Spectroscopy 17 (2003) 693–698
IOS Press

Protein characterization using Liquid Chromatography Desorption Ionization on Silicon Mass Spectrometry (LC-DIOS-MS)

Jessica E. Prenni [a], Zhouxin Shen [b], Sunia Trauger, Wei Chen [b] and Gary Siuzdak [a,*]

[a] *The Scripps Research Institute, Center for Mass Spectrometry, Department of Molecular Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*

[b] *Mass Consortium Corporation, 3030 Bunker Hill Street, Suite 104, San Diego, CA 92109, USA*

Abstract. This paper presents a novel combination of off-line liquid chromatography (LC) separation with desorption ionization on silicon mass spectrometry (DIOS-MS) as an alternative to the traditional on-line coupling of LC and electrospray ionization (ESI). In this work, electrospray deposition is used to generate a spatially preserved linear track of the separated sample on a specially designed DIOS chip. The total sequence coverage analysis of two model protein systems was evaluated by both LC-DIOS-MS and nanoLC ESI tandem MS (MS/MS). LC-DIOS-MS yielded improved sequence coverage for both of the model systems (between 99.5 and 100%) compared with traditional LC-ESI-MS/MS analysis (between 82 and 87.6%). In addition to improved sequence coverage determination, LC-DIOS-MS also offers the potential for high-throughput protein characterization.

1. Introduction

Liquid chromatography (LC) coupled with mass spectrometry (MS) has become an indispensable technology in biochemical research where LC-MS and LC-MS/MS (tandem mass spectrometry) are extensively used in protein characterization and drug discovery [1–4]. The use of a separation technique, such as reverse phase LC, greatly reduces signal suppression of low-abundance ions in the analysis of complex mixtures such as protein digests. Traditionally, LC-MS techniques have been coupled with electrospray ionization (ESI) in a real-time analysis format. While such systems are easy to implement and widely used, the real-time nature of the analysis imposes restrictions on throughput and the sensitivity toward low intensity ions [3,5,6]. As an alternative, several researchers have recently reported advancements in the off-line coupling of LC separation with solid-state desorption/ionization, such as matrix-assisted laser desorption/ionization (MALDI). Peters et al. presented an approach in which an intermittent voltage was applied to a MALDI target plate resulting in the deposition of sample droplets of controlled size from an LC column (the matrix could be applied before, during, or after LC separation) [5,6]. The plate was moved in the x–y plane underneath the column for collection of the entire LC effluent, and was patterned with small hydrophilic regions to facilitate sample and matrix migration and concentration. Wall et al., utilized a heated nebulizer to deposit LC effluent as linear tracks on a MALDI target plate which was pre-coated with matrix [7]. Similar experiments have also been performed coupling high-resolution capillary electrophoresis with MALDI-MS analysis, in which off-line deposition was done under vacuum conditions [8].

*Corresponding author. Tel.: +1 858 748 9415; Fax: +1 858 784 9496; E-mail: Siuzdak@scripps.edu.

0712-4813/03/$8.00 © 2003 – IOS Press. All rights reserved

The requirement of a matrix, such as alpha-cyano-4-hydroxycinnamic acid, imparts significant limitations to the LC-MALDI-MS approach that include relatively high flow-rates, sample heterogeneity, and an effective mass range above approximately 700 m/z. Desorption/ionization on silicon (DIOS) is a solid-state ionization technique similar to MALDI, yet DIOS is matrix-free. In DIOS, the analyte molecules are trapped on a porous silicon surface and desorbed/ionized through irradiation with ultraviolet light [9–11]. The absence of matrix in DIOS allows for a low practical mass range, well below 300 m/z.

