On-Chip CGE Protein Separations with Off-Line MALDI-MS Analysis

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Abstract

The off-line coupling of polymer microfluidic chips to mass spectrometry (MS) through electrospray deposition of analyte onto matrix-assisted laser desorption/ionization (MALDI) targets for MALDI-MS analysis is presented. Using polycarbonate (PC) microfluidic chips with integrated hydrophobic membrane electrospray tips, selected peptides and proteins were eluted onto a stainless steel MALDI target via electrospray deposition, followed by off-line MALDI-MS analysis. A variety of matrix compositions were evaluated, and the resulting film morphology was studied via scanning electron microscopy. Compared to the pipetting or spotting methods of MALDI target preparation, microchip electrospray deposition is found to yield excellent homogeneity of peptide crystals, and exhibits significantly improved reproducibility during MALDI-MS analysis. Peptide identification down to 15 fmol is readily achieved, and deposition of whole proteins from the electrospray chip is also demonstrated. Furthermore, in-line protein digestion using the electrospray tip as a real-time proteolytic membrane reactor is decsribed, and the coupling of electrospray deposition to on-chip CGE is discussed.

Introduction

Several approaches to coupling microfluidic systems with mass spectrometry have been explored. Direct electrospray from planar microfluidic chips is particularly attractive as an efficient, straightforward interface between microfluidic separations and MS analysis. Despite the potential for direct ESI-MS analysis from microfluidic systems, it is often desirable to decouple on-chip separations from MS analysis. For example, there is often an incompatibility between the time scales for biomolecular separations and MS data acquisition. Another demand for off-line analysis arises from the ability to perform multiple parallel on-chip separations in high throughput microfluidic systems, in which simultaneous ESI-MS from each separation channel is not feasible. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) is a powerful analytical technique commonly employed for off-line MS analysis following capillary separations (1-3). Preparation of MALDI targets is typically carried out by the dried-droplet method, in which pipetting or spotting of an aliquot containing a mixture of sample and matrix solution is followed by air-drying of the deposited spot (4). However, the dried-droplet approach is not ideal for microfluidic systems, in particular for arrays of closely-spaced microchannels in which crosstalk between eluted analytes can occur during droplet deposition. Furthermore, the quality of MALDI data is highly dependent on the way that analytes are prepared on the MALDI target plate (5). Liquid-phase deposition methods including dried-droplet (4), fast solvent evaporation (6), sandwich (7), and two-layer (8) preparation tend to suffer from poor homogeneity of crystallized sample, since matrix and analyte tend to partition during the solvent evaporation process (6,9), resulting in significant variations in mass resolution, intensity, and selectivity, and preventing meaningful quantitative analysis (4,10,11).

As an alternative to mechanical pipetting or spotting, a number of studies have investigated the use of electrospray deposition of analytes onto MALDI targets followed by MALDI-MS analysis. Capillary electrospray has been reported for MALDI target preparation following capillary electrophoresis (3,12), reverse-phase liquid chromatography (13), and size-exclusion chromatography (2). As a sample preparation technique, electrospray has been reported to yield uniform films for plasma desorption mass spectrometry (14-16), and secondary ion mass spectrometry (17). A number of studies have shown that electrospray deposition can markedly improve the homogeneity of sample on the MALDI target surface by reducing segregation of matrix/analyte components, leading to greatly enhanced repeatability (9,18-21), thereby enabling improved quantitative analysis (9,20). Furthermore, electrospray deposition has been shown to significantly improve the precision of molecular mass measurements during MALDI-MS (21).

While interfacing microfluidic systems with MALDI-MS has not been explored as extensively as ESI-MS, several examples exist. Ekstrom et al. used a microchip-based immobilized enzyme reactor for protein digestion followed by a microfabricated piezoelectric flow-through dispenser to deposit the protein digest onto a high-density nanovial MALDI target plate (22,23). Gustafsson et al. described the use of centrifugal forces in a disc format to elute protein digest from a microfluidic reverse-phase liquid chromatography column to an on-chip MALDI target, with sub-fmol mass sensitivity achieved (24). Murray et al. have also discussed the coupling of microfluidic chips to a continuous-flow MALDI system using a frit-based elution method (25).

