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Research Article

Interface solution isoelectric focusing with in situ MALDI-TOF mass spectrometry

This paper describes a simple and reusable microfluidic device combining solution IEF (sIEF) with MALDI-TOF MS for rapid proteomic and metabolic analysis of microliter samples. The device contains two glass plates with nanoliter microwell arrays, which can be assembled to form a fluidic path for sIEF separation, and reconfigured for dividing separated bands. One microliter samples can be loaded and separated by sIEF into static bands in 10∼30 min. After a slipping operation, the static IEF bands can be divided into nanoliter droplets in microwells without mobilization, and the device can be opened for in situ MALDI-TOF MS detection without loss of separation resolution. The performance of the device is characterized by separating and identifying intact proteins. The applicability in metabolic analysis is demonstrated by preliminary experiments on profiling small molecular metabolites in cerebrospinal fluid microdialysates from rat brain.

Keywords:
MALDI / Microfluidics / Proteomics / SlipChip / Solution IEF
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1 Introduction

This paper describes a simple microfluidic device, which combines solution IEF (sIEF) with in situ MALDI-TOF MS imaging for rapid proteomic analysis with microliter samples. Proteomics, the large-scale profiling of proteins of living organisms, is expected to impact broadly on molecular biology, clinical diagnosis, and medicine [1, 2]. MS is regarded as the most powerful and widely used tool for proteomics and metabolomics research [3, 4], with which biomolecules, such as proteins and peptides, can be quantitatively determined with detailed information, including intact molecular weight, peptide fragment weight, and the amino acid sequence. Two complementary soft ionization techniques have been developed to analyze proteins with MS: ESI produces highly charged ions directly from solution, and can be integrated with separation systems such as HPLC [5] and CE [6]. Alternatively, MALDI generates ions predominantly singly charged, and is often used off-line with 2D-PAGE [7]. Compared with ESI, MALDI has high tolerance to nonvolatile buffers and impurities, and is suitable for analyzing intact proteins [8].

Integration of separation or pretreatment systems with MS is essential for analysis of complex biological samples. Among various separation methods, IEF has been widely used for separation and enrichment of proteins or peptides based on differences in their p*I*s [9]. Conventionally, IEF is performed on gel-based strips with immobilized pH gradient, which is time-consuming and limits its direct interfacing with MS. The last few years have seen a rapid increase in the interest of sIEF, using capillary or microfluidic devices [10] for fast and high-resolution separation of protein samples in small volumes. From a practical perspective, the combination of sIEF and MS is valuable in helping to identify protein or metabolite substances in complex mixtures of biological and clinical origin, providing combinatorial information including p*I*s, molecular weights, and relative abundances of the substances in minute amount of samples. sIEF allows rapid enrichment of low-abundant proteins or peptides at their specific p*I*s, and also helps to eliminate interfering substances and ions that may affect MS detection [11]. Since sIEF is performed in solution phase, it does not require digestion and elution steps as gel-based system does, and can provide molecular weight information of intact proteins. However, to

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Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; **CSF**, cerebrospinal fluid; **GFP**, green fluorescent protein; **sIEF**, solution IEF

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Colour Online: See the article online to view Figs. 1–4 in colour.

conjugate sIEF with MALDI-TOF MS, it usually requires mobilization of focused bands to the outlet of the channel [12,13], which causes band broadening and loss of resolution [14]. To avoid this problem, several recent research efforts have been carried out to promote in situ MALDI detection without mobilization, using microfabricated grooves with or without removable top cover layer as the separation channel [15–17]. sIEF bands were dried or frozen-dried on chips, and subjected to direct MALDI-TOF MS analysis in the grooves. This effectively reduced loss of resolution due to the mobilization of sIEF bands.

