating 1% acetic acid), respectively, by capillary flow. After filling, the sample solution in the sample inlet (SI) was replaced by the catholyte (1% ammonium hydroxide), whereas the sheath liquid also served as the anolyte during cIEF. Focusing of the peptides was performed at 2400 V cm^{-1} applied between the SI and sheath liquid inlets (SLI) for 60 s until an electrical current drop and stable reading was obtained. After focusing, the catholyte in the SI was replaced by the background electrolyte (BGE, 20 mM ammonium acetate with 25% methanol) and the peptides were mobilized by applying electric field strength of 800 V cm^{-1} between the SI and the SLIs. During mobilization, the excess electrical current was grounded through a 50 MΩ resistor coupled in parallel with the electrospray voltage power supply [15]. MS detection was performed on an ion trap 6330 (Agilent Technologies, Santa Clara, CA, USA) instrument.

Before the multiplex cIEF-tITP-ESI/MS analysis, the separation and the auxiliary channels (Fig. 1B) were filled with BGE (30 mM ammonium acetate with 30% methanol) and sheath liquid, respectively, by capillary flow and vacuum suction. After filling, the solutions in the buffer inlet (BI), the SI, and the SLI, were filled with BGE, sample solution (peptides in 2% ampholyte solution with 0.33% TEMED), and sheath liquid, respectively. The catholyte (CA)- and anolyte (AN) inlets were filled with 0.5–1% ammonium hydroxide and 1% formic- or acetic acid, respectively. Then, the cIEF channel was loaded with the sample solution using electrokinetic flow at 500–1000 V cm^{-1} (SI → BI) for 100 s. Next, cIEF was performed by switching the potential difference (E = 500–2000 V cm^{-1}) between the CA and the AN. Typically, a stable current reading was obtained in 200–270 s, which was considered sufficient for the formation of the pH gradient and focusing of the peptides. Last, the focused peptide zones were mobilized toward the MS by applying potential difference between the BI (6 kV) and the SLI (3.5 kV). The voltage applied to the SLI simultaneously served as the ESI voltage (2.5 kV relative to MS). The MS experiments were performed on an API365 triple-quadrupole MS (Perkin-Elmer Sciex, Concord, ON, Canada) instrument.

Details about both setups can be found in the Supplementary data.

3. Results and discussion

The performances of the two microchip cIEF-ESI/MS setups were examined with the help of peptide standards and by monitoring



Steps	SI	SLI	SLI
Focusing (60 s)	Ground	6.0 kV	6.0 kV
Mobilization	4.0 kV	2.0 kV	2.0 kV

Steps	BI	CA	SI	AN	SLI
Loading (100-120 s)	Ground	Float	1.5-3 kV	Float	Float
cIEF (200-270 s)	Float	Ground	Float	1.5-6 kV	Float
Mobilization	6 kV	Float	Float	Float	3.5 kV

Fig. 1. Schematic views and photographs of the SU-8 microchips used for direct coupling of cIEF to on-chip ESI/MS (A and C, upper images) and for multiplex cIEF-tITP separation coupled to on-chip ESI/MS (B and C, lower images) together with high voltage sequences used. In (A), filling of the separation channel with the sample solution was done by capillary flow from the SI, after which the focusing potential was applied between the SLI (anolyte/sheath liquid) and the SI (catholyte). Mobilization of the peptides toward the MS was done by changing the solution in SI to the background electrolyte (BGE) and switching on the mobilization voltages. In (B), loading, cIEF, and mobilization were performed electrokinetically with the voltages given in the table. The separation and sheath liquid channel dimensions of the design (A) were 50 μm × 50 μm × 25 mm (w × h × L). In design (B), the length of the cIEF and mobilization channels were 20 mm and 35 mm, respectively, while both had channel cross-sections of 50 μm × 50 μm (w × h). The dimensions of the sheath liquid channels were 150 μm × 50 μm × 12 mm (w × h × L). BI = buffer inelt, SI = sample inlet, CA = catholyte, AN = anolyte, SLI = sheath liquid inlet.