Here we investigate electrospray deposition for MALDI-MS sample preparation as a new approach for efficiently coupling microfluidic systems to MS. The off-line coupling of microfluidic devices with MALDI-MS offers the potential to address key mismatches between on-chip separations and MS analysis, both temporal mismatch, particularly during the analysis of complex samples, and spatial mismatch when multiplexing parallel on-chip separations. The use of electrospray as the deposition method provides several advantages over other approaches to chip-based MALDI interfacing. It does not require the addition of relatively complex external microfabricated elements, as in the case of piezoelectric deposition, and unlike centrifugal deposition it is a generic method which can be applied to a wide range of microfluidic systems. Electrospray deposition also provides excellent uniformity in on-target sample morphology and distribution, while also offering the potential to enable simultaneous deposition from high-density arrays of electrospray tips on a single chip with no crosstalk for multiplexed analysis.

Experimental

Robust electrospray emitters were integrated into polymer microfluidic chips through the addition of a porous hydrophobic membrane at the channel exit. The fabrication process is detailed elsewhere (26). Briefly, planar microchannels were fabricated in polycarbonate (PC) using polymer hot embossing with a silicon template patterned by bulk Si micromachining. The resulting open microfluidic channels with trapezoidal cross section (122 μm wide at the top, 80 μm wide at the bottom, 30 μm deep, 4.5 cm long) were enclosed by thermally bonding with a blank PC substrate with reservoir holes drilled for electrical and fluidic access at one end of each channel. Capillary connections (Nanoport, Upchurch Scientific, Oak Harbor, WA) were bonded via epoxy on top of the reservoir to provide liquid supply from a syringe pump. A platinum electrode contacting the liquid through a capillary T-junction provides the required electrical contact for electrospray deposition. The resulting chip is cut to expose the microchannels at their ends opposite the

reservoirs using a CNC milling system. A 50 μm thick PTFE membrane with 70% porosity and 0.22 μm average pore size is thermally bonded to the exposed microchannel outlet surface. Fig. 1 gives an example of a well-defined Taylor cone without lateral spreading established at the channel exit using an applied flow rate of 120 nL/min, chip-to-counterelectrode spacing of 2 mm, and applied voltage of 3.9 kV. The highly hydrophobic and porous structure of the PTFE membrane effectively constrains liquid spreading at the channel exit. A potential ranging from 2-3 kV is applied to the analyte solution to achieve electrospray, with a stable total ion current of between 42-55 nA maintained for all tests.

MADLI mass spectrometry measurements were performed with a Bruker Autoflex MALDI-time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser and operated in the linear mode. Each mass spectrum was produced by averaging 30 laser shots. All analyses were performed at 65% of maximum laser power.

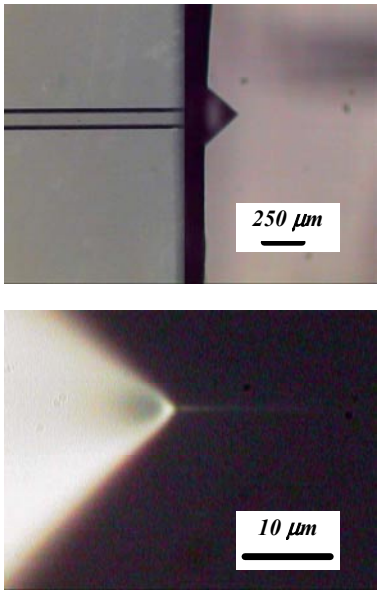


Fig. 1 Photomicrographs of stable Taylor cones established at the microchannel exit.