SlipChips are microfluidic devices made of two or more microfabricated plates sealed with a thin layer of lubricating oil. It contains reconfigurable fluidic networks, and can easily manipulate nanoliter experiments precisely and simultaneously by the slipping operation [18]. Various SlipChip devices for protein crystallization screening [19, 20], multiplex PCR detection of pathogens [21], digital PCR analysis of HIV and hepatitis C viral load [22], nanoliter immunoassays [23] had been developed. Recently, we and another group proposed similar SlipChip-based IEF approaches for protein separation in two MicroTAS conference proceeding papers [24–26]. Zhao et al. developed a device fabricated with two 2-mm thick hot-embossed acrylic plates with 140 50-nL microwells for capillary IEF in the gel polymer solution, and introduced two delicate methods for collection divided droplets in serial or in parallel for microchip gel electrophoresis using an Agilent 2100 Bioanalyzer [23, 24]. At the same time, we reported a glass SlipChip which interfaced sIEF with on-chip MALDI-TOF MS analysis [25]. To make it compatible with on-chip MALDI-TOF MS detection, we chose 0.7 mm soda-lime glass as the substrate, and avoided using gel polymer solutions. In this paper, we further optimized the device for better interfacing sIEF with MALDI-TOF MS, and improved its performance and sensitivity in separation and in situ identification of intact proteins. In addition, we extended the applicability of this system from protein analysis to profiling of low abundant metabolites using cerebrospinal fluid (CSF) microdialysates.

2 Materials and methods

2.1 Chemicals and materials

The protein/ampholyte solution was a mixture of 3.5 pmol/μL green fluorescent protein (GFP), 11 pmol/μL FITC-labeled ribonuclease A from bovine pancreas (RNase A), 1% pharmalyte/carrier ampholyte (pH 3 to 10), 0.1% TEMED and 0.7% glycerol. The preparation of 2.2 pmol/μL FITC-labeled BSA was the same as described above. CSF microdialysates from the brain of an adult male Sprague-Dawley rat were kindly provided by Professor Lanqun Mao from the Institute of Chemistry, Chinese Academy of Sciences, with the collecting procedure published previously [27]. Briefly, the microdialysates were collected with a microdialysis probe (CMA, dialysis length, 4 mm; diameter, 0.24 mm) at a flow rate of 1 μL/min. The membrane of the probe was only permeable to water and small soluble molecules. Then carrier ampholyte, TEMED and glycerol were added to the CSF, which was tenfold diluted by DI water. The working anolyte and catholyte were 20 mmol/mL phosphoric acid (pH 3) and 20-mM sodium hydroxide (pH 10), respectively. MALDI matrix solutions for protein samples and the CSF sample were 10 mg/mL sinapic acid and 5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA), respectively, which were dissolved in the solution containing 50% ACN, 49.9% DI water, and 0.1% TFA. A solution of 2.5% methylcellulose was prepared in boiled DI water to prevent EOF.

2.2 Fabrication and assembly of the device

The device was made by standard photolithography and wet chemical etching techniques using two thin glass plates (2.5 × 7.5 cm in size, 0.7 mm thick) [28]. The glass plates were cleaned and then their surfaces were silanized with 1H,1H,2H,2H-perfluorooctyl trichlorosilane [18]. The top plate contained 101 microwells with a volume of 4.47 nL (400 × 260 μm in size, 50 μm in depth, 150 μm spacing) and two access holes (0.035 inch in diameter); the bottom plate contained 100 microwells and two ducts (260 μm in width, 50 μm in depth, 1.75 mm in length) for connecting access holes on the top plate. Both plates contained an etched open area of 80 μm in depth. The top and bottom plates were precisely aligned under stereoscope and tightly clamped by clips, forming a fluidic path 58 mm in length. Five microliters of fluorinated oil (FC-40 with 5% PFO v/v) was injected through the fluidic path via the access holes, and spread into the gaps between two plates for lubrication of slipping and prevention of leakage. Extra FC-40 oil in the fluidic path was removed by applying vacuum via the access holes. The Reservoirs were produced from 10 mm-long top sections of 1 mL, 9 mm id pipette tips, affixed with epoxy glue on the top plate, surrounding the access holes.

2.3 Process of sIEF on device

A schematic illustration of operating sIEF on the device was shown in Fig. 1. Samples were pipetted into the fluidic path, and then the access holes were loaded with 10 μL of methylcellulose solution. Three hundred microliters of anolyte and catholyte were loaded into either reservoir over the access holes. Two needle electrodes were positioned in the center of the corresponding reservoir filled with anolyte or catholyte. A separation voltage of 50 V/cm was applied across the fluidic path for sIEF. For 2.2 pmol/μL FITC-labeled BSA, the stable fluorescence band was observed after 30 min. Focused fluorescence bands were visualized by a transilluminator (Zeesan Biotech, Xiamen) with an excitation wavelength of 470 nm and an emission wavelength of 510 nm. After focusing, the top plate was slipped against the bottom plate, cutting the focused bands into microwells on both plates. The device was frozen at −30°C for 10 min, and plates were slipped to bring microwells onto the etched open area without

