A novel nanoflow LCMS limited sample proteomics approach using micro pillar array columns (µPAC™)

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ABSTRACT

Bottom-up proteomics using 50 to 100 µm C18 packed capillaries coupled to high resolution mass spectrometers is now established as the common workflow to analyze protein samples from tissues, body fluids or cell lysates. Typically, micrograms of samples are separated in 30 min to 4 hour nano LC gradients resulting in the identification of 4000 to 5000 protein groups [1]. However, ease-of-use and reproducibility of nanoflow LCMS using packed capillaries does not yet allow novice and routine use.

PharmaFluidics' µPAC™ technology (micro Pillar Array Column) is a unique and novel approach to a chromatographic support structure and builds upon micromachining chromatographic separation beds into silicon. The low 'on-column' dispersion obtained by the resulting perfect order separation bed virtually eliminates axial peak dispersion, resulting in higher column plate numbers with sharper peaks and higher concentration of compounds. The freestanding nature of the pillars also leads to much lower backpressure allowing the use of very long columns (Figure 1). These exceptional properties result in excellent chromatographic performance with high-resolution and high sensitivity [2].

Here, we are presenting data on testing this novel approach to a nanoflow column in a bottom-up proteomics workflow. Coupling a 2 m long µPAC™ column via a nanoflex source to a Thermo Fisher nLC1200 pump and a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (Figure 2), we used standard proteomics separation and electrospray conditions, e.g. a flow rate of 300 nl/min with a 3 to 32% gradient of 0.1% formic acid to acetonitrile/0.1 % formic acid in 2 and 4 hours, an ESI voltage of 1900 V with a 10 µm silica emitter. The Fusion™ Lumos™ Orbitrap™ mass spectrometer was operated at a resolution of 120000.

Injecting a dilution series of 1 µg, 100 ng and 10 ng, we observed in triplicate runs at the highest concentration 5400 protein groups in a 4 hour gradient run. Interestingly, when injecting only 10 ng HeLa cell digest, corresponding to the content of 50 cells [3], we still see over 3000 protein groups. Thus, this workflow using µPAC™ columns and a Fusion™ Lumos™ MS is suitable to proteomics experiments where the sample amount is very limited to a small number of cells and therefore opens up a new tool for biologists.

MATERIALS AND METHODS

Sample Preparation: Lyophilized tryptic Hela cell digest was purchased from Thermo Fischer and dissolved in 20 µl of solvent A (0.1% formic acid in water) resulting in a concentration of 1000 ng/µl. 1/10 and 1/100 dilution of this stock resulted in samples with a respective peptide concentration of 100 and 10 ng/µl.

Instrumentation: A 200 cm long PharmaFluidics µPAC™ C18 nano LC column was operated with a Thermo Fisher nLC1200 pump and coupled via a nanoflex source to a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer.

Test Method(s): Standard proteomics separation and electrospray conditions were used; e.g. a flow rate of 300 nl/min with a 3 to 32% gradient of water (0.1% formic acid) to acetonitrile (0.1% formic acid) in 120 and 240 minutes. 1 µl of sample was injected using a direct injection method without trapping. A voltage of 1900V was applied at the end of a 10 µm ID picotip™ silica emitter and the Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer was operated at a resolution of 120000.

Data Analysis: Protein and peptide identification was performed using Thermo Scientific™ Proteome Discoverer™ software, Chromatographic chachterization of the raw data was performed using Thermo Scientific™ Excalibur™ software.

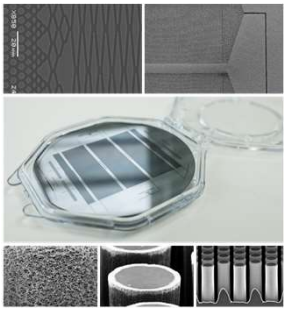

Figure 1. Overview of the PharmaFluidics µPAC™ technology. Top: SEM images of flow distributor structures used to make low dispersion turns. Center: A 4 inch Si wafer with 3 x 200 cm and 2 x 50 cm long µPAC™ columns. Bottom: SEM images of the micro pillar array bed and outer mesoporous pillar shell.