Here, we present a novel combination of off-line LC separation with DIOS-MS (LC-DIOS-MS), which utilizes electrospray deposition (ESD) to generate a spatially preserved linear track of a sample mixture. In contrast to the previously discussed LC-MALDI-MS approaches [5–8], LC-DIOS-MS is a simple one step procedure that does not require matrix deposition, offers the advantages of off-line LC separation, utilizes low flow rates (~150–200 nanoliters/min), and offers the potential for high-throughput analysis. In addition, Go et al. have recently demonstrated the application of electrospray deposition on DIOS chips without LC separation for quantitative measurements [12]. In our work, the application of LC-DIOS-MS is shown for the identification of the precursor coat protein in the flock house virus (FHV) and the standard protein, bovine serum albumin (BSA). Our results illustrate that LC-DIOS-MS offers reduced signal suppression, resulting in higher sequence coverage as compared to traditional LC-ESI-MS/MS analysis.

2. Experimental

Flock house virus (FHV) and bovine serum albumin (BSA, sigma) proteolytic digests were performed with trypsin (Promega), thermolysin (Roche), and V8 (Roche) at a 50 : 1 protein to enzyme ratio by mass. The proteins were denatured at 90°C for 20 minutes, followed by reduction and alkylation with dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Trypsin digests were incubated overnight at 37°C, V8 digests were incubated overnight at room temperature, and thermolysin digests were incubated for 1 hour at 60°C.

Nano LC-ESI-MS/MS experiments were performed on an Agilent quadrupole ion trap mass spectrometer. LC-DIOS-MS experiments were performed on a Micromass MALDI-MS. Mobile phases utilized for LC-ESI-MS/MS consisted of buffer A: 100% H_2O (Burdick and Jackson, high purity solvent) + 0.1% formic acid (J.T. Baker) and buffer B: 90% acetonitrile (Fisher, optima grade) + 0.1% formic acid. The mobile phases used for LC-DIOS were identical with the addition of 3 mM ammonium citrate (Aldrich, 98% purity). Both experiments utilized a 70 minute LC separation with a gradient running from 0–80% buffer B. DIOS-MS and MALDI-MS experiments were performed on a Perspective Biosystems Voyager STR time-of-flight (TOF)-MS.

DIOS chips were prepared by electrochemical etching of a low resistivity (0.005–0.02 Ω-cm) n-type Si(100) wafer in a 25% v/v HF/ethanol (Acros and Sigma, respectively) solution under white light illumination for 2 minutes (current density of ~22 mA/cm^2 for LC-DIOS-MS and ~5 mA/cm^2 for DIOS-MS). Photopatterning in the previous step resulted in either a pattern of lanes for the LC-DIOS-MS experiments, or in a 5 × 5 or 10 × 10 grid for multiple sample analysis in DIOS-MS. Following the first etching, the surface was oxidized with ozone and rinsed with 5% v/v HF/H_2O solution. Before and after each step in the etching procedure, the chips are rinsed with ethanol and dried under N_2 (g).

Reverse phase fused silica nanoelectrospray columns were constructed from 100 μm id capillary (Agilent) pulled to a diameter of ~5 μm and then pack with C_{18} stationary phase (XDB-C_{18}, Eclipse). These columns were utilized for both the LC-MS/MS and LC-DIOS-MS experiments at flow rates of 150–200 nanoliters/minute. For LC-DIOS, a voltage of 1.8–2.0 kV was applied to the column to induce the