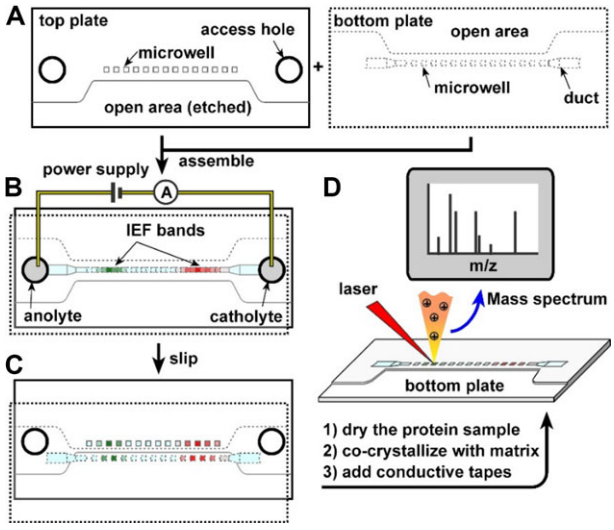

Figure 1. Schematic operation of the sIEF-MALDI device: (A and B) Assembly and setup of the device for sIEF; the red and green bands in the fluidic path were focused protein bands during sIEF; (C) Slip the device to divide the sIEF bands into array of microwells; (D) Disassemble the device for in situ MALDI-TOF MS detection.

disrupting the samples. Finally, the glass plates were disassembled and placed in sterile Petri dishes, and samples were dried at room temperature for 10 min.

2.4 In situ MALDI-TOF MS analysis

Matrix droplets (10 nL) were deposited into each microwell using a 100 μm id Teflon tubing connected with an 11 Elite syringe pump (Harvard Apparatus, Holliston, MA). The matrix cocrystallized with the sample in microwells, and dried at room temperature. Three conductive copper tapes (7.5 cm in length, 0.5 cm in width) were attached to the surfaces of the plates beside and under the microwell array. The glass plates were fixed on a target plate with 1 × 3 inch glass slide adapters. MALDI-TOF MS analysis of samples was carried out on a Bruker ultrafleXtreme instrument (Bruker Daltonics, USA).

3 Results and discussion

3.1 Design of the microfluidic system

By far, many efforts have been made to interface sIEF with MALDI-TOF MS; usually focused bands need to be deposited as segments onto the MALDI target plate [6]. This process inevitably causes band dispersion and loss of separation resolution, and adds difficulty when the channel size and sample volume decrease. In this work, we solved these problems by introducing a simple strategy: We utilized slipping operation to divide sIEF bands into nanoliter droplets embedded in microwells, and dehydrated the sample before opening the device to expose it directly for in situ MALDI-TOF MS detection. The whole process did not involve sample transfer and therefore maintained the sIEF resolution. The operation of the sIEF-MALDI device was illustrated in Fig. 1. A fluidic path for sIEF was assembled by aligning two microwell arrays on the opposite glass plates (Fig. 1A). Microliter sample was pipetted into the fluidic path, anolyte and catholyte were loaded into the reservoirs, and a separation voltage was applied across the fluidic path for sIEF (Fig. 1B). After formation of sIEF bands, the top plate was slipped to divide the separated sample into nanoliter droplets (Fig. 1C). These droplets were exposed in etched open areas and dried in microwells. Finally, the device was disassembled and matrix was applied into microwells for MALDI-TOF MS analysis (Fig. 1D).

We consider reusability of the device as an important quota since fabrication of glass device requires specialized facilities. After disassembling, we cleaned the glass plates with detergents, concentrated sulfuric acid, and ethanol sequentially, and then, the surfaces of glass plates were plasma cleaned and resilanized with fluorinated silane. After these treatments, the device could be reused repeatedly without affecting its performance, making it more affordable for common laboratories in the case that the device will be commercialized in the future.

We utilized a manual deposition method with a syringe pump for applying the matrix solution in each microwell (see Supporting Information for details). The deposition of 100 matrix droplets on a glass plate with 100 microwells took only around 10 min. Instead, commercially available matrix sprayers could be employed to apply the matrix automatically.

