Introduction

The practice of bottom-up proteomics relies to a large extent on the separation performance that can be achieved with state-of-the-art nano LC-MS/MS equipment. Depending on the sample complexity or the instrument time that can be dedicated to a certain sample, different LC columns and corresponding LC-MS/MS methods are often required. When aiming for comprehensive proteome analysis with deep coverage, relatively long columns (lengths up to 75 cm) are typically operated with long and shallow solvent gradients, delivering the highest chromatographic performance. This is indeed a good strategy if very complex samples need to be analyzed and when as much information as possible needs to be retrieved from these samples. However, daily routine proteome analysis often deals with much less complex samples or demands increased sample throughput, making total analysis times above 120 min undesirable or even impossible.

As an alternative to the conventional packed bed nano LC columns, PharmaFluidics offers micromachined nano LC chip columns known as micro pillar array columns (µPAC™). The inherent high permeability and low ‘on-column’ dispersion obtained by the perfect order of the separation bed makes µPAC™ based chromatography unique in its kind. The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated during separation (1). The freestanding nature of the pillars also leads to much lower backpressure allowing the use of very long columns with exceptional peak capacities (2).

Complementary to its landmark 200 cm long column which is ideally suited to perform comprehensive and sometimes time consuming proteome research, PharmaFluidics now also offers a 50 cm long µPAC™ column which can be used in a more routine research setting. With an internal volume of 3 µL, this column is perfectly suited to perform high throughput analyses with shorter gradient solvent times (30, 60 and 90 minute gradients) and it can be used over a wide range of flow rates, between 100 and 2000 nL/min.
Experimental

The separation performance of two state-of-the-art packed bed (PB) nano LC columns (150 x 0.075 mm, sub-2 µm porous silica particles obtained from 2 different vendors) and a 50 cm long µPAC™ column were evaluated by analyzing 500 ng of a tryptic digest originating from a human cell lysate. For every column, separation was performed using three different gradient times (30, 60 and 90 min), and this in triplicate as illustrated in Table 1.

Sample preparation and experimental set-up

Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N 88328) was obtained from Thermo Scientific™ in lyophilized form. 20 µg of lyophilized peptide material was reconstituted in 40 µL of 0.1% formic acid in LC-MS grade water to a concentration of 500 ng/µL. Samples were spiked with Thermo Scientific™ Pierce™ Retention time calibration mixture (0.5 pmol/µL; P/N 88320) to a final concentration of 50 fmol/µL. Freshly prepared protein digest standard was used for each column type.

All columns were positioned in the column compartment of a Thermo Scientific™ Ultimate 3000 nano RSLC system and maintained at 50°C during the entire experiment. The nano LC system was configured to perform direct injection of 1 µL sample onto the column. All columns were operated at a flow rate of 300 nL/min. In addition, the 50 cm µPAC™ was operated at a flow rate of 1000 nL/min as much lower flow resistance is observed for this type of columns. A non-linear gradient from 1% to 50% of solvent B (0.1% formic acid in 80% LC-MS grade acetonitrile) was applied in respectively 30, 60 and 90 min.

For these experiments, the nano LC system was coupled to a Thermo Scientific™ Orbitrap Elite™ hybrid Ion Trap-Orbitrap mass spectrometer by using a nanoFlex ion source. All columns were connected to a New Objective PicoTip™ emitter and for the 50 cm µPAC™ column, a grounded connection was provided between the outlet union and the mass spectrometer. For all columns, the voltage required for electro spray ionization (ESI) was applied on a 50 µm through-bore stainless steel union through a liquid junction. LC-MS parameters can be found in Table 2.
One of the main goals of this experiment was to benchmark the separation performance of the 50 cm µPAC™ column against two commonly used packed bed columns obtained from different vendors. Rather than aiming for extremely deep proteome coverage, the 50 cm µPAC™ column is designed for proteome research where improvements in reliability and throughput are needed aside excellent chromatographic performance.
**Elution profiles for tryptic digest sample**