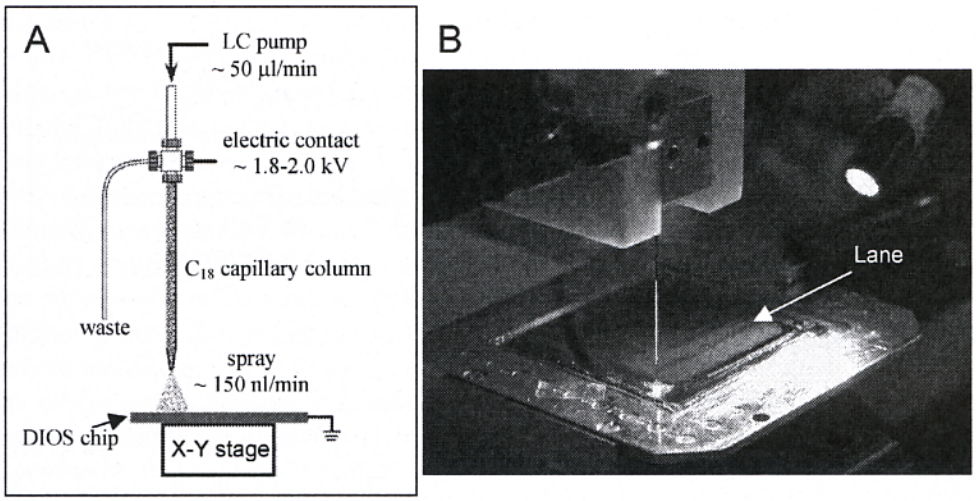

Fig. 1. (A) Schematic of LC-DIOS sample deposition. (B) Photo of LC-DIOS sample deposition showing the DIOS chip with the etched lane pattern and the C_{18} packed capillary column.

electrospray deposition. The tip of the column was brought ~1 mm away from the DIOS surface for deposition as shown in Fig. 1. The DIOS chip was directly adhered to a customized MALDI plate with tape and placed on the flat arm of a syringe pump traveling at 0.6 mm/min to allow for deposition along the length of the lane (24 mm). For DIOS-MS experiments, the sample is pipeted directly onto the DIOS chip and allowed to dry at 37°C. Subsequent experiments not presented in this paper utilized a modified MALDI prep station (Waters) for LC-DIOS deposition. In this set-up, the DIOS chip x–y translation is automated through a computer interface, which is essential for high-throughput applications.

3. Results and discussion

The traditional method of DIOS-MS sample preparation involves direct deposition of a 0.5 μl droplet of sample onto DIOS chip grid (either 5 × 5 or 10 × 10). The sample is then allowed to air dry before analysis. While this method is fast, and effective for many applications, there is a compromise in DIOS signal reproducibility due to the inhomogeneity of the sample. To address this problem, ESD can be utilized to generate a more homogeneous sample spot. Sample homogeneity is especially significant in studies where quantitative information is desired. Recently, Go et al. conducted a thorough investigation of DIOS sensitivity and reproducibility in samples that were traditionally spotted (with pipette) versus those prepared by ESD [12]. They found that the %RSD for the samples prepared by ESD was consistently <5%, while analysis of the spotted samples yielded %RSD values ranging from 6–20%. These results indicate that ESD yields superior sample-to-sample reproducibility and is therefore the method most suited for quantitative measurements using DIOS.

ESD alone increases reproducibility and sample homogeneity for DIOS analysis, however, without separation there is no improvement in signal suppression effects. In this work, the utility of LC separation combined with DIOS-MS is demonstrated by the sequence coverage analysis of various proteolytic digests of FHV (a well characterized model virus system [13]) and BSA. Comparative LC-ESI-MS/MS experiments were also performed on the same samples. A combination of the LC-ESI-MS/MS results

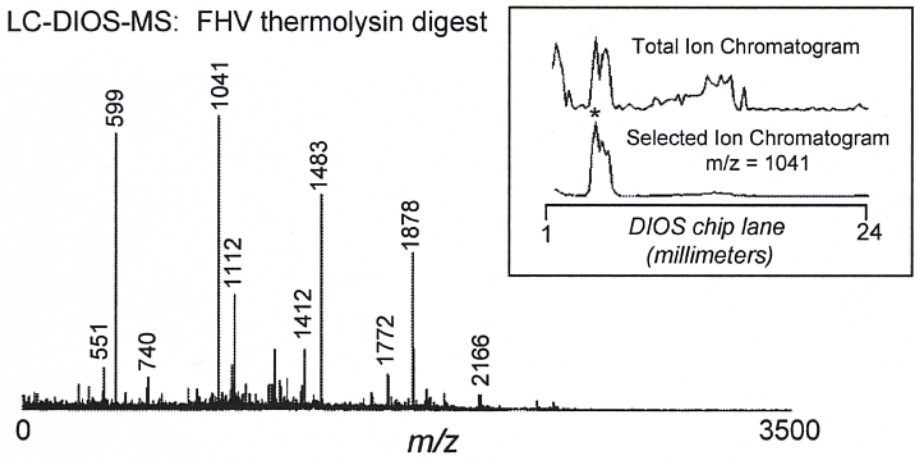

Fig. 2. Inset: Total ion chromatogram representing one lane of an LC-DIOS chip and a selected ion chromatogram illustrating peptide separation. Main figure: LC-DIOS-MS spectrum of a FHV thermolysin digest at the stared position (~30% buffer B) of the selected ion chromatogram.