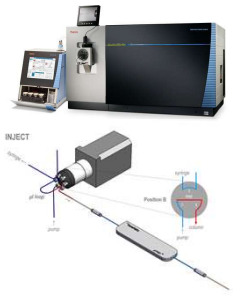

Figure 2. Schematic overview of the set-up used for the experiments. Top: Thermo Fisher nLC1200 pump and Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer. Bottom: Direct injection configuration used to inject 1 µl.

RESULTS

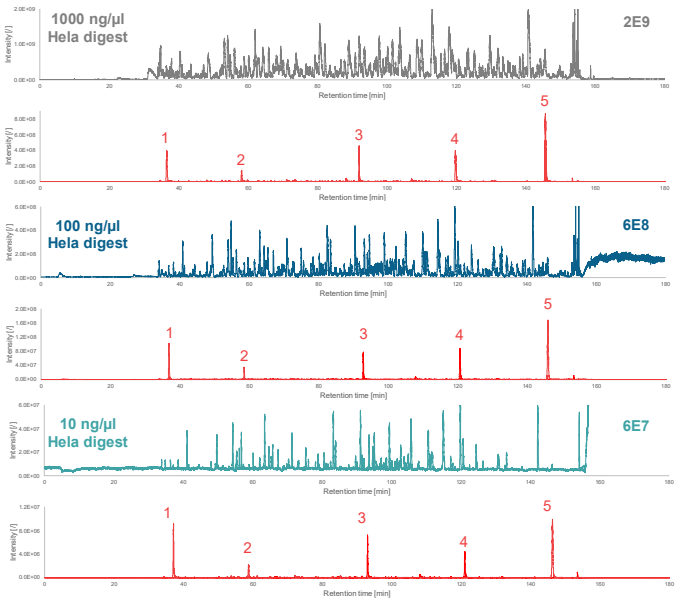

Figure 3. Basepeak chromatograms for 120 min gradient separations of tryptic Hela cell digest, Top: 1000 ng/µl, Center: 100 ng/µl, Bottom: 10 ng/µl. Combined extracted ion chromatograms for the peptides used to evaluate chromatographic performance are shown underneath in red. 1: m/z 393.72 , 2: m/z 617.30, 3: m/z 638.32, 4: m/z 640.36, 5: m/z 807.45.

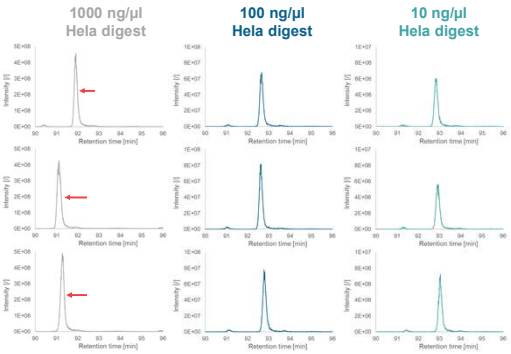

Figure 4. Extracted ion chromatograms for a peptide with m/z 638.32. Triplicates for each concentration (1000 ng/µl, 100 ng/µl and 10 ng/µl) are compared.

Table 1. Average chromatographic metrics for 120 min gradient separations (n=3).

	Results from 5 selected peptides Hela digest		
	1000 ng/µl	100 ng/µl	10 ng/µl
Average Retention Time Variation (CV)	0.8%	0.2%	0.1%
Average Area Variation (CV)	9.8%	8.2%	10.2%
Average Peak FWHM (min)	0.26	0.20	0.21

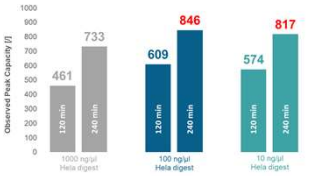

Figure 5. Peak capacity values calculated according to equation 1 using the average peak width of 5 peptides distributed over the elution window. Extracted ion chromatograms of these peptides shown in Figure 3.

