Mixed-mode electrokinetic and chromatographic peptide separations in a microvalve-integrated polymer chip†

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A cycloolefin polymer chip supporting the concatenation of isoelectric focusing (IEF) and reversed-phase liquid chromatography (RPLC) is demonstrated for high throughput two dimensional peptide separations. A unique benefit of the mixed-mode platform is the ability of IEF to act as a highly concentrating electrokinetic separation mode for effective isolation of sample components prior to RPLC. The thermophilic chip contains integrated high pressure microvalves, enabling uniform sample transfer from the IEF channel to multiple parallel RPLC channels, gradient elution from each RPLC column, and hydrodynamic isolation between the separation dimensions. The reusable system is shown to provide efficient 2-D separations together with facile interfacing with MALDI-MS, suggesting a new path towards effective peptide analysis from complex samples.

Introduction

Due to the large range of relative abundance typical of biological specimens, estimated to be greater than 6 orders of magnitude for the case of isolated cell populations and over 10 orders of magnitude for blood plasma, the global analysis of proteins remains a great challenge for modern analytical methods. While mass spectrometry is a highly successful technique for proteomic identification and quantification, the complexity and dynamic range of proteins within biological samples far exceeds its analytical capability. Thus, effective fractionation of intact proteins or peptides from biological samples is necessary to reveal a deeper view of the proteome, particularly for low-abundance components.

When selecting a front-end separation method for proteomic studies, peak capacity is an important criterion. While relatively high peak capacities can be achieved with single dimensional separation methods such as capillary isoelectric focusing (CIEF) and ultra-high pressure liquid chromatography, multidimensional separations generally offer greater resolving power. According to the theoretical prediction of Giddings, the maximum peak capacity of a multidimensional separation is the product of peak capacities of the individual separation dimensions, provided that the separation mechanisms are completely orthogonal. Two dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a multidimensional separation technique commonly used for the analysis of intact proteins. To overcome the intrinsic disadvantages of 2-D PAGE, including poor reproducibility, low analysis speed, and difficult automation, a variety of on-line liquid-phase multidimensional separation techniques have been developed by hyphenating different chromatographic or electrokinetic separations. Link et al. developed a 2-D LC method for multidimensional protein identification technology (MudPIT) employing strong cation exchange (SCX) in the first dimension and RPLC in the second dimension for the separation of proteolytic digests. Moore et al. combined RPLC and capillary zone electrophoresis (CZE) to separate complex peptide mixtures, and later added a size exclusion chromatography (SEC) front end to the RPLC-CZE platform, resulting in an on-line three dimensional separation system. Other examples include the hyphenation of capillary sieving electrophoresis (CSE) and micellar electrokinetic chromatography (MEKC), IEF and transient isotachophoresis/zone electrophoresis (ITP/CZE), and IEF coupled with capillary gel electrophoresis (CGE). In addition to these on-line techniques, various off-line multidimensional separations have also been developed, in which resolved species eluted from the first separation dimension are collected in multiple fractions and sequentially loaded into the second dimension. Examples of this off-line approach include ion exchange (cation/anion) chromatography-RPLC, RPLC-CZE, SEC-SCX-RPLC, and strong anion exchange chromatography-RPLC-PAGE.

Rational selection of separation modes for each dimension is necessary to maximize overall separation performance. Lee and co-workers demonstrated that when used as the first separation dimension of a multidimensional system, highly concentrating electrophoretic modes including IEF and ITP may be used to efficiently isolate sample components in exceptionally small peak volumes, thereby reducing or eliminating sample overlap between adjacent fractions collected for analysis in the second dimension. By maintaining high analyte concentrations in small peak volumes via electrokinetic focusing or stacking in the first dimension, and using a fully orthogonal RPLC separation in the second dimension, both the complexity and dynamic range of down-stream fractions analyzed by MS were substantially reduced. This concept has been successfully demonstrated using both on-line and offline capillary systems. However, the throughput of these capillary systems is relatively low, with analysis times on the order of 6–8 h to complete a full 2-D separation.