Table 1
Summary of sequence coverage experiments performed by LC-DIOS-MS, LC-MS/MS, and DIOS-MS for both FHV and BSA

Enzyme	FHV			BSA		
	LC-DIOS-MS	LC-ESI-MS/MS	DIOS-MS	LC-DIOS-MS	LC-ESI-MS/MS	DIOS-MS
Thermolysin	100%	64.6%	12%	69%	47.4%	52.2%
Trypsin	–	34.6%	11%	85%	71%	54.6%
V8	–	16.7%	2%	–	37%	15%
Total	100%	82.3%	24.3%	99.5%	87.8%	71.4%

for trypsin, thermolysin, and V8 digests of FHV yielded 82.3% sequence coverage. However, the LC-DIOS-MS experiment of a thermolysin FHV digest alone yielded 99.8% sequence coverage, missing only 1 amino acid. The missing amino acid in this sequence is the N-terminal methionine, which is often naturally cleaved especially if the second amino acid is small and uncharged (in FHV the second amino acid is valine) [14,15]. Therefore, our results, in all probability, indicate 100% sequence coverage of present amino acids. In a typical LC-DIOS-MS experiment, a 70 min LC separation corresponds to sample deposition on two lanes on a DIOS chip, however, the majority of peptides are eluted on the second lane. The total ion chromatogram for the second lane of an FHV LC-DIOS-MS analysis, the selected ion chromatogram for a representative peptide, and the spectrum corresponding to the selected ion are shown in Fig. 2.

Complementary experiments were conducted for BSA digests to further show the effectiveness of LC-DIOS-MS. For BSA, a combination of LC-ESI-MS/MS results from the same three enzymes mentioned above, yielded 87.8% sequence coverage. In this case, a combination of LC-DIOS-MS results from trypsin and thermolysin BSA digests yielded 99.5% sequence coverage, missing only three amino acids. The results from these two experiments are summarized in Table 1.

In comparison to the LC-ESI-MS/MS results, it is clear that LC-DIOS-MS yielded superior sequence coverage for both protein systems analyzed. While high sequence coverage is not a requisite for accurate protein identification, this information is extremely useful for the identification and study of post-translational modifications. However, it is important to note that the LC-DIOS-MS results reflect only

MS analysis and thus there is a sacrifice in the amount of structural information generated using this technique. Recently, DIOS has been coupled with a tandem time of flight mass analyzer (DIOS-TOF/TOF) to obtain high quality fragment ion information of a BSA trypsin digest [16]. Therefore, the powerful combination of a TOF/TOF mass analyzer with LC-DIOS would yield both improved sequence coverage and structural information.

The improved sequence coverage obtained by LC-DIOS-MS can be primarily explained by the nature of the ionization process and the off-line LC separation. In the LC-ESI-MS/MS experiments there are a variety of factors contributing to the lower sequence coverage. For example, there will be some degree of ionization suppression in the process of electrospray ionization, which ultimately will favor the more basic (i.e. higher proton affinity) peptides [3]. In LC-DIOS-MS, the electrospray process is used for deposition and not ionization; therefore ionization suppression should be greatly reduced. The LC-MS/MS experiments utilize a data dependent scanning protocol in which the software picks the peptides for MS/MS analysis based on the relative peak intensity. Because of the on-line nature of the LC separation in these experiments, there is only a limited window of time for the analysis of each peptide. Thus, the data dependent scanning will routinely miss the lower intensity peptides for MS/MS analysis, especially in the analysis of highly complex mixtures. This issue is somewhat overcome by the use of the Dynamic Exclusion/Active Exclusion feature of the software that will select a given peak only once in a set time interval. The off-line LC separation in LC-DIOS-MS generates a spatially preserved linear track of the sample and consequently there are no time limitations imposed on peptide analysis. In addition, signal suppression is less significant with desorption/ionization techniques such as DIOS and MALDI, which results in a larger number of peptides available for analysis.