3.2 Elimination of EOF

Many studies have been carried out for suppressing EOF and improving the reproducibility of sIEF in microfluidic channels [29, 30]. Here, we used a combinatory strategy to achieve more reproducible sIEF on SlipChip: first, the plate surface was silanized with fluorinated silane to restrain EOF and provide good stability at different pH; second, 0.7% glycerol was added into the sample to increase the viscosity and further reduce EOF; finally, 20 μL 2.5% methylcellulose was added in the access holes to prevent pH gradient compression.

3.3 Device disassembly

To disassemble the device without leakage, we developed a simple approach: the top plate was slipped on the opposite direction to divide the fluidic path, and placed at −30°C for 10 min to freeze the sample. Next, the top plate was slipped backward to bring the frozen sample over the etched open area (see Supporting Information Fig. S1). Then, the device was opened without disrupting droplets in the microwells, and dried at room temperature for 10 min. This procedure only requires a common −30°C refrigerator that is almost available in all laboratories. As the top plate and the bottom plate contained complementary and slightly different

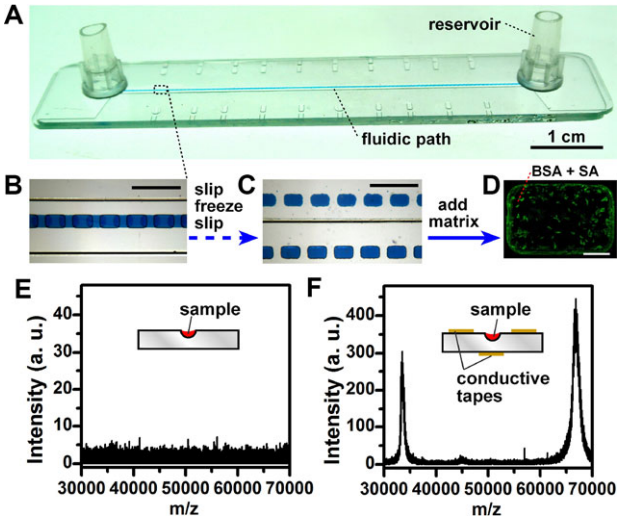

Figure 2. The procedure of sample treatment in the sIEF-MALDI device prior to MS detection. (A) Photograph of the device with a fluidic path loaded with the blue dye solution. (B) Zoom-in view of the fluidic path composed of microwells from the top and bottom plates. (C) Food dye solution was divided into segments in microwells after freezing and slipping steps. (D) The fluorescence microphotograph of 2.2 pmol/μL BSA cocrystallized with sinapic acid. (E) Mass spectra of BSA without copper tapes in the microwell. (F) Mass spectra from the same microwell after applying copper tapes.

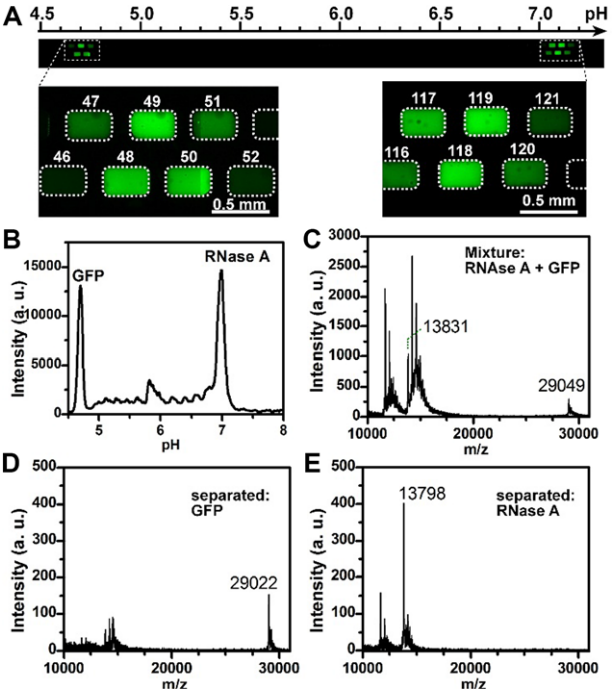

Figure 3. Separation and MALDI-TOF MS identification of proteins. (A) Montage of fluorescence microphotographs of the fluidic path during sIEF with the zoom-in view of the bands for GFP and RNase A. (B) The average fluorescence intensity of the microwells plotted against pH values. (C) Mass spectra of the protein mixture directly spotted on chip. (D) Mass spectra in the microwell of GFP band. (E) Mass spectra in the microwell of RNase A band.

segments, we need to analyze both plates to completely cover the sample separated by sIEF. Besides, we can also generate two glass plates with identical sIEF segments by slipping the device along the fluidic path to overlay the top microwells to the bottom ones after sIEF (see Supporting Information Fig. S2). The two daughter glass plates can be subjected to MALDI-TOF MS analysis with the same matrices for evaluating the reproducibility, or different matrices for selective ionization.