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Together with these advances in capillary separations, multidimensional systems based on microfluidics technology have been explored to leverage potential benefits including small sample and reagent consumption, high analysis speed, and high levels of integration. Microfluidic platforms are particularly well suited for multidimensional separations due to their ability to perform parallel analyses with a single device, together with seamless and low dead volume fluidic interfacing to hyphenate multiple separation dimensions. Microfluidic systems have been developed with coupled separation modes including capillary electrochromatography (CEC)-CZE, MEKC-CZE, CGE-MEKC, IEF-CZE, and IEF-CGE/CZE. In these examples, a single second dimension separation channel was used to sample sequential fractions eluted from the first dimension. Multidimensional microfluidic systems using multiple parallel second dimension separations have also been reported, combining IEF with multiplexed CGE as a microfluidic analog of traditional 2-D PAGE. It is notable that in each of these aforementioned systems, electrokinetic modes are used for both separation dimensions. However, electrokinetic separations are susceptible to variations in factors such as separation channel surface conditions, buffer pH and ionic strength, which can be difficult to repeatably control in a microfluidic system. In addition, the second dimensions of these systems have insufficient loading capacity to effectively resolve some highly concentrated fractions from the first dimension. Replacing one or both electrokinetic dimensions with pressure-driven LC can potentially avoid these issues and thus improve 2-D separation performance.

Here we describe a microfluidic cyclic olefin polymer (COP) chip combining IEF and pressure-driven multiplexed RPLC (RPLC) as the first and second separation dimensions, respectively. To our knowledge, this work represents the first example of a microfluidic platform combining an electrokinetic separation with pressure-driven liquid chromatography. Leveraging our recent efforts on microfluidic IEF-CGE and single-dimension RPLC, the IEF-mRPLC chip contains an individual IEF channel intersecting an array of RPLC micro-columns. High-pressure microfabricated shut-off valves integrated on the chip serve to isolate bulk solvent flow during the mRPLC separations, allowing uniform sample transfer and acetonitrile gradient delivery to each RPLC column. The IEF-mRPLC chips inherit the multiplexing advantages of microfluidic IEF-CGE, while overcoming several disadvantages of this earlier work including limited reproducibility and challenging MS interfacing.

The utility of the microfluidic system towards bottom-up proteomic studies is demonstrated by chip IEF-mRPLC separations and MALDI-MS analysis of a label-free protein digest. Results obtained with the prototype IEF-mRPLC chips suggest that this technique can potentially provide both high resolving power and high separation throughput, essential to the proteomic analysis of complex biological samples.

**Experimental**

**Materials**

Angiotensin fragment 1–7, bovine serum albumin (BSA), cytochrome c, urea, dithiothreitol (DTT), iodoacetamide (IAM), fluorescein isothiocyanate (FITC), 40% ampholyte pH 3.0–10.0 solution, hydroxypropyl methyl cellulose (HPMC), ammonium bicarbonate, trypsin, trifluoroacetic acid (TFA), dimethylsulfone (DMSO), acetonitrile (ACN), HPLC grade, z-cyano-4-hydroxy cinnamic acid (CHCA), butyl methacrylate (BMA), ethylene glycol dimethacrylate (EDMA), 2,2′-dimethoxy-2-phenyl-acetophenone (DMPA), poly(ethylene glycol) (PEG), 1,4-butanediol, and 1-propanol were purchased from Sigma-Aldrich (Saint Louis, MO). Pre-cast 2.0 mm thick Zeonor 1020R cyco-olefin polymer (COP) plaques were procured from Zeon Chemicals (Louisville, KY). Isopropyl alcohol (IPA), deionized water (HPLC grade), acetone, methanol, cyclohexane, sodium hydroxide, and phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA). Sylgard 184 polydimethylsiloxane (PDMS) silicone elastomer was purchased from Dow Corning (Midland, MI).

