Chip 2-D LC/MS: Seamless Integration Using a Microfluidic Multilayer Structure

Poster–ASMS 2004

Hongfeng Yin, Kevin Killeen, Reid Brennen and Tom van de Goor

Agilent Labs, Palo Alto, CA
Introduction

Multi-dimensional LC offers the ultimate separation power that many complex proteomic samples demand. Currently SCX column can be coupled with RP capillary column through a low dead volume rotary valve in order to achieve on-line 2-D LC separation. It often involves three packed columns and up to 12 standard LC fittings. At sub µL/min flowrate, fluidic leaks and blockages are the main failure mechanisms for such a setup. We have developed polymer µ-fluidic devices capable of 2-D LC separation to eliminate all fittings between the separation columns and the nanospray tip.

LC chip/MS technology was first described at the 51st ASMS (Montreal, June 2003). The µ-fluidic chip has an integrated sample enrichment column, a RP LC separation column, an electrical contact for electrospray and a nano-electrospray tip. The chip is hydraulically interfaced to a nano-HPLC pump and an autosampler through a face seal rotary valve, thus eliminating conventional LC fittings and connections. The rotary valve also serves as flow switching mechanism for sample introduction. The potential for a high-degree of integration based on such a µ-fluidic platform allows us to further integrate additional sample processing capabilities on this device. Two-dimensional liquid chromatography on µ-fluidic chip is the first application of such a device described here.

Device Fabrication

Laser ablation of the polyimide film is performed using a direct write process with a solid state laser (Coherent Avia-355-1500) operating at 355 nm in combination with a fixed optics train and a high precision x-y table controlled from a personal computer.

Platinum electrical contacts for the electrospray are deposited on one or multiple layers in the interior of the device, and then directly laminated into the final structure. The electrical contact intersects the fluid channel and continues to an access hole cut into the top layer of polyimide.

Column dimensions:

1st dimension LC: 50 µm x 250 µm x 8 mm; SCX (PolyLC)

Online enrichment column: 40 nL; 5 µm Zorbax SB-C18

2nd dimension LC: 50 µm x 75 µm x 45 mm; 3.5 µm Zorbax SB-C18

Chip-Valve Interface

The chip is sandwiched between the stator and the rotor of a two-position HPLC rotary valve (p/n 7900-000, Rheodyne, Rohnert Park, CA). The chip is mounted on a support plate in which the LC valve has been mounted such that the valve rotor mates and aligns to the inlet ports on the back of the chip (Fig 1). It is critical that the stator aligns accurately to the chip so that the fluidic communication ports on the chip are aligned with the stator ports and rotor grooves. The valve stator is mounted in a clamping arm mechanism such that when the arm is closed, the stator ports match to the ports on the top of the chip and the rotor channels to the ports on the bottom of the chip. The valve position is controlled through the LC control software and a contact closure board mounted in the LC system.

Experimental

Device Fabrication

Laser ablation of the polyimide film is performed using a direct write process with a solid state laser (Coherent Avia-355-1500) operating at 355 nm in combination with a fixed optics train and a high precision x-y table controlled from a personal computer.

Platinum electrical contacts for the electrospray are deposited on one or multiple layers in the interior of the device, and then directly laminated into the final structure. The electrical contact intersects the fluid channel and continues to an access hole cut into the top layer of polyimide.

Column dimensions:

1st dimension LC: 50 µm x 250 µm x 8 mm; SCX (PolyLC)

Online enrichment column: 40 nL; 5 µm Zorbax SB-C18

2nd dimension LC: 50 µm x 75 µm x 45 mm; 3.5 µm Zorbax SB-C18

LC/MS Analysis

An Agilent 1100 CapPump running at 4 µL/min was connected to an Agilent 1100 µ-well plate autosampler for sample loading on the µ-chip, as well as injections of salt steps. The RP LC mobile phase gradient was delivered by an Agilent 1100 nanoPump. The experimental condition for the on-chip LC channel was a 40 minute gradient from 2% B to 42% B. The RPLC flowrate was set to 300 nL/min. The 2-D LC chip was interfaced with an Agilent 1100 LC/MSD Trap XCT through an on-chip nanoelectrospray tip.

A human plasma sample (Sigma) was treated with the Agilent immunoaffinity depletion system (5185-5984) to remove the six most abundant proteins. The sample was then reduced, alkylated and digested with trypsin.
Flow path mapping

It is important to note that the µ-fluidic device described here has two fluidic layers, one on top of the other. The 1st dimension SCX channel is in the 1st layer. The enrichment column and RPLC column are in the 2nd layer.

