Interfacing Capillary Gel Microfluidic Chips with Infrared Laser Desorption Mass Spectrometry

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We report on the fabrication and performance of a gel microfluidic chip interfaced to laser desorption/ionization (LDI) mass spectrometry with a time-of-flight mass analyzer. The chip was fabricated from poly(methylmethacrylate) with a poly(dimethyl siloxane) cover. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in the channel of the microfluidic chip. After electrophoresis, the cover was removed and either the PDMS chip or the PMMA cover was mounted in a modified MALDI ion source for analysis. Ions were formed by irradiating the channel with 2.95 μm radiation from a pulsed optical parametric oscillator (OPO), which is coincident with IR absorption by N-H and O-H stretch of the gel components. No matrix was added. The microfluidic chip design allowed a decrease in the volume of material required for analysis over conventional gel slabs, thus enabling improvement in the detection limit to a pmol level, a three orders of magnitude improvement over previous studies in which desorption was achieved from an excised section of a conventional gel. (J Am Soc Mass Spectrom 2006, 17, 469–474) © 2006 American Society for Mass Spectrometry

The rapid expansion of the field of proteomics has created an increased demand for selective, sensitive, and high-throughput methods of analysis. Soft ionization methods, such as matrix-assisted desorption/ionization (MALDI) [1, 2] and electrospray (ESI) [3, 4] are suitable for achieving the goal of identification and structural determination of proteins. Mass spectrometry (MS) has become the primary analysis method in this area because of its high throughput, sensitivity, and high mass accuracy. However, the large number and broad concentration range of the proteins present in the expressed proteome of a typical organism requires that one or more fractionation or separation steps be performed on the sample before mass spectrometry analysis.

One of the most commonly utilized techniques for protein separation is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [5–9]. The molecular weight of a protein is determined by the migration distance after electrophoretic separation together with several marker proteins of known molecular weights that bracket the protein of interest. Currently, most proteome analyses are based on the separation of complex protein mixtures by 1D or 2D gel electrophoresis, followed by mass spectrometric analysis of proteolytic digests of gel spots to identify individual proteins [10–13]. However, this protocol requires intermediate sample processing steps such as extraction of the protein from the gel [13], blotting the protein onto a membrane [14], or electroelution [15], which introduces a number of laborious and time-consuming manual steps that are difficult to automate.

Various efforts have been directed toward direct analysis of gel and planar chromatography separations by laser desorption ionization (LDI) mass spectrometry. The mass spectrometric analysis of peptides and proteins by UV-MALDI directly from polyacrylamide gels has been reported previously [16]. Here, ultrathin gels (less than 10 μm) were dried and affixed to the MALDI target and the matrix was added by pipette. Direct analysis of thin-layer chromatography (TLC) plates has been reported using UV [17, 18] and IR lasers [18, 19]. Crystalline matrices were used for IR and UV MALDI [17, 18], and a sprayed-on glycerol liquid matrix was used for IR MALDI of the TLC plates [19]. The advantage of the IR laser in these studies is the greater depth of penetration compared to the UV laser [20]. Direct IR LDI mass spectrometry from polyacrylamide gels without the addition of a matrix has been reported using a 5.9 μm free electron laser [21] and (from the author's laboratory) using a 3 μm OPO [22]. Here, the "matrix" is thought to be the gel or water within the gel. MALDI analysis of standard size gel and TLC plates requires a relatively large quantity of material due to the mismatch in size between the laser spot, which is typically a few tenths of a millimeter in diameter, in comparison

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to the separated material, which is several millimeter to as much as a centimeter in diameter.

One approach to reducing the size of the separation spot is microfluidics [23]. There have been a number of reports of coupling microfluidic chips to MALDI-MS, both on-line and off-line. For example, open channel capillary electrophoresis (CE) has been coupled off-line with MALDI [24]. After the separation, the solvent evaporates, matrix is added, and the sample analyzed in the mass spectrometer. In other off-line approaches, the sample is dispensed from the chip using a piezoelectric dispenser [25], pumped through a spinning microfluidic disk [26], or sample droplets moved on a dielectric chip surface by electrowetting [27, 28]. In a unique on-line approach, the vacuum of the mass spectrometer source is used to pull fluid through a microfluidic chip where it is subjected to UV MALDI as it exits the device [29, 30]. A second on-line approach uses a rotating stainless steel ball coupled to an on-chip capillary electrophoresis for the separation of peptides [31].