MS basepeak chromatograms obtained for 60 min gradient separations (i.e. 90 min total run time) of 500 ng HeLa protein digest standard are shown in Figure 1. Even though very similar relative abundances were found for the conventional packed bed columns \((1.5E+08)\), the highest value \((2.5E+08)\) was found for the 50 cm µPAC™ operated at a flow rate of 300 nL/min. A slightly lower overall relative abundance was found for the 50 cm µPAC™ column when operated at 1000 nL/min \((1.0E+08)\), but a substantial reduction of the column void time can be achieved by increasing the flow rate (Table 3). Because of the low column backpressure that is inherent to µPAC™ column backbones, chromatographic separations on 50 cm µPAC™ columns can be performed at elevated flow rates up to 2000 nL/min. As shown in Figure 2, the maximum column backpressure of the µPAC™ column (even when operated at 1000 nL/min) is exceptionally low compared to those observed for the packed bed columns. With stated backpressures around 40 bar, this represents a more then 6-fold reduction of the column backpressure for the 50 cm µPAC™ column.

**Figure 1:** Basepeak MS chromatograms of 500 ng HeLa cell digest for 60 min gradients on 50 cm PharmaFluidics µPAC™ column operated at 300 nL/min (green), 50 cm PharmaFluidics µPAC™ column operated at 1000 nL/min (blue), state-of-the-art 150 mm x 75 µm packed bed column 1 (PB1) (yellow) and state-of-the-art 150 mm x 75 µm packed bed column 2 (PB2) (grey). See experimental section for LC gradient and MS conditions.
Table 3: Column void times and accompanying backpressures observed for three different LC columns.

<table>
<thead>
<tr>
<th></th>
<th>Flowrate (nL/min)</th>
<th>Void time (min)</th>
<th>Backpressure (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB column A</td>
<td>300</td>
<td>11,4</td>
<td>249</td>
</tr>
<tr>
<td>PB column B</td>
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<td>296</td>
</tr>
<tr>
<td>50 cm µPAC™</td>
<td>300</td>
<td>18,1</td>
<td>40</td>
</tr>
<tr>
<td>50 cm µPAC™</td>
<td>1000</td>
<td>5,4</td>
<td>122</td>
</tr>
</tbody>
</table>

Figure 2: Column pressures (bar) of each replicate (n=3) for 30, 60 and 90 min gradient separations for 50 cm PharmaFluidics µPAC™ column operated at 300 nL/min (green), 50 cm PharmaFluidics µPAC™ column operated at 1000 nL/min (blue), state-of-the-art 150 mm x 75 µm packed bed column 1 (PB1) (yellow) and state-of-the-art 150 mm x 75 µm packed bed column 2 (PB2) (grey). Dotted lines show the evolution of the backpressure for each of the columns over the entire experiment.
**Chromatographic performance observed for peptide retention time standards**

Besides a high flow rate flexibility (100 – 2000 nL/min) and low column backpressure, excellent peak shapes were found for the 50 cm µPAC™ column. Based on the 15 reference peptides from the Pierce™ Retention time calibration mixture, average peak widths ranging from 0.13 to 0.22 min (measured at 13.5% height or 4σ) were observed, which is remarkably lower if compared to both packed bed column types (Figure 3). This trend is even more pronounced when running the µPAC™ column at a flow rate of 1000 nL/min.

Calculation of the peak capacity (nC) according to (3) revealed higher numbers for the µPAC™ column compared to both conventional nano LC columns, and this for all gradient durations that were tested (Figure 4). The exceptional peak capacity value of 226 obtained for a 30 min gradient separation at a flow rate of 1000 nL/min highlights the position of this 50 cm µPAC™ column as a valid improvement to the traditional packed bed columns that are frequently used in daily routine proteomics research.