Load position (with rotor grooves at red position):
- Autosampler connection to top side at port #1
- Through 1st D column (pink line in the 1st layer) and reaches #6 on the bottom side
- Connect to on-chip enrichment column (yellow line in the 2nd layer) via rotor groove 6-1 (red line)
- To waste via rotor groove 4–5 (red line)

Run position (with rotor grooves at green position):
- nanoPump connects to enrichment column via rotor groove 2-1 (green)
- Connects to RP LC column via rotor groove 4–3 (green)

Sample loading and salt steps

A 5 µL (50 µg total protein) aliquot of immunoaffinity depleted human serum was loaded onto the SCX column by the autosampler. Breakthrough from the SCX column was captured by the sample enrichment column and then separated by the reverse phase column before nanospray MS. After the first reverse phase gradient was completed, the rotary valve interface was switched to the load position and 5 µL of 10 mM KCl was injected into the SCX column.

This 10 mM KCl step elutes the less retained peptides from the SCX column. These peptides are then trapped by the enrichment column before the 2nd reverse phase LC gradient separation starts.

Subsequent salt steps are 20mM, 50mM, 75mM, 100mM, 150mM, 200mM, 500mM and 1M KCl respectively. The first LC/MS run (sample loading to SCX/SCX breakthrough fraction) and the following nine LC/MS runs after the salt steps elutions from SCX are plotted in Fig. 3.
The data from these ten LC/MS/MS runs were searched against the NCBI database with Spectrum Mill MS proteomics workbench software. Proposed matches were validated within the Spectrum Mill environment using a combination of automatic and interactive results review. A total of 54 proteins were confidently identified from the sample. The most abundant proteins in the sample are listed in Table 1. One should note that the common abundant proteins in human plasma sample (HSA, IgG, IgA, heptoglobin, transferrin, and antitrypsin.) were successfully removed by the Agilent immunoaffinity column.

Table 1. The most abundant proteins identified

<table>
<thead>
<tr>
<th>Group</th>
<th>Score</th>
<th>Protein ID</th>
<th>Sequence MH</th>
<th>Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>111</td>
<td>627.480</td>
<td>(K)RTIFIDEAHITQALIWLSQR</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>218.321</td>
<td>(K)DTVIKPLLVEPEGLEK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>767.380</td>
<td>(K)FEVQVTVPK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>225.140</td>
<td>(K)HNVYINGITYTPVSSTNEK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>717.719</td>
<td>(K)IAQWQSFQLEGGLK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>145.410</td>
<td>(K)FEVQVTVPK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
<td>148.071</td>
<td>(K)KDNSVHWERPQKPK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>8</td>
<td>47</td>
<td>190.510</td>
<td>(K)DTVIKPLLVEPEGLEK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>184.061</td>
<td>(K)KSAAQGGFSSTQDTV</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>110.340</td>
<td>(K)TEHPFTVEEFVLPK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>198.912</td>
<td>(K)VDLSFSPSQSLPASHAHLR</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>12</td>
<td>34</td>
<td>125.992</td>
<td>(K)VSVQLEASPAFLAVPVEK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>188.061</td>
<td>(K)VVSMDENFHPLNELIPLVYIQDPK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>14</td>
<td>22</td>
<td>122.552</td>
<td>(K)YDVENCLANK</td>
<td>Human interleukin-1, alpha</td>
</tr>
<tr>
<td>15</td>
<td>32</td>
<td>2105.781</td>
<td>(K)YSDASDCHGEDSQAFCEK</td>
<td>Human interleukin-1, alpha</td>
</tr>
</tbody>
</table>

Table 2. Peptide distribution among salt steps (alpha 2 macroglobulin precursor)

Total of 21 peptides have been identified for alpha 2 macroglobulin precursor. These peptides are first separated by SCX and then by RP column. The distribution among different salt steps is listed in Table 2. The large amount of peptides found in the first file (break through fraction) indicates that there is a need to increase sample capacity of the SCX column.

Conclusions
- Polymer µ-fluidic device capable of integrating two dimensional separation columns and nanospray tip.
- Multi-layered µ-fluidic chips
- No fittings between the separation channels and the nanospray tip
- Leak free interface and flow switching with a rotary valve
- 2-D LC separation demonstrated with immunoaffinity depleted Human serum samples.

Future Work
- Optimize SCX channel dimensions for sample capacity to reduce break through
- Optimize SCX separation material to reduce overlapping between salt steps
- Investigate linear gradient v.s. salt steps for SCX separation
- Other orthogonal separations
- Affinity media in the 1st dimensional channel

Acknowledgments
The Authors thank Christine Miller for supplying the immunoaffinity depleted serum samples. Debbie Ritchey has greatly contributed through her work in device fabrication.

Table 2. Peptide distribution among salt steps (alpha 2 macroglobulin precursor)