**Chip fabrication**

The IEF-mRPLC chip design is outlined in Fig. 1. The design was fabricated by direct micromilling of COP plaques using a MDX-650A computer numerical control (CNC) machining system (Roland ASD, Lake Forest, CA). The RPLC columns are 254 μm wide, 130 μm deep and 42 mm long. With the same width as that of the RPLC columns, the IEF channel has a depth of 180 μm and a total length of 70 mm. To accommodate the on-chip microvalves, the width and depth of two 2.7 mm long IEF channel sections were reduced to 130 μm and 100 μm, respectively, with their centers locating 10 mm away from the anolyte or catholyte reservoir. The upper flow splitter network is composed of 130 μm wide and 100 μm deep channels. The RPLC columns and the upper channel network are connected to the IEF channel through short constrictions consisting of 700 μm long, 85 μm wide and 60 μm deep channel sections which serve to...
minimize sample dispersion during IEF. The connecting channels between the RPLC column exits and the waste reservoirs have the same channel width and depth, while the average length is 8 mm.

The micromilled COP substrate was bonded to COP cover plates pre-drilled with 650 μm diameter needle access holes, 533 μm diameter microvalve ports, 533 μm diameter anolyte/catholyte reservoirs and 1.8 mm diameter waste reservoirs using a solvent-bonding process. Before bonding, threads were created on the inner surface of the microvalve ports using a threaded needle with a pitch of 140 μm, which was prepared from a hypodermic needle segment (710 μm o.d., 150 μm i.d., 2.54 cm long; Hamilton Company, Reno, NV) using a miniature jeweler die (American Science and Surplus, Skokie, IL). Both the micromilled and the cover plates were then thoroughly rinsed with acetone, methanol and water, and dried with nitrogen gas and degassed overnight in a vacuum oven at 80 °C. The cover plate was cooled to room temperature, then incubated in a glass container partially filled with cyclohexane, with the bonding side positioned 5 cm from the solvent surface. The container was heated to 30 °C to generate a controlled cyclohexane vapor pressure to solubilize the exposed COP surface. After 8.5 min, the cover plate was removed from the container and manually aligned and pressed to the micromilled substrate. The chip assembly was immediately placed in a hydraulic press (Auto-Four/15; Carver, Wabash, IN) at 300 psi for 2 min at room temperature to achieve a permanent bond. High pressure world-to-chip interfaces and microvalves were fabricated on the COP chips following protocols reported previously.

**RPLC column array preparation**

Commercial C\textsubscript{18} silica particles (5 μm Zorbax C\textsubscript{18}; Agilent Technologies, Santa Clara, CA) were used as a stationary phase for the RPLC column array. While our previous efforts to develop microfluidic RPLC columns focused on the use of polymethacrylate monoliths as a stationary phase, silica particles were employed here to speed the development process. To retain the particles in the columns, a 1 mm long polymethacrylate monolith frit (Fig. S1) was fabricated at each column exit by UV photopolymerizing a pre-filled monomer solution containing 23.5 wt% BMA, 15.5 wt% EDMA, 34 wt% 1,4-butanediol, 26 wt% 1-propanol and 1 wt% DMPA through a mask using a UV source (PRX-1000; Tamarack Scientific, Corona, CA) with a power of 23 mW/cm\textsuperscript{2} and exposure time of 10 min. After thoroughly rinsing the IEF channel and upper channel network with IPA and water to remove the residual monomer solution, the two on-chip microvalves were simultaneously closed by hand. The chip was connected to an analytical HPLC pump (PU-2089; Jasco, Easton, MD) to condition the RPLC columns with acetonitrile for 5 h followed by water for 3 h at a constant pressure of 3 MPa.

**Protein digest preparation**

Denatured protein stock solution was prepared by dissolving the sample in 50 mM ammonium bicarbonate buffer with 8 M urea and 10 mM DTT to a final concentration of 25 mg/mL. Reduction of the protein was facilitated by keeping the solution at 50°C for 15~20 min. After cooling to room temperature, IAM was added to the reduced protein solution to a concentration of 20 mM to quench the reduction and alkylate the denatured protein with free sulphydryl ends. The alkylation reaction was allowed to proceed in dark at room temperature for 1 h, then 200 μL of denatured protein solution was mixed with 1.8 mL of 50 mM ammonium bicarbonate solution to decrease the urea concentration below 1 M. Trypsin was added to the solution to start digestion, with a weight ratio of 1 : 30 (trypsin/protein). The solution was incubated at room temperature for 16 h and stored at −20°C before use.