In this work, we report on matrix-free IR LDI of polypeptides and proteins directly from a polyacrylamide gel after electrophoretic separation in a microfluidic chip fabricated from polymethylmethacrylate (PMMA). PMMA is well suited for use as a substrate for microfabricated devices because of its high dielectric constant, thermal conductivity (comparable to silica), low cost, and ease of fabrication [32]. We use a cross-linked gel that is compatible with off-line coupling to MALDI MS. Samples are analyzed off-line following electrophoresis by IR-LDI, using the compounds in the gel itself as the matrix.

Experimental

Materials

Bradykinin, bovine insulin, and cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, MO). PMMA sheets were purchased from Goodfellow Co. (Berwyn, PA). The PDMS used in this experiment was purchased from General Electric (Waterford, NY). A set of RTV 615A (pre-polymer) and RTV 615B (curing agent) were mixed with a 10:1 weight ratio and degassed using a vacuum pump to remove air bubbles entrapped during mixing. The polymer was cast against the glass mold and polymerized at 100°C for 1 h. Except when otherwise noted, all chemicals used for gel electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA). All chemicals were used without further purification.

Microchip Fabrication

The microfluidic chip layout is shown in Figure 1 and was a standard "tee" format designed using AutoCAD (Autodesk Inc., San Rafael, CA). Microfluidic chips were fabricated from PMMA wafers using a micromilling machine (Kern Micro und Feinwerktechnik GmbH and Co., KG, Murnau, Germany). The channels were 300 μm in width and 150 μm in depth and the diameters of reservoirs were 1 mm. Two chip types were manufactured from 3 mm thick PMMA wafers: a short channel and a long channel version. The short-channel chip was 15 $\times$ 20 mm with a 5 mm short arm and 16 mm long channel and the channels crossed such that the separation channel was 14 mm long. The long-channel chip was 15 $\times$ 55 mm with 5 and 50 mm short and long arms, respectively. The separation channel was 50 mm long. Before assembly, 1 mm diameter holes were drilled through a 1.5 mm PDMS cover to serve as reservoirs. After cleaning of the embossed microfluidic chip, a 0.5 mm PDMS cover was thermally annealed to the chip by clamping the assembled microfluidic chip between two glass plates and heating to 105°C for 12 min. in a GC oven.

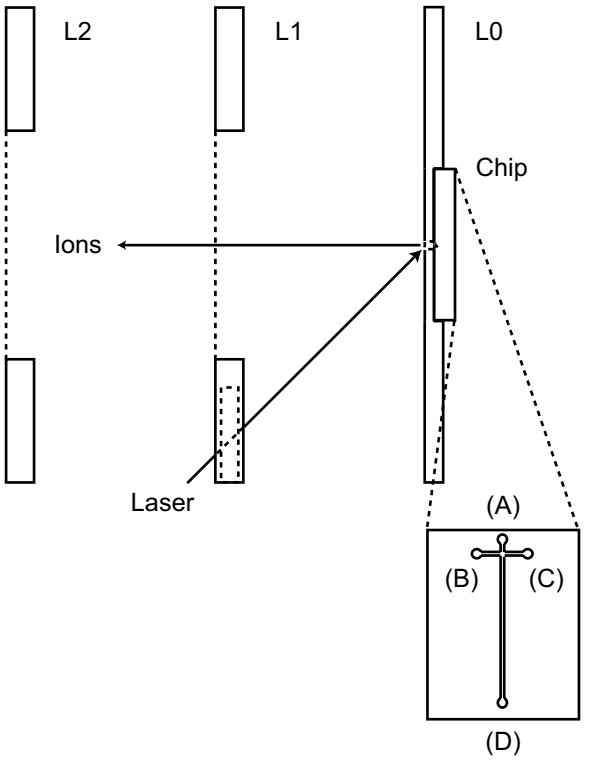

Figure 1. Schematic of the gel microfluidic chip ion source. The chip is shown below inset with buffer reservoir A, analyte reservoir B, analyte waste C and buffer waste D. Ions are desorbed from the chip held behind the target L0 and ions are accelerated into the flight tube by acceleration grids L1 and L2.

