CHROMATOGRAPHY APPLICATION NOTE

A Microdispenser for LC Separation and MALDI MS Analysis

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Peptide mass fingerprinting (PMF) is a mass spectrometry protein identification technique based on peptide mass detection and is useful for differential expression analysis in the search for drug targets and for biomarker discovery.[1,2] The efficiency of PMF experiments can be related to the number of proteolytic peptide fragments that are identified. Maintaining the protein identification confidence level becomes more challenging when concentrations of protein in a sample are reduced, as in the case of a limited number of cells or tissue. The problem of reduced peptide detection is further exacerbated by proton competition between the high- and low-abundance analyte ions.[3,4] This can result in ion suppression and loss of detection of low-abundance peptides or proteins, thus reducing the sequence coverage for the protein of interest. Small-scale liquid chromatography separation can significantly enhance the detection of peptide and protein samples during MS analysis.[5–7] The LC separation partitions the coeluting low- and high-abundance analyte ions and reduces ion suppression effects.[8] Ultimately, this increases the sequence coverage for the protein of interest. A variety of small-scale LC separation modes can be performed either separately or in combination and include affinity capture, ion exchange, size exclusion chromatofocusing, and reversed phase.[9–11]

The PiezoLC (**MicroFab Technologies, Inc.,** Plano, TX, patent pending) is an inkjet system that delivers microvolumes (0.1–100 nL) of chromatographically separated proteolysed peptides onto matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS targets for subsequent MS analysis. A porous polymer monolith is located within the glass capillary of the inkjet dispensing device to provide chromatographic separation of peptides. The peptide sample is loaded onto the integrated chromatography column (reversed phase) and an elution buffer separates the peptides as the buffer passes through the column. The eluted peptides exit the orifice of the piezoelectric device in the form of drops and land on a MALDI-TOF MS target plate, preserving the chromatographic separation. The analysis of the peptides can then be performed at a later date and multiple times.

The quantity of protein and peptide materials available for PMF analysis is often limited, and miniaturization of the fluid handling for the mapping process is desired. The PiezoLC inkjet device utilizes a polymeric monolithic column and requires a small volume (<10 µL) of proteolysed material. Methacrylate-based polymeric separation media do not require the packing of beads or frits, which are difficult to incorporate in microfluidic devices and can interfere with fluid flow.[12] The large pore size of the polymeric monolithic material results in low backpressure, which is important for the efficient operation of the system's dispensing device. Polymerization by UV irradiation enables patterning and positioning of the monolithic column. Multiple chemistries/functions can be combined in one monolith, i.e., strong cation exchange (SCX) and reversed phase (RP). Additionally, these columns are robust and have fast separation due to rapid convective mass transfer.[13]

Figure 1 Left: Cross-section and porous structure of monolith. Right: fluorescein isothiocyanate (FITC)-labeled angiotensin II captured on monolith in glass capillary.

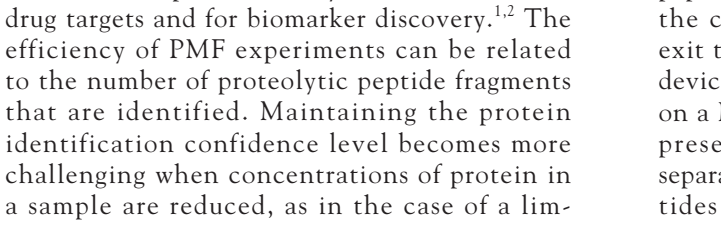

Figure 2 Schematic of the PiezoLC microdispensing device showing location of monolithic column "C" in proximity to orifice at distance "A."

The PiezoLC can be a useful tool for the identification and characterization of proteins from finite samples. The chromatographic separation of peptides by the inkjet system can reduce ion suppression and improve the resolution of MALDI-TOF MS analysis for applications such as peptide mass fingerprinting. The separation of tryptic digests of bovine serum albumin (BSA) resulted in a larger number of peptides identified in comparison to the control. This resulted in a higher degree of amino acid sequence coverage and improved protein identification.