3.4 In situ MALDI-TOF MS analysis of protein

The ionization efficiency of MALDI on nonconductive substrates is generally very low [15]. However, we have to use nonconductive substrates for applying the separation voltage during sIEF. In this work, thin glass plates (0.7 mm thick) were used and conductive tapes were applied to enhance the efficiency of MALDI [31]. To further improve the performance, we taped both sides of microwells array and the back of the glass plates with copper adhesive tapes. As shown in Fig. 2E and F, with the help of applying conductive tapes, the mass spectra signal intensity at m/z value of ~67 000 increased from baseline (Fig. 2E) to 450 (Fig. 2F) for FITC-labeled BSA (loading concentration: 2.2 pmol/μL) in the same microwell at the center of the sIEF band. The LOD for FITC-labeled BSA based on 3 times the SD of the blank values was 1.6 fmol/μL (3σ). The detection limit for small peptides or metabolites should be much lower than FITC-labeled BSA. To improve the sensitivity, we can use longer channel to increase the separation efficiency of sIEF. The thickness of the nonconductive substrate will dramatically affect the effective electric field received by the protein sample during ionization, thus we can also make the device with thinner plates to further improve the performance.

3.5 Protein separation

A mixture of 3.5 pmol/μL GFP and 11 pmol/μL FITC-labeled RNase A was used as a model sample to evaluate the performance of separation. After 30 min of sIEF at 50 V/cm, the device was slipped to divide the focused sIEF bands. As shown in Fig. 3A, stable fluorescence bands for GFP and FITC-labeled RNase A were localized at No. 49 microwell (pH~4.7) and No. 119 microwell (pH~7.1), respectively. The average fluorescence intensities of all microwells after slipping were plotted against pH values as shown in Fig. 3B. The calculated pI values were consistent with pIs provided in product specifications from the manufacturers (GFP, pI = 4.7~4.9; RNase A, pI = 7.8). The theoretical minimum resolvable difference in pI was about 0.07 based on the measurement of the bandwidths of sIEF bands. SlipChip divided sIEF bands at the optimal condition without band mobilization, hence avoided