Results and Discussion

During microfluidic electrospray deposition, analyte is sprayed from the surface of the porous hydrophobic PTFE membrane along the exit face of the microfluidic chip, resulting in a circular deposition pattern on the stainless steel MALDI target plate. For the samples used in this study, the diameter of the resulting spot varies as a function of chip-to-target spacing, with a typical diameter of 0.5 mm for a 1 mm spacing. Stability of the electrospray current is dependent on a number of factors, including bias voltage, counterelectrode spacing, flow rate, and solvent conditions, as described previously for the microfluidic membrane-based electrospray emitters (26). The goal of the present study is not to detail the optimal electrospray conditions, but to evaluate the performance of the deposition process, deposited sample morphology, and MALDI-MS analysis under a set of nominal electrospray conditions.

Matrix Selection and Film Morphology. Sample preparation for MALDI-MS plays a critical role in determining the sensitivity and quality of the mass spectral data (5). Using angiotensin peptide sample, six different matrix solution preparations were first evaluated to determine a suitable matrix for further characterization. Solutions were prepared with CHCA at 10 μg/μL in a solvent solution with an acidic component. Variations in the primary organic solvent component (acetonitrile or methanol), solvent content (70% or 50%), and acidic component (acetic acid or trifluoroacetic acid) were considered. Different matrix solutions were found to result in vastly different crystal morphologies. Overall, matrix solutions with higher organic solvent content resulted in greater inhomogeneity in the resulting peptide/matrix film, presumably due to enhanced resolvation and resulting segregation of deposited peptides. Compared to methanol as the solvent, acetonitrile was found to result in more homogeneous films. The best matrix solution evaluated was 50% acetonitrile, 40% water, 10% acetic acid, which provided consistently uniform films with nearly constant film morphology across the deposited spot as shown in Fig. 2 for a solution

of 50 pmol/μl angiotensin deposited at 100 nL/min for 3 min, for a total of 15 pmol angiotensin.

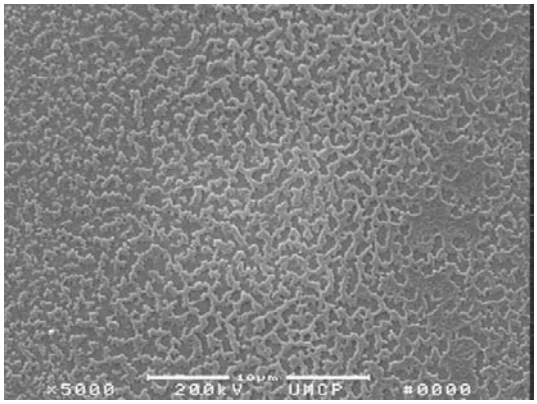


Fig. 2 SEM of electrospray-deposited angiotensin at 50 pmol/μL.

Peptide Concentration. To evaluate the effects of local peptide concentration on ion signal intensity, the total amount of angiotensin deposited by electrospray deposition from the microfluidic chip was varied, followed by MALDI-MS analysis. Angiotensin was diluted to concentrations from 0.05 to 50 pmol/μL in buffer solution (50% methanol, 49% water and 1% acetic acid), and deposited at a flow rate of 100 nL/min for 3 min with a chip-to-target spacing of 1 mm. The corresponding amount of angiotensin deposited on the target ranges from 15 fmol to 15 pmol. Due to the larger total fluid volume eluted onto the target compared to the previous 3 s deposition, all deposited spots down to 15 fmol could be visually located for MALDI-MS analysis. Again, ion intensity remains relatively constant across and between each spot, down to a concentration of 2.5 pmol/μL (total peptide loading of 750 fmol), beyond with the average signal begins to drop and inhomogeneities across the sample spot appear. For the 15 fmol spectrum shown in Fig. 3, the signal-to-noise ratio is between 8-10, suggesting that further reductions in total peptide deposition could be tolerated.

Protein Analysis. The analysis of proteins deposited from the electrospray chip was also investigated. Stable operation of the microfluidic electrospray emitters requires the use of a PTFE membrane as an extremely hydrophobic layer at the channel exit (26). In order to deposit whole proteins from the chip, the significantly larger and more hydrophobic molecules must traverse the porous PTFE membrane without becoming bound to the membrane surface. To explore this issue, bovine serum albumin (BSA) was loaded on an electrospray chip at a concentration of 1 mg/mL and deposited onto the MALDI target at a flow rate of 100 nL/min with a tip-to-target spacing of 1 mm for 120 min. The chip was moved to a new deposition location in 5 min increments, for a total of 0.5 μg BSA deposited per spot. For the first 5 min, no BSA signal was observed. At 10 min the BSA peak was clearly visible, and from 20 min to 120 min the peak intensity saturated with an average value approximately 20% higher than the initial value at 10 min, indicating that the majority of available binding sites became saturated within the first 10 min of deposition.