**Sample labeling**

Labeling for on-chip fluorescence detection was performed using FITC dissolved in DMSO to a concentration of 20 mM. The digest prepared from the solution with a protein concentration of 2.5 mg/mL (∼38 μM) was mixed with FITC stock solution at a molar ratio of 20 : 1 (FITC/protein). The labeling reaction was kept in dark at room temperature for 48 h. Before loading onto the chip, the FITC-protein digest solution was diluted 2.5-fold using an aqueous solution containing 2% ampholyte (pH 3.0–10.0) and 0.4% HPMC. The same labeling procedure was used to prepare FITC-peptides.

**IEF-mRPLC separations with on-chip fluorescence detection**

The experiment setup for on-chip IEF-mRPLC separations is shown in Fig. 2. In a typical experiment, the IEF channel and upper flow splitter network were filled with water after RPLC column conditioning, with both microvalves open. The off-chip shut-off valve was then closed and the selection valve switched to position 2 to seal the inlet port, followed by vacuum withdrawing of a 0.4% HPMC coating solution to the IEF channel. The coating solution was kept in the channel undisturbed for 30 min to allow HPMC to adsorb onto the COP channel surface in order to reduce non-specific interactions between the channel walls and sample components. The coating procedure is required in every experiment because HPMC can be rinsed off the IEF channel during gradient mRPLC. Immediately after coating, the sample solution was withdrawn from the anolyte reservoir to the catholyte reservoir by vacuum, and catholyte (35 μL of 0.5 M H\textsubscript{3}PO\textsubscript{4}) were added to their respective reservoirs. A high-voltage power supply (CZE 1000R; Spellman High-Voltage Electronics, Plainview, NY) was used to apply a voltage of 1000 V through two platinum electrodes inserted into the anolyte and catholyte reservoirs to perform the IEF separation. After 30 min, the high voltage was removed and the two microvalves were simultaneously closed by hand. The
The selection valve was switched to position 3 and the HPLC pump was turned on to deliver water at 2 μL/min through the splitter network, allowing uniform injection of peptide bands focused in the IEF channel into the RPLC columns. Following 1 min sample injection, the off-chip shut-off valve was opened to allow flow splitting, and a 30 min long solvent gradient (0–40% ACN) was delivered from the HPLC pump at a total flow rate of 0.6 mL/min. The columns were swept with neat ACN for 1 min after completing the gradient. The individual column flow rate, determined by measuring the elution volume over a fixed period of time, varied with the progress of gradient elution from 2.9 μL/min to 2.2 μL/min, resulting in an average splitter flow ratio of 240 : 1.

An inverted epi-fluorescence microscope (TE-2000S; Nikon, Melville, NY) and an Amixa CFR MALDI-TOF mass spectrometer (Shimadzu Scientific Instruments, Columbia, MD) were employed for detection of separated FITC labeled and label-free peptide sample respectively. The IEF-mRPLC chip shown in Fig. 1 was used for fluorescence detection. Waste reservoirs in the chip were replaced with the needle interfaces (710 μm o.d., 400 μm i.d., Hamilton Company) to collect fractions for MALDI-TOF MS analysis.

The fluorescence detection was performed by fastening the chip on the automated XY microscope stage (Nikon) and moving the device to position the detection point in the center of the detection region of the RPLC column array. An excitation wavelength within the range of 465–495 nm was selected using a B-2E/C blue filter (Nikon) to detect the analytes. A 4×, 0.20 N.A. objective (Nikon) was used for simultaneous imaging and detection. Fluorescence intensity was recorded using a CoolSnap HQ2 CCD camera (Roper Scientific, Tucson, AZ) installed on the microscope and chromatograms were generated using Nikon Advanced Elements image analysis software.