SDS-PAGE in Microchips

The solutions and gels for Tris/Tricine SDS-PAGE were prepared according to the procedure of Schägger and von Jagow [8]. The concentration of acrylamide in the resolving gel was 16.5% T and the acrylamide-to-bisacrylamide ratio was 30:1. Here, %T denotes the weight percentage of total monomer (acrylamide and bisacrylamide) whereas %C denotes the percentage of the

cross-linker relative to the total monomer mass. Then the sieving gel was introduced into the channel in the microfluidic chip and was polymerized for 1 h. After polymerization, the reservoirs were flushed with Tris/Tricine running buffer. The same solution was used as a sample buffer and buffers for other reservoirs. The device was exposed to an electrical field of 150 V/cm for 5 min. before sample loading. The samples were incubated for 30 min. at 40°C in 4% SDS, 12% glycerol, 50 mM Tris-HCl (pH 6.8), and 0.02% peptide and protein samples were loaded by applying voltage across the twin-T injector for 9 min with buffer reservoirs floating. During separation, 30 V/cm was applied to both side channels to prevent leakage of excess sample into the separation channel. Electrophoresis was performed on bradykinin and bovine insulin in SDS PAGE microchips using a field of 150 V/cm that was applied for 9 min.

Mass Spectrometry

The MALDI-TOF mass spectrometer used in this work has been described in detail previously [22]. The instrument is a 1 m linear time-of-flight instrument with delayed ion extraction. After electrophoresis, the PDMS cover was peeled off of the PMMA microfluidic chip and either the chip or the cover was mounted in a modified MALDI target holder (Figure 1). The target holder consisted of a 5 cm diameter, 2 mm thick circular piece of stainless steel with a 1.6 by 1 cm recess milled across the center of the holder to accommodate the microfluidic chip. A circular piece of stainless steel with the same diameter as the holder held the chip from the rear. A 20 × 1 mm slit was cut into the center of the holder to allow the laser light to irradiate the channel of the chip and allow passage of ions into the flight tube.

The IR source was a Nd:YAG pumped OPO that is tunable from 1.45 to 4.0 μm and has a pulse width of 6 ns. All experiments were run at 2.95 μm wavelength at a repetition rate of 2 Hz. The OPO output was focused onto the sample to a spot size of ~200 by 300 μm as determined using laser burn paper and a measuring magnifier. At this spot size, IR fluence ranged from 7000 to 10,000 J/m^2. A new spot on the microfluidic chip channel was irradiated by each laser shot by moving the chip across the path of the IR laser either manually or using a micrometer on a linear motion feedthrough that was driven by a variable speed motor. Alignment of the laser with the center of the gel filled microfluidic channel was achieved by observing ablation of the gel on a video camera.

Data Acquisition

A 500 MHz digital oscilloscope (model LT372, LeCroy, Chestnut Ridge, NY) was used to record mass spectra that were later downloaded to a computer for processing. Mass spectra were obtained in either manual or automated acquisition modes. Mass spectra were obtained manually by continuously moving the laser down the length of the channel while observing the fluorescence of the target on a video camera. Spectra were saved after 5 to 10 laser shots that generated analyte signal on the oscilloscope. Alternatively, mass spectra were obtained in automated mode by moving the chip under the laser beam using the micrometer drive described above and saving all data obtained every 5 laser shots for the entire length of the gel chip channel. The chip was moved at a rate of 1.3 mm/min and mass spectra were averaged over 5 laser shots with 50,000 data points and a flight time resolution of 1 ns per data point. The mass spectra were downloaded and stored as the experimental data collection progressed in real time. Fifty to 100 spectra were downloaded over the course of a typical experiment.

Results and Discussion

In our previous study [22], IR LDI mass spectrometry of peptides and proteins separated using conventional SDS-PAGE was performed using an 80 × 73 × 0.75 mm gel. A visualization dye was necessary to locate the position of the migrated analyte. Once located, the spot was excised and attached to the sample target using double-sided conductive tape. A wavelength of 2.94 μm from an OPO was used to desorb and ionize intact peptides and proteins without the addition of a matrix. In the current method, the entire gel chip is loaded into the mass spectrometer and irradiated by the IR laser. No visualization dyes are necessary and the size of the gel is greatly reduced. With the exception of the visualization dye and its solvents, the same material is used in the current study and in the previous study.