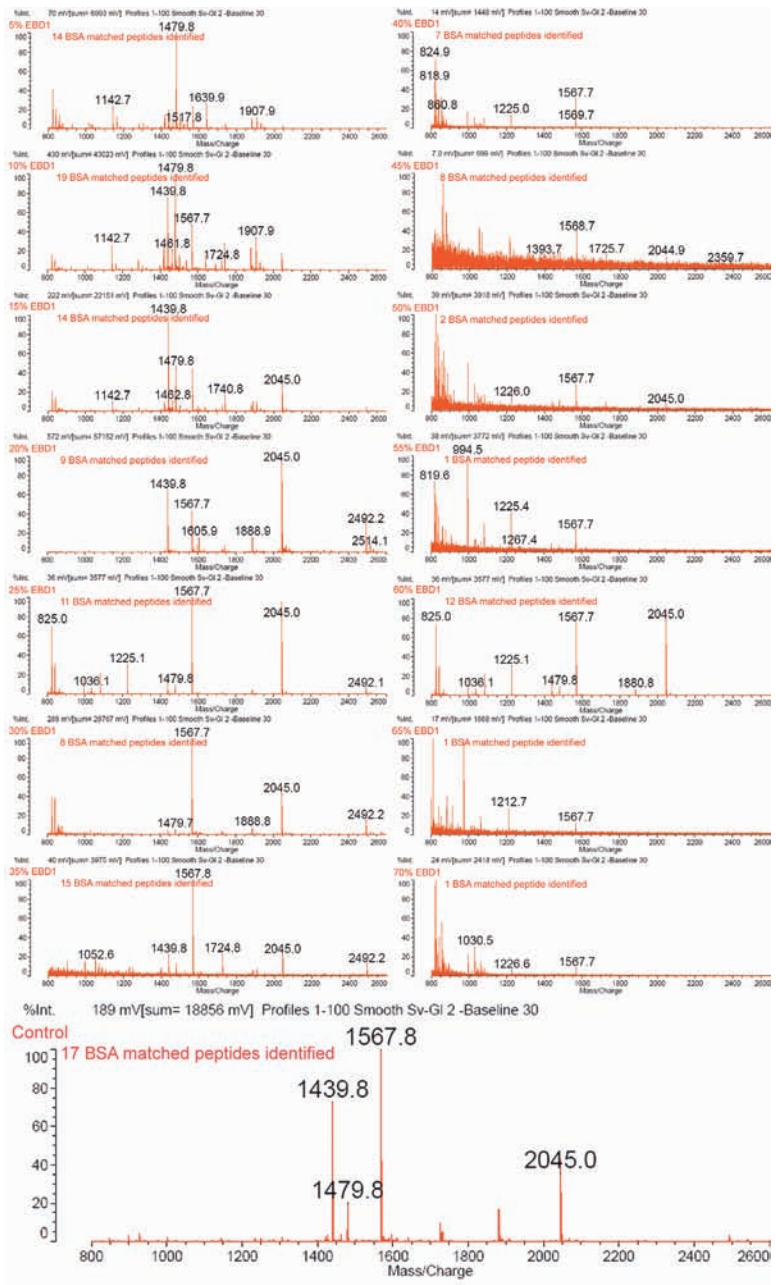

Figure 3 MALDI-TOF MS spectra of the BSA digest eluted from the PiezoLC device during a stepwise gradient elution of 5%–70% concentration. The number of BSA matched peptides during PMF is indicated. The bottom spectrum is from the 70% isocratic elution control.

Methods

BSA in solution digest

In-solution digests of BSA were performed as described by Kinter et al.[14] The BSA was mixed with 20 µg of trypsin (**Promega**, Madison, WI) and was incubated at 37 °C overnight.

Monolithic columns

1. *Borosilicate glass capillary vinylization.* Monolithic poly(butylmethacrylate-co-ethylene dimethacrylate)

Nominal mass (M_r): **69248**; Calculated pI value: **5.82**
NCBI BLAST search of P02769-00-00-00 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Bos taurus **Isocratic Control**

Variable modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **17**
Number of mass values matched: **17**
Sequence Coverage: **30%**

Matched peptides shown in **Bold Red**

```
    1 MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
   51 FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
  101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
  151 KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
  201 LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
  251 FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
  301 CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
  351 GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
  401 KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
  451 RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
  501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
  551 ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
  601 STQTALA
```

Nominal mass (M_r): **69248**; Calculated pI value: **5.82**
NCBI BLAST search of P02769-00-00-00 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Bos taurus **PiezoLC Stepwise Separation**

Variable modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **38**
Number of mass values matched: **38**
Sequence Coverage: **56%**

Matched peptides shown in **Bold Red**

```
    1 MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
   51 FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
  101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
  151 KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
  201 LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
  251 FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
  301 CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
  351 GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
  401 KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
  451 RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
  501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
  551 ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
  601 STQTALA
```

Figure 4 *MASCOT search results displaying matched peptides for* Bos taurus *from the PiezoLC isocratic elution and stepwise gradient separation of a BSA digest.*

capillary columns were prepared as described by Lee et al.[15] The internal wall surface of the PiezoLC borosilicate glass capillaries was vinylized to enable the covalent attachment of the monolith.