IEF-mRPLC separations coupled with MALDI-MS

In chip IEF-mRPLC-MALDI-MS analysis, both water and acetonitrile were acidified with 0.1% (v/v) TFA and used in columns conditioning and RPLC separations. To avoid the impacts of TFA to IEF, the acidic mobile phase in upper channel network and IEF channel was replaced with water at a flow rate of 20 μL/min using a syringe pump connected to position 1 of the selection valve (Fig. 2). The shut-off valve was closed and the two microvalves were open during the 15 min rinsing. LC separation conditions were the same as those used during fluorescence detection experiments, and the digest sample was diluted to 1.0 mg/mL using a solution containing 2% ampholyte pH 3–10 and 0.4% HPMC before loading.

Sample collection for MALDI-MS analysis was performed by bringing the spotting needle tips in close proximity to a MALDI target plate to allow the eluents from mRPLC separation to be directly deposited onto the target every 6 min over a 36 min period. After air drying the sample droplets at room temperature, 1 μL of a matrix solution prepared by saturating CHCA in 0.1% (v/v) TFA acidified acetonitrile was deposited on top of each sample spot. After matrix/peptide co-crystallization, the target plate was loaded to the MS instrument. All MS spectra were recorded in the linear, positive ion mode using a 337 nm nitrogen laser with a 3 ns pulse width and an effective laser spot size of 200 μm. Each MS spectrum was obtained by averaging spectra generated by 400 laser pulses using Kompact mass spectrum analysis software (Shimadzu). The MS data was visualized using custom code developed in MATLAB 6.5 (Math-Works, Natick, MA) software.

Results and discussion

IEF separations

To avoid dispersion of sample into the upper channel network and RPLC column heads during sample injection into the IEF channel, which would result in interference during the following LC separations, the channels connecting the upper channel network and RPLC columns to IEF channel were fabricated with a cross sectional area 9-fold smaller than that of IEF channel, while the cross sectional area of the upper channels was reduced by 3.5-fold in comparison to IEF channel. Similar connecting interface has been fabricated by Emrich et al. in their microfluidic 2-D electrophoresis chips, with a cross sectional area 65-fold smaller than the separation channels to provide excellent dispersion resistance during reagent loading. Wider interfaces were used in this work to avoid obstruction of silica bead transport in slurry packing. To prevent sample dispersion to
upper channel network through the interfaces, vacuum pressure was adjusted below 25 kPa during sample introduction. Sample intrusion to packed columns was difficult since the columns had high fluidic resistance. The effectiveness of this design can be seen in Fig. 3A, which shows sample nearly completely constrained within the IEF channel following vacuum introduction of FITC labeled peptides. An additional benefit of this design is that electrical resistance of the upper splitter network is increased, thereby minimizing electrical crosstalk during IEF.

During early chip tests, the strongly hydrophobic COP channel surface was found to provide multiple non-specific adsorption sites for FITC labeled peptides, causing sample loss and significant decreasing IEF resolution, capacity and reproducibility. Therefore, surface modification is necessary to deactivate the COP surface before separations. A common surface treatment for cycloolefin polymer/copolymer channels involves the use of high energy UV radiation to graft functional groups or fouling-resistant coatings onto the channel surface. Unfortunately, grafted coatings can be easily damaged by the harsh environment of repeated solvent rinses and LC separations, requiring re-grafting following each separation and column regeneration step. Instead, dynamic coating approach is preferable for many thermoplastic chip systems because of its simplicity, robustness and ease of replenishing the coating after each run. A series of common surface active regents for dynamic coating such as polyvinyl alcohol, polyethylene oxide and celluloses were tested in the 2-D chips. We found that 0.4% HPMC gave repeatable IEF results and did not significantly affect the following LC separations, while the others either cannot effectively prevent nonspecific adsorption of focused peptides to COP channel surface during IEF, or became so viscous in the solvent gradient that the packed columns was clogged.