All of the capillary gel microfluidic mass spectra were obtained after the analytes had been electrophoretically transported through a closed chip channel. Operation with a closed channel requires that the PDMS cover be removed from the PMMA microfluidic chip before insertion of the chip into the mass spectrometer. When removing the PDMS cover from the PMMA chip, sections of the gel tended to adhere to the surface of the PDMS. With some amount of care, the entire gel lane could be extracted intact from the chip, and initial mass spectra were obtained by mounting the PDMS cover in the mass spectrometer. Figure 2a shows the IR-LDI mass spectrum of the peptide bradykinin (M_r = 1060.2) desorbed and ionized from the PDMS cover. The bradykinin was loaded into the chip at a concentration of 2.5 mM and injected into the chip channel using a field of 150 V/cm for 9 min. The spectrum is the result of 10 laser shots at 2.95 μm wavelength. The base peak of the spectrum is assigned to the singly-protonated bradykinin molecule, $[M + H]^+$. The sodium adduct, $[M + Na]^+$, is present in the spectrum, but at low intensity; less than 5% as intense as the protonated peptide. An adduct of acrylamide with bradykinin was observed 71 *m/z* to the high *m/z* side of the base peak. Several peaks were observed below *m/z* 400 that were

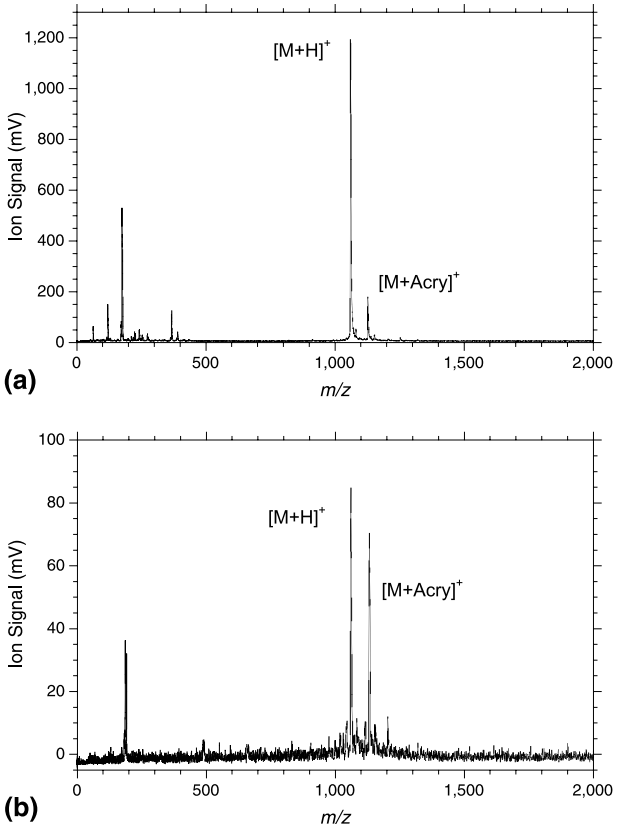

Figure 2. Mass spectra of bradykinin in a gel microfluidic chip obtained by 2.95 μm infrared laser desorption/ionization of (**a**) the PDMS cover and (**b**) the PMMA microfluidic chip.

tentatively assigned to ionized gel components. No visualization dyes were used, which may account for the lower number of peaks in the low *m/z* region compared to the previous study [22].

Although IR-LDI from the PDMS resulted in excellent mass spectra, it was difficult to obtain an intact gel along the full length of the channel. Furthermore, the flexible cover could not be easily translated with respect to the laser desorption spot, which prevented interrogation of the full length of the gel. With sufficient care, it was possible to remove the PDMS cover and leave the gel intact in the channel. Laser desorption/ionization from the PMMA gel microfluidic chip is shown in Figure 2b. The sample loading and electrophoresis conditions were identical for Figure 2a and b. The spectra in Figure 2 are similar in that the protonated bradykinin is the base peak and the low *m/z* region of the mass spectrum is nearly free of interfering peaks. However, the analyte signal is approximately ten times lower in Figure 2b and the intensity of the acrylamide adduct peak is nearly identical to the $[M + H]^+$ peak intensity. From observing the ablation of the gel using the video camera and later inspection of the irradiated gel, the depth ablated by each laser shot is estimated to be ~50 μm. Because the laser is continuously moved across the surface of the gel, the entire volume of gel in the 150 μm deep microchannel is not removed by the IR