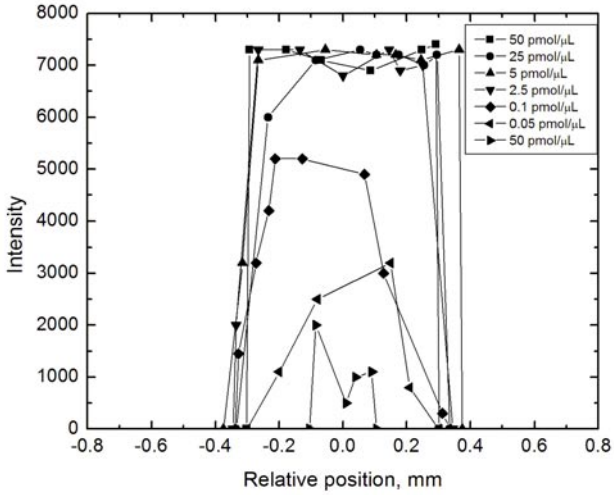
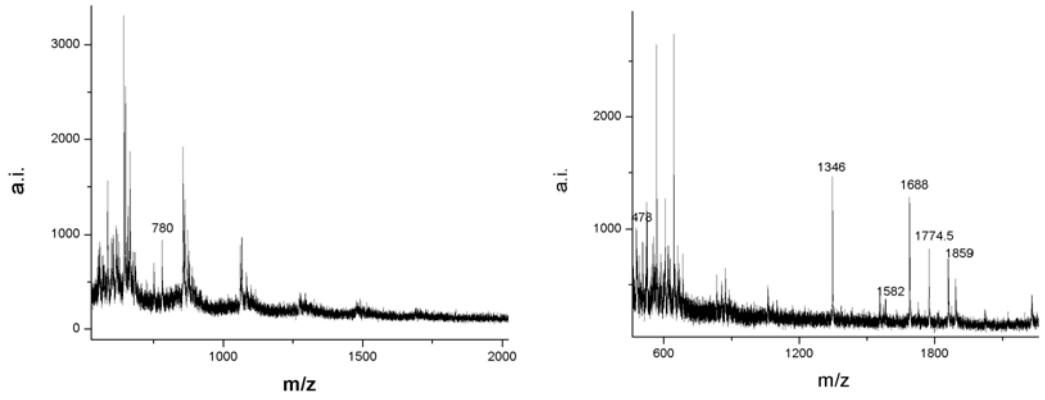


Fig. 3 Signal intensity profiles across deposition spot for angiotensin deposited at 100 nL/min flow rate for 1 min.

A study was also performed to evaluate the ability to digest proteins in-situ as they traverse the PTFE electrospray membrane. Following previous work by Cooper et al. towards the development of immobilized-trypsin membrane reactors for in-line capillary protein digestion (27), a solution of trypsin solution at 2 mg/mL was prepared in 10 mM ammonium acetate

buffer and flushed through the electrospray membrane at a flow rate of 50 nL/min for 1 h, allowing the trypsin to bind to the membrane surface. The reactor was then flushed with an ammonium acetate buffer for 20 min to remove any unbound trypsin. Denatured and reduced cytochrome *c* and ovalbumin at varying concentrations were deposited through the membrane onto a MALDI target. Examples of MALDI-MS spectra from these samples are shown in Fig. 4. Overall trypsin activity remains constant for at least 3 h of electrospray in repeated tests.



(a) (b)

Fig. 4 MALDI mass spectra for (a) 20 μg/ml cytochrome c at a flow rate of 0.1 μl/min for 1 min deposition, and (b) 1mg/ml ovalbumin at a flow rate of 0.1 μl/min for 1 min deposition.