![Figure 3: Average peak widths measured at 4σ, obtained for 15 reference peptides (Thermo Scientific™ PRTC mix) that have been spiked at 50 fmol/µL into 500 ng HeLa cell lysate for 30, 60 and 90 min gradient separations. The 50 cm PharmaFluidics µPAC™ column operated at 300 nL/min is shown in green, 50 cm PharmaFluidics µPAC™ column operated at 1000 nL/min in blue, state-of-the-art 150 mm x 75 µm packed bed column 1 (PB1) in yellow and the state-of-the-art 150 mm x 75 µm packed bed column 2 (PB2) is shown in grey.](image1)

![Figure 4: Peak capacities (nC) of respectively 30, 60 and 90 min gradient separations for a 50 cm PharmaFluidics µPAC™ column operated at 300 nL/min (green), 50 cm PharmaFluidics µPAC™ column operated at 1000 nL/min (blue), state-of-the-art 150 mm x 75 µm packed bed column 1 (PB1) (yellow) and state-of-the-art 150 mm x 75 µm packed bed column 2 (PB2) (grey).](image2)
**Increased proteome coverage**

Acquired MS2 spectra were screened against the human reference database (UniProt) with a false discovery rate (FDR) of 0.1% by using the Thermo Scientific™ Proteome Discoverer 2.2 platform. More than 1,700 protein groups (based on 6,100 peptide groups) could be identified with the 50 cm µPAC™ column by using a 60 min gradient separation profile (Figure 5). Compared to the two conventional packed bed columns, this is an average increase of 40% in protein and 60% in peptide IDs. In line with the improved chromatographic performance (reduced peak width and increase in peak capacity) that is observed when working with the µPAC™ column, higher proteome coverage, both at the protein and the peptide level, was observed for all conditions tested within this experiment.

**Figure 5:** Average numbers of identified protein and peptide groups for 30, 60 and 90 min gradient separations performed on a 50 cm PharmaFluidics µPAC™ column operated at 300 nL/min (green), 50 cm PharmaFluidics µPAC™ column operated at 1000 nL/min (blue), state-of-the-art 150 mm x 75 µm packed bed column 1 (PB1) (yellow) and state-of-the-art 150 mm x 75 µm packed bed column 2 (PB2) (grey).
Conclusions

µPAC™ technology clearly offers several benefits regarding robustness, high operational flexibility and excellent separation performance compared to conventional packed bed column technology. When aiming for comprehensive proteome analysis with deep coverage, the 200 cm long µPAC™ column which delivers unprecedented separation performance is the best choice. However, the true benefits of using a long 200 cm µPAC™ column will only come into full play when working with long solvent gradient times (>120min). With an internal column volume of approximately 3 µL and an increased operational flexibility (flow rates up to 2000 nL/min), the 50 cm µPAC™ column serves those who are looking for increased separation performance in daily routine proteome analysis settings where shorter gradient times (<120min) and increased throughput are desired.

**High flow rate flexibility:** 100 to 2000 nL/min — corresponding column backpressures of respectively 12.5 and 250 bar — maximum operating pressure is 350 bar.

**Increased column robustness:** Each column has been manufactured by etching channels out of a solid piece of silicon, the column is perfectly bidirectional and contains no particles nor frits.

**Increased column to column reproducibility:** Each column is manufactured using the same lithographic mask, making every column identical.

**Increased separation performance:** Peak capacity values above 200 can be obtained with short (30-90 min) gradient separations.

**Improved proteome coverage:** Higher peak capacity translates into better separation of tryptic peptides and hence allows for more proteins to be identified within a single run.

References


µPAC™ driven separations – Better by Design

Conventionally LC columns are fabricated by stacking (packed beds) or depositing (monoliths) material into a capillary. PharmaFluidics’ µPAC™ technology (micro Pillar Array Column) is unique in its kind as it is built upon the precise micromachining of designed chromatographic separation beds into silicon. This approach brings along three crucial and unique characteristics:

Perfect Order.
µPAC™ beds are designed with a high degree of order, eliminating heterogeneous flow paths otherwise present in conventional columns (so called Eddy dispersion). Flow through µPAC™ columns adds very little dispersion to the overall separation. As a result, peaks remain sharper and sensitivity is increased.

High Permeability.
µPAC™s operate at moderate pressures, typically lower than 300 bar. Separation channels with exceptional length (50 cm to 200 cm) are therefore possible. These are folded onto a small footprint by a interconnecting concatenating bed segments.

Solid Backbone.
The micromachined backbone of the separation bed forms a rigid structure that is not influenced by pressure. There are no obstructions by touching surfaces, and there is no risk for perturbations by pressure fluctuations.