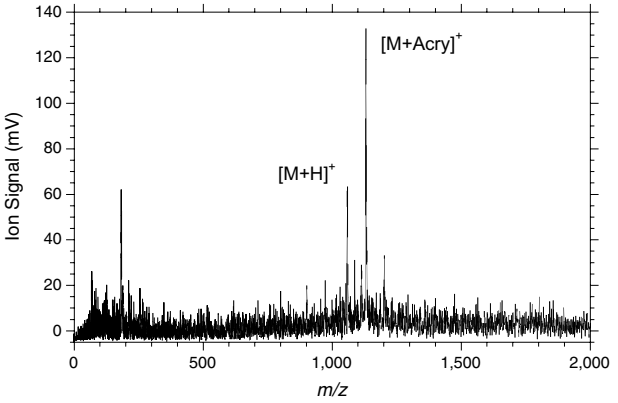

Figure 3. Single shot 2.95 μm infrared laser desorption/ionization mass spectrum of 1 pmol bradykinin injected into the gel channel.

laser. The OPO penetration depth, together with the better quality of the mass spectra obtained from the PDMS cover slip, suggests that the analyte is migrating to the bottom of the gel channel and is more accessible to the desorption laser when the gel is removed from the channel. Another possibility is the difference in the ion extraction field between the recessed gel channel and the protruding gel on PDMS cover slip, which may affect the efficiency of ion extraction.

An estimate of the detection limit for the microfluidic gel chip was obtained using a 100 μM bradykinin solution loaded onto the chip using 150 V/cm for ~9 min. and electrophoretically driven through the microfluidic chip channel. The mass spectrum corresponding to this injection is shown in Figure 3 and represents the minimum quantity of peptide required to obtain a mass spectrum from the gel chip channel. When the quantity of analyte is reduced, the ratio of $[M + H]^+$ to the acrylamide adduct drops. The relative intensity of the b_8 fragment ion at *m/z* 906 is also proportionally larger, possibly due to the higher laser energy used with the lower concentration sample. The volume of the channel intersection is ~10 nL. If it is assumed that the volume is filled with analyte solution, then a 1 pmol quantity of analyte was injected. This represents greater than 1000-fold improvement over the previously reported detection limit for a full sized gel [22]. This estimate does not include the volume of the channel displaced by the gel medium (which would decrease the estimate) or the possible effects of stacking in loading the channel (which would increase the estimate).

The IR-LDI mass spectra of bovine insulin electrophoretically run through the gel microfluidic chip is shown in Figure 4. The concentration of the solution that was loaded onto the chip was 2.5 mM and the material was injected into the channel using 150 V/cm for 9 min. The base peak in the mass spectrum is assigned to the singly protonated insulin molecule $[M + H]^+$. An adduct of acrylamide with bovine insulin molecule is observed as well as several other adducts in

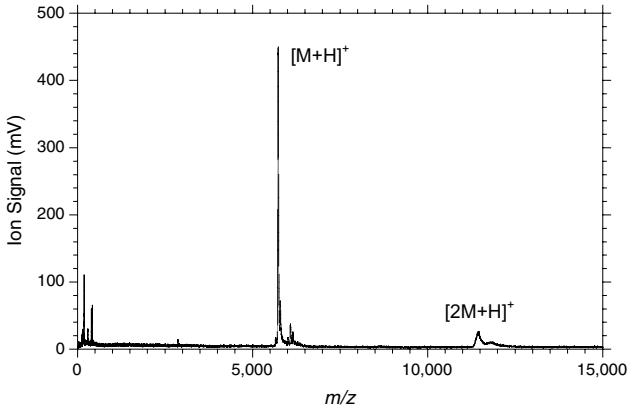

Figure 4. Infrared laser desorption/ionization mass spectrum of bovine insulin injected into a capillary gel chip.