Using HPMC dynamic coating, focused peptide bands were repeatably generated during IEF, as shown in Fig. 3B for the case of BSA digest. This figure also reveals limited dispersion of analyte into the constrained channels linking the RPLC columns, presumably due to electrokinetic mobilization from fringing fields at the channel junctions. However, the amount of the trapped samples was much smaller than that of the focused analytes in the IEF channel, and significant carryover of peptides between the LC fractions was not observed. It is notable that besides providing sample enrichment and efficient isolation of peptide species, IEF also serves to provide sample cleanup. Since FITC is not zwitterionic but negatively charged, free dye can migrate out of the sampling region toward the anode during IEF, thus preventing interference within the RPLC columns.

### IEF-mRPLC separations

An obvious difference between the current 2-D system and other reported chip 2-D separations is that pressure-driven mRPLC is utilized as the second separation dimension. The major advantage of this practice is that truly orthogonal 2-D separation can be performed to maximize the overall peak capacity. In a typical IEF-mRPLC chip, the effective peak capacity of the first IEF dimension is determined by the total number of RPLC columns, which is limited by the available chip area. Therefore, it is important to acquire high peak capacity in the second dimension to increase the total peak capacity of the chip. The peak capacity of the 4.2 cm long RPLC columns was evaluated by separating FITC-cytochrome c digest in a 30 min solvent gradient (1.33% ACN/min; starting from 0% ACN; no IEF). A typical chromatogram is shown in Fig. 4A. Based on the separation results, the apparent peak capacity of the column is estimated to be 43 by the method of Dolan et al. Since the same LC separation conditions were employed in 5 column IEF-mRPLC experiments, the overall peak capacity of the chip is calculated as 215, comparable to the peak capacity of commercial narrow bore RPLC columns packed with 3.5 μm particles. However, the number of LC channels, column length, stationary phase selection, column preparation, and gradient LC conditions are not optimized for the proof-of-concept device. There is still much space for further improvement of the performance of IEF-mRPLC chips.

For the 2-D separations, it is also crucial to maintain equal flow rates and gradient profiles for all columns in the parallel separation system. We adopted a symmetrical flow splitter design.
to ensure that LC pump pressure and the mobile phase can be distributed equally to each RPLC column during slurry packing and gradient separations. To evaluate the flow uniformity of the IEF-mRPLC chip, we measured column flow rates using the optimized gradient conditions and found the column-to-column flow rate variation was 4.8% RSD. FITC-labeled angiotensin-(1–7) was injected to the chip and eluted with the same solvent gradient (Fig. 4B) without performing a first-dimension IEF separation. The column-to-column retention time variation was calculated to be 1.4% RSD according to the retention time of the labeled peptide. Together this data implies that a uniform gradient flow was obtained in the 2D chip, important to achieve reproducible and efficient IEF-mRPLC separations. The chromatograms shown in Fig. 4B also reveals that the peaks in columns 1 and 5 have a height approximately half of those in columns 2 to 4. This result was expected, since the injection volume for columns 1 and 5 is half that of columns 2–4 due to the current design of the IEF-mRPLC chip.

Chromatograms from each of the LC channels following a full 2-D separation of FITC-labeled BSA digest are shown in Fig. 5. Nearly all bands were eluted within 25 min. Note that the free dye peak found in 1-D μRPLC separation of BSA digest does not appear in 2-D separation since the free dye has been removed in IEF. Peptides in the 20–25 min interval were not observed in the 1-D separation, possibly because these peptides were present at low abundances and could not be detected in the 1-D separation without preconcentration. We estimate that about 25 components can be recognized in 1D μRPLC separation, compared with 66 in the IEF-mRPLC separation, corresponding to a 2.6-fold improvement in overall peak yield within the same separation time frame. However, unlike the μRPLC separation where all sample components were injected into the column, only those peptides with isoelectric points (pl) within the pH gradient established in the IEF channel were analyzed in IEF-mRPLC.