2. *Polymerization mixture*. The vinylized borosilicate glass capillaries were masked using opaque electrical tape and filled with the following polymerization mixture: 16% (v/v) ethylene dimethacrylate, 24% (v/v) butylmethacrylate, 59% (v/v) 1-decanol, and 1.0% (v/v) 2,2-dimethoxy-2-phenylacetophenone (**Sigma-Aldrich**, St. Louis, MO). The filled capillaries were irradiated for 15 min using a 365-nm 15-mW/cm^2 UV light source ELC 4001 (**Electro-Lite Corp.,** Danbury, CT). The pore size of the RP mode polymeric monolith was measured to be 2.2 µm.
3. *PiezoLC microdispensing device assembly*. The glass capillaries containing monoliths were used in the assembly of the PiezoLC inkjet microdispensing devices (*Figure 1*). Various dispensing tests were performed with the monolithic column in different locations relative to the dispensing orifice. The location of the column in the glass capillary shown in *Figure 2* was based on the least amount of interference with drop formation and dispensing.

Elution experiments

1. *Matrix solution*. Recrystallized α-cyano-4-hydroxycinnamic acid (αCHCA) (**LaserBio Labs**, Sophia-Antipolis, France) was dissolved in a 35% (w/v) solution containing equal parts of 1-propanol, methanol, 1-butanol, and acetonitrile (**Sigma-Aldrich**). The αCHCA solution containing 0.1% (v/v) trifluoroacetic acid (TFA) was deposited onto a stainless steel MALDI target plate (PN DE1271TA, **Shimadzu Scientific Instruments**, Columbia, MD) at 100 nL per spot using a piezoelectric dispensing device having a 55-µm orifice diameter (PN MJ-AT-01-55, **MicroFab Technologies, Inc.**).
2. *Column loading*. The PiezoLC device was flushed with 200 µL of 70% (v/v) acetonitrile and 0.1% (v/v) TFA, followed by a 200-µL flush of 0.1% (v/v) TFA. The BSA digest (50 fmol/µL) was loaded onto the column. The column was then flushed with 200 µL of 0.1% (v/v) TFA.
3. *Stepwise elution*. A stepwise gradient elution in 5% steps between 5% and 70% was performed using a solution containing equal parts of 1-propanol, methanol, 1-butanol, and acetonitrile (**Sigma-Aldrich**) and 0.1% (v/v) TFA (EBD1 elution buffer). A volume of 100 nL of eluent was overprinted onto 12 matrix spots per elution step at a flow rate of 6.0 µL/min. The control was an isocratic elution of the BSA digest using a 70% concentration of EBD1 elution buffer.
4. *MALDI-TOF MS and PMF*. MALDI-TOF MS analysis was performed in reflectron mode using an Axima CFR MALDI-TOF MS (**Shimadzu Scientific Instruments**) with 10 laser shots per profile and 100 profiles per sample spot. Spectra were internally calibrated using peptide masses of 1439.8117 Da and 2045.0279 Da. The peak smoothing method was Savitsky-Golay with peak picking method gradient–centroid. Peak lists of monoisotopic masses generated by the Axima software were uploaded to the MASCOT PMF search engine.[16] The SwissProt database was searched using the variable modification carbamidomethyl (C) and peptide tolerance of ±0.2 Da.

Results

MALDI-TOF MS spectra

The LC separation of the BSA digest peptides is visible when comparing the MS spectra for each elution buffer concentration in *Figure 3*. This figure displays the MALDI-TOF MS spectra from each gradient elution of BSA digest from the PiezoLC device using EBD1 elution buffer 5%–70% concentration.

The sequence coverage and the number of peptides identified for the isocratic-eluted control was 30% and 17, respectively. The PiezoLC separation of the same BSA digest using a stepwise gradient elution resulted in sequence coverage of 56% with 38 peptides being identified (*Figure 4*). This is an 87% increase in the sequence coverage and 124% increase in the number of peptides identified over the control percentage and number. No significant matches to BSA peptides were identified during the MASCOT search of the mass peak lists from the stepwise gradient elutions of 50%, 55%, 65%, and 70%. These results were consistent with other PiezoLC BSA digest elution experiments.

Conclusion

A drop-on-demand piezoelectric device containing a polymeric monolithic column has been used to dispense eluents of proteolysed material onto a MALDI-TOF MS target for PMF. The LC separation of the BSA digest peptides generated during stepwise gradient elution improved sequence coverage and protein identification. This microfluidic approach to protein digest sample separation can significantly facilitate the PMF of low-concentration proteins and offers a new instrument for proteomic analysis.

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