CGE Integration. In order to integrate the electrospray deposition method with microfluidic gel electrophoresis for protein separation prior to MALDI-MS, a modified device was fabricated. Since bulk flow of solution is prevented by the introduction of a gel media in the microchannel, a secondary channel was added to provide makeup solution to enable stable electrospray. The basic configuration is shown in Fig. 5. To reduce electroosmotic flow and prevent protein adsorption on the surface of the microchannel walls, a 2 mg/mL solution of BSA was flushed through the entire chip before starting. A solution of 1.5% poly (ethylene oxide) (PEO, MW 600,000) gel in Tris buffer at pH 8.0 and 0.05% sodium dodecyl sulfate (SDS) as the separation medium was pumped from the gel-loading reservoir (A) to the waste reservoir (D). Gel was kept only within the separation section by flushing extra gel from undesired areas (i.e. the cross-injector for sample loading and the ESI makeup flow region). Visualization of the gel regions was performed by loading Rhodamine dye within the initial gel solution, as shown in Fig. 5. Running buffer was injected from reservoir E through the electrospray tip. Using this configuration, stable electrospray was realized by controlling the makeup solution flow rate and voltage applied at reservoir E, while simultaneously applying a separation voltage at reservoir A to define the electric field within the CGE region. With this configuration, single protein species have been successfully mobilized through the gel, electrospray deposited, and identified by MALDI-MS. Efforts to characterize complex samples using this approach are ongoing.

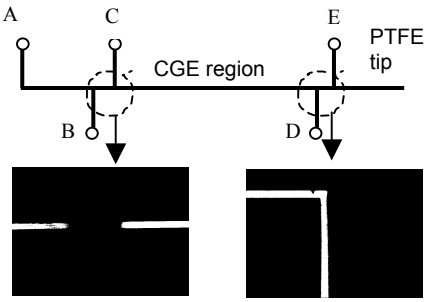


Fig. 5 Schematic of CGE chip, with fluorescence images showing the gel-filled chip regions.

Conclusion

The combination of electrospray target preparation with off-line MALDI-MS is a powerful technique for integrating microfluidic systems with mass spectrometry, particularly for multichannel devices performing parallel sample preparation and separation. One advantage of the technique is that the morphology of deposited sample exhibits significantly improved homogeneity compared to mechanical spotting of sample. While this observation has been widely reported for capillary-based electrospray deposition of pre-mixed matrix and sample solutions, the present work demonstrates that excellent MALDI-MS signal uniformity can be achieved by microchip electrospray deposition of sample, followed by the addition of matrix solution post-deposition. This feature is particularly important for microfluidic analytical platforms, since it can potentially eliminate the broadening of narrow sample bands which would otherwise result from the on-chip matrix mixing process. Furthermore, since the deposition process is not discretized, an arbitrarily small volume of analyte solution can be eluted from the microfluidic chip onto each target spot, providing improved compatibility with high-resolution separations where mixing of separated species is undesirable prior to MALDI-MS analysis.

Off-line coupling of microfluidic devices with MALDI-MS through electrospray deposition provides useful benefits compared with previously-reported piezoelectric and centrifugal elution methods. Because electrospray deposition eliminates the need for complex external microfabricated elements which introduce additional plumbing and dead-volume limitations, the technique should minimize band broadening from on-chip separations compared with piezoelectric deposition. Unlike centrifugal elution, the method is widely applicable to the vast majority of microfluidic systems which employ non-centrifugal pumping for on-chip sample manipulation. Furthermore, the PTFE membranes support the ability to integrate in-line and real-time proteolytic digestion directly into the electrospray tips themselves.

The essential elements required for combining on-chip CGE protein separations with MALDI-MS interfacing within a microfluidic platform have been described. While additional efforts will be required to optimize separation efficiency in the coupled system, the core building blocks have been demonstrated. Future work will be directed towards optimization of size-based separations for complex sample via on-chip CGE, and scaling the system to high-density arrays of parallel on-chip separation channels for high-throughput analysis by leveraging back-end MALDI-MS for protein identification.

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