Interfaceing IEF-mRPLC with MALDI-TOF MS

Protein digests were labeled with fluorescent dyes such as FITC to allow the use of sensitive fluorescence detection for characterizing separation performance in the 2-D system. Unfortunately, the quantum yield of FITC decays significantly below pH 4,46 excluding the use of common mobile phases acidified with formic acid or trifluoroacetic acid for the 2nd dimension ion-pairing RPLC separations. Another disadvantage of using FITC is that analytes with multiple labels can be introduced to the sample during conjugation reactions, leading to confusing separation results, and uneven labeling efficiencies of different peptides also poses a problem. Therefore, methods enabling label-free detection are highly advantageous. In particular, MS analysis of unlabeled peptides can provide high resolution mass discrimination, enabling the identification of peptides and peptide modifications on the basis of molecular weight, and ultimately the identification of parent proteins through peptide mass fingerprinting.

Electrospray ionization (ESI)-MS has been routinely hyphenated to both capillary and microfluidic LC columns for peptide analysis.50,51 However, ESI-MS is not suitable for multiplexed separations due to practical limitations on realizing multiple mass analyzers to sample the parallel eluents simultaneously. In contrast, MALDI-MS is an off-line method that may be readily interfaced with multiplex separations by parallel deposition of eluents onto a MALDI target surface for later serial analysis,51,52 making this technique well suited for the IEF-mRPLC chips. After completing the deposition of all LC fractions, a 1 μL droplet of CHCA matrix solution was dispensed on top of each target spot for co-crystallization with the deposited peptides.

Using unlabeled BSA digest as a model system, MALDI-MS analysis was performed on a set of 6 sequential fractions deposited from each of the LC channels. Comparing the results obtained with and without the front-end separation, 48 unique BSA digest peptides were identified following IEF-mRPLC separations with 34% sequence coverage and a score of 91 as determined by Mascot (Matrix Science, Boston, MA), while only 18 unique peptides were identified by direct MALDI-MS detection of the whole digest deposited on the same target. Data obtained from the IEF-mRPLC fractions were converted to a set of 2-D cluster maps to show the distribution of the 30 highest intensity peptides (Fig. 6). Each map reveals the fraction and channel number in which the particular peptide was identified, with color saturation proportional to the integrated signal intensity for each identified peak. The cluster maps reveal that the majority of peptides were confined to specific channels and fractions with little overlap, demonstrating that the IEF-mRPLC separations are effective at isolating peptides by pI and hydrophobicity.

Using the ExPASy pI calculator,53 the pH gradient established within the IEF channel was estimated by determining the average
pI for all peptides observed within each LC column. The resulting pH plot, shown in Fig. 7, shows that peptides within each RPLC column have been efficiently segregated by pI. The trend deviates from the desired linear gradient, with a profile that covers a relatively narrow pH range from approximately 4.5–6, likely due to a combination of anolyte and catholyte ion deple-
tion within the IEF reservoirs, and voltage drops within the narrow channels used to define the elastomeric valves near the ends of the IEF channel. These issues can be addressed by enlarging the IEF reservoirs and modifying the channel design to reduce the voltage drop outside the desired focusing region, thereby enabling the mRPLC array to sample fractions from a wider pI range.

Conclusion

The combination of electrokinetic and parallel chromatographic separations in a seamlessly integrated chip opens the door to new separation modalities in a high throughput format. Separation of protein digests reveals that the peak capacity of IEF-mRPLC is enhanced when compared with 1—D μRPLC under the same conditions, without significantly impacting the speed of the overall separation. The use of IEF-mRPLC as a front end for MALDI-MS resulted in improved peptide identification, enhancing the success of protein database searching. Further improvements in resolving power and first dimension reproduc-
bility can be realized through microvalve automation, optimized IEF design and RPLC gradient conditions, and increased density of channels in the mRPLC array. Compared to previous capillary-based multidimensional separations,39–24 chip IEF-mRPLC simplifies the separation process by integrating both dimensions and key valving elements within a single integrated device, thus reducing sample loss during the analysis of low-quantity analytes and offering potential for the automation of complex analyses. Furthermore, due to the use of pressure-driven LC in the second dimension, various alternatives to RPLC may be readily imple-
mented in the chip format, such as hydrophilic interaction liquid chromatography or ion exchange chromatography, offering a range of options for realizing efficient, versatile and economic separations for proteomic studies and related bioseparations.

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References