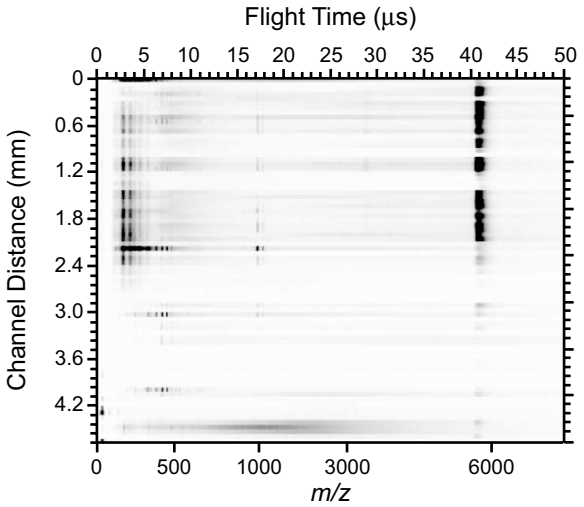

Figure 5. A two-dimensional representation of a 2.95 μm laser desorption/ionization mass spectrum scan of a gel microfluidic chip loaded with a mixture of bradykinin and bovine insulin. The chip was translated past the laser and the mass spectra were recorded automatically.

a small cluster of peaks centered around 6100 Da. The peaks at *m/z* 6037, 6110, and 6178 are most likely adducts of SDS (M_r = 288.5), SDS + acrylamide (M_r = 359.6) and SDS + bisacrylamide (M_r = 442.7), respectively. As is typical of spectra obtained with no added matrix, there are few peaks in the *m/z* region below 500 Da. Insulin was the largest mass protein that could be detected reliably on the gel chip. Mass spectra of the protein cytochrome *c* (M_r = 12,362) could be obtained with difficulty, but the mass resolution and signal were poor (data not shown).

A separation of the bradykinin and bovine insulin was accomplished in a gel microfluidic chip, and the chip was analyzed in an automated fashion in the mass spectrometer. The chip was driven by the motor at a rate of 1.3 mm/min, and mass spectra were recorded continuously by the oscilloscope and downloaded to the computer in real time. A total of 78 mass spectra were recorded and these are represented by the two-dimensional contour plot in Figure 5.

The x-axis in Figure 5 corresponds to ion flight time, whereas the y-axis corresponds to distance scanned on the chip channel. The *m/z* scale is indicated at the bottom of the plot. Intensities in the mass spectra are represented by grayscale shade: dark regions correspond to high intensities and light areas correspond to low intensities. Peaks from the gel in the *m/z* region below 500 appear as vertical stripes between 2 and 5 μs flight time. Because these peaks appear in all mass spectra, regardless of the presence of analyte, their intensity gives an indication of the overall efficiency of ionization at a given point on the gel channel. It can be seen that the efficiency between 0 and 6 mm is good, but then drops off. This may be due to the gel being partially removed from the channel in this region. The bradykinin is observed at a flight time of 17 μs and the insulin at a flight time of 40 μs. Both bands are spread out over several mm, probably due to the analytes sticking on the channel wall as they migrate through. The bradykinin is observed from 1 to 3 mm and the insulin is highest in intensity between 0 and 2 mm. The substantial electroosmotic flow (EOF) as well as potential interactions between the polypeptides and the channel walls can induce band spreading over the distance of the microchannel. The effect of solute-wall interactions and a biopolymer's electoosmotic flow has been reported for DNA separations in polymer microchips [33–36]. Dispersion of the analytes over a large distance in the microchannel translates into less material under the laser at any given position, which adversely affects the achievable detection limit.

Conclusions

Soft laser desorption ionization from a capillary gel microfluidic chip has been demonstrated using a 2.95 μm pulsed infrared laser and no added matrix. The gel chips were machined in a simple tee structure from PMMA and covered with an annealed PDMS cover. After electrophoresis, the PDMS cover was removed and the gel inserted into vacuum and ablated with the pulsed laser to form ions. The gel could be analyzed when attached either to the PDMS cover or in the PMMA chip. Ionization from the PDMS resulted in approximately 10 times larger signal, possibly due to the larger quantity of analyte on the exposed area resulting from the removal of the gel from the microfluidic channel. Excellent mass spectra were obtained from pure mass standards bradykinin and insulin using pmol quantities of material injected into the gel channel. The mass range for this approach is limited to less than 10 kDa, as has been reported previously [22]. An automated analysis of an off-line separation shows broad electrophoretic bands, suggesting that the analyte is sticking to the polymer as it migrates through the chip. Ongoing work is focused on surface coatings to reduce analyte sticking and minimization of the dead

volume between the gel and cover to reduce band spreading. Further reduction in the size of the gel channel coupled with multiple gel lanes per chip will improve the limit of detection and reduce the throughput time for each sample.

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