While LC-DIOS-MS clearly improves sequence coverage for the study of post-translational modifications, an additional advantage is the potential for high-throughput analysis. Peters et al. demonstrated the automated deposition of sample and/or matrix from multiple LC columns for MALDI-MS analysis [5, 6]. Here, all of the experiments presented utilized one column depositing on one lane of the DIOS chip. However, it is feasible that a single LC pump could be used to run multiple LC columns and deposition of these columns could occur simultaneously and by automation. The average time for MS analysis of the DIOS chip is $\sim$2 min/lane. Therefore, one could easily perform 10 or more protein identification experiments in the same time it takes to run only one such analysis on a conventional LC-ESI-MS/MS system. This could drastically reduce the overall time, instrument costs, and labor for high-throughput protein analysis and identification.

4. Conclusion

The combination of LC separation with DIOS yielded improved sequence coverage of two model protein digests compared with traditional LC-ESI-MS/MS analysis, which is greatly beneficial in the study of post-translational modifications. LC-DIOS-MS offers the advantage of off-line separation resulting in reduced signal suppression as well as the potential for high-throughput analysis. In addition, the combination of LC-DIOS with a high resolution tandem mass analyzer, such as a TOF/TOF system, would result in higher quality structural information.

Acknowledgement

The authors would like to gratefully thank the NIH for funding (GM 55775-05).

References

[1] D.I. Papac and Z. Shahrokh, *Pharmaceut. Res.* **18**(2) (2001), 131–145.
[2] R.D. Smith, *Trends Biotechnol.* **20**(12) (2002), S3–S7 Suppl. S.
[3] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stien and T. Olah, *J. Am. Soc. Mass Spectrom.* **11** (2000), 942–950.
[4] G. Siuzdak, *Mass Spectrometry for Biotechnology*, Academic Press, San Diego, CA, 1996.
[5] E.C. Peters, A. Brock, D.M. Horn, Q.T. Phung, C. Ericson, A.R. Salomon, S.B. Ficarro and L.M. Brill, *LCGC Europe* **July 1** (2002), 2–7.
[6] E.C. Peters, A. Brock, Q. Phung, J. Fitchett, D.M. Horn, C. Ericson, S.B. Ficarro and A. Salomon, *American Pharmaceutical Review* **Fall** (2002), 2–6.
[7] D.B. Wall, S.J. Berger, J.W. Finch, S.A. Cohen, K. Richardson, R. Chapman, D. Drabble, J. Brown and D. Gostick, *Electrophoresis* **23** (2002), 3193–3204.
[8] T. Rejtar, P. Hu, P. Juhasz, J.M. Campbell, M.L. Vestal, J. Preisler and B.L. Karger, *J. Proteome Res.* **1** (2002), 171–179.
[9] Z. Shen, J.J. Thomas, C. Averbuj, K.M. Broo, M. Engelhard, J.E. Crowell, M.G. Finn and G. Siuzdak, *Anal. Chem.* **33** (2001), 179–187.
[10] J. Wei, J.M. Buriak and G. Siuzdak, *Nature* **399** (1999), 243–246.
[11] J.J. Thomas, Z. Shen, J.E. Crowell, M.G. Finn and G. Siuzdak, *PNAS* **98**(9) (2001), 4932–4937.
[12] E.P. Go, Z. Shen and G. Siuzdak, in preparation.
[13] A. Schneemann, V. Reddy and J.E. Johnson, *Adv. Virus Res.* **50** (1998), 381–446.
[14] C. Giglione and T. Meinnel, *Trends Plant Sci.* **6**(12) (2001), 566–572.
[15] S. Chen, J.A. Vetro and Y.-H. Chang, *Arch. Biochem. Biophys.* **389**(1) (2002), 87–93.
[16] E.P. Go, J.E. Prenni, J. Wei, A. Jones, S.C. Hall, E. Witkowska, Z. Shen and G. Siuzdak, *Anal. Chem.* in press.