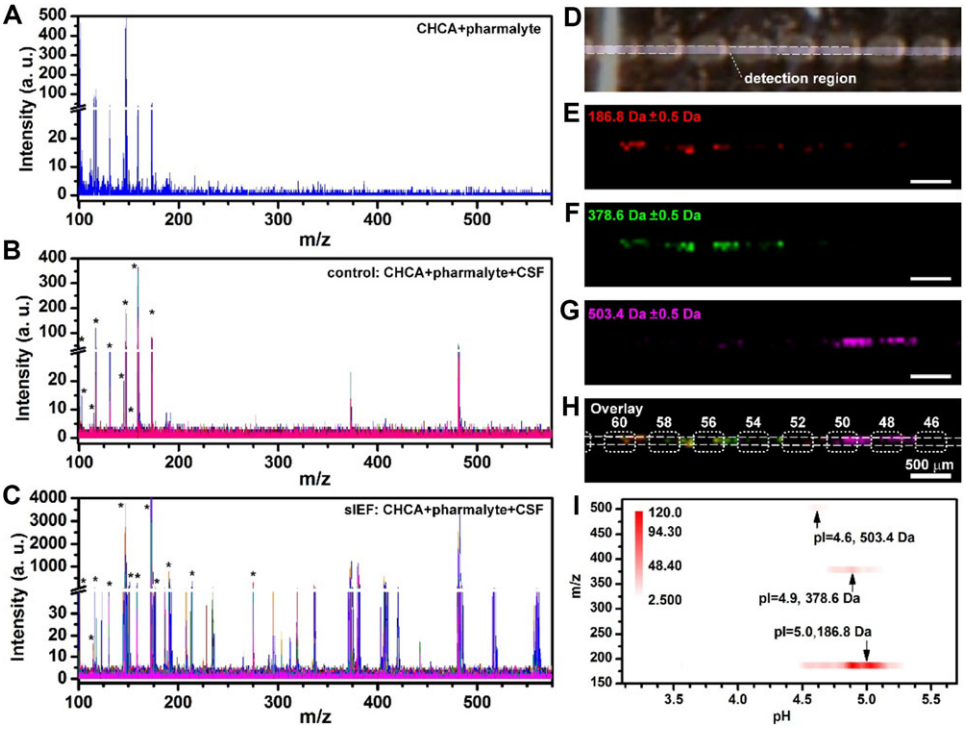

Figure 4. Profiling of CSF microdialysates on sIEF-MALDI device. (A) Mass spectra of background pharmalyte and CHCA. (B) Overlay of nine reproducible Mass spectra of the CSF deposited on the glass plate. (C) Overlay of 201 mass spectra from 201 microwells obtained with MALDI imaging of the sIEF separated sample; peaks from matrix and carrier ampholyte are marked with "*". (D) The scanned image of the bottom plate with outline of MALDI-TOF MS imaging region. (E–G) Ion intensity maps showing sIEF bands distribution of intensity for peaks with *m/z* of 186.8, 378.6, and 503.4; scale bar = 500 μm. (H) The overlapped ion intensity map. (I) 2D mapping of p*I* and *m/z* of substances with obvious sIEF bands in the region.

dispersive band broadening and diffusive dilution. Mass spectra after separation in respective microwells (Fig. 3D and E) were in agreement with the fluorescence microphotographs, indicating that GFP and RNase A were successfully separated. Compared with conventional "bottom-up" strategies which analyzed proteolytic peptides from complex mixture of proteins [32] the capability to directly detect the intact proteins without digestion provided a "top-down" approach, and may be used for identification of multiple proteins with overlapped p*I*s in complex biological samples.

To investigate if the device can improve the performance of MALDI-TOF MS in analysis of complex biological samples, we tested a CSF microdialysate sample. We did sIEF separation with a separation voltage of 50 V/cm for 10 min, and subsequently performed automatic MALDI imaging of the microwell array. The mass spectra of blank solution prepared the same as CSF sample were shown in Fig. 4A; mass spectra of the CSF sample deposited on the glass plate were shown in Fig. 4B (also see Supporting Information Fig. S4); overlay of 201 mass spectra of the CSF sample after sIEF separation in microwell arrays were shown in Fig. 4C. All mass spectra indicated that there were no detectable metabolites with molecular weight larger than 600 Da in the sample using CHCA as the matrix. With the help of our device, the peaks derived from the CSF microdialysate increased from 3 to 29 after excluding background peaks from ampholytes and CHCA (see Supporting Information Table S1). The signal enhancement at *m/z* of 372.4, 406.7 and 480.8 were 172.8, 83.7, and 65.9 times, respectively. In addition, three sIEF bands at *m/z* of 186.8, 378.6, and 503.4 were observed on both top and bottom plates in the same p*I* regions of 5.0, 4.9, and 4.6 (Fig. 4D–I). We did a searching of CSF metabolites in the Human Metabolome Database (HMDB, http://www.hmdb.ca/), and 11 possible candidates were listed in Supporting Information Table S2. The further confirmation of metabolites and biomarkers in CSF will be investigated for future research.

4 Concluding remarks

In summary, we developed a simple, portable, low-cost, and reusable microfluidic device interfacing sIEF with MALDI MS for fast separation and identification of proteins or metabolites using only 1 μL sample. In addition to rapid sIEF separation, the device can divide the focused bands into nanoliter segments without loss of resolution, and be directly opened for in situ MALDI-TOF detection with volume as low as 4.47 nL. We believe that our methods can be employed in proteomics and metabolomics analysis of precious samples and widely used in clinical diagnoses and the discovery of biomarkers.

The advantage of MS is often compromised when facing with complex biological samples with high salt concentration, which could dramatically suppress the signal [33, 34]. It is difficult to use conventional desalting methods when the available volume of sample is extremely low, such as aqueous humor [7] and tears [35]. In the experiments of CSF microdialysates, the enhancement of signal and detection of new species might be partially due to desalting effect of sIEF [11]. However, a comprehensive characterization has to be carried out to quantify the desalting effect and evaluate the performance with various biological and clinical samples.

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