Figure 6. Average number of identified protein and peptide groups.

Loadability

Direct injection of 1 µl tryptic Hela cell digest sample was used to investigate the loadability of the µPAC™ C18 column. 1000, 100 and 10 ng of tryptic Hela cell digest was injected and retention time, peak width and peak area were monitored for five selected peptides which elute over the entire elution window. A consistent shift in retention time (Figure 4) was observed for the 1000 ng injections compared to the 100 and 10 ng injections. This shift in retention time is due to column overloading which is backed up by the fact that average peptide peak widths (FWHM) increase up to 25% from 0.2 to 0.26 minutes when going from 100 to 1000 ng of injected tryptic digest sample amount.

Excellent analytical performance and reproducibility

Aside having very high analytical performance, reproducibility is one of the key requirements for the chromatographic separation in bottom-up proteomics experiments because it allows analytical scientists to retrieve reliable quantitative information about the proteome. Average chromatographic performance parameters have been determined for triplicate runs and are listed in Table 1. These results clearly indicate very good reproducibility in terms of peptide retention time and peak area.

Average retention time variation (CV) as low as 0.1% can be achieved when injecting 10 ng of sample. However, a significantly higher variation (0.8%) is observed when injecting 1000 ng of sample, which can be attributed to column overloading.

The analytical performance of a column under certain conditions is often expressed as peak capacity (n_c), which takes both gradient time (t_g) and peak width at half maximum (w_p) into consideration and is defined in equation 1.

Equation 1: $$n_c = 1 + \frac{t_g}{(\sum_1^n w_p)/n}$$

A substantial increase in peak capacity can be observed when extending the gradient from 120 to 240 minutes, and this for all sample concentrations. Using a 240 minute gradient and injecting 100 ng of tryptic Hela cell digest, peak capacity values well above 800 have been obtained.

Flow rate versatility

The optimal flow rate to operate µPAC™ nano LC columns is 300 nl/min, resulting in a backpressure of only 90 bar for a 200 cm long column. This low backpressure allows chromatographers to use a variety of flow rates depending on the separation needs. Flow rates up to 1000 nl/min can for instance be used to speed up sample loading or to perform relatively short gradient separations, with total run times in the order of 60 minutes.

Proteome coverage for limited sample amounts

The main goal of bottom-up proteomics experiments is of course to identify as many peptides and proteins as possible in a given analysis time. As can be seen in Figure 6, these numbers are affected by gradient conditions as well as sample loading conditions.

Extending gradient times from 120 to 240 minutes allows identifying 15% more proteins and 20% more peptides when injecting 100 or 1000 ng tryptic Hela cell digest. At the highest concentration, this resulted in the identification of over 5400 protein groups for a single 240 minute gradient run.

Interestingly, when injecting only 10 ng tryptic HeLa cell digest, corresponding to the content of 50 cells [3], we still see over 3000 protein groups. Thus, this workflow using µPAC™ columns and a Fusion™ Lumos™ MS is suitable for proteomics experiments where the sample amount is limited to a small number of cells and therefore opens up a new tool for biologists.

CONCLUSIONS

- Highly reproducible chromatographic separation of tryptic digest samples can be achieved using the combination of a 200 cm long PharmaFluidics µPAC™ C18 nano LC column, a Thermo Fisher nLC1200 pump and a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer.
- Excellent analytical performance with peak capacities up to 846 for a 240 minute gradient can be obtained using this combination.
- Injecting a dilution series of 1000, 100 and 10 ng tryptic Hela cell digest, over 5400 protein groups were identified in a 240 minute gradient run at the highest sample concentration.
- When injecting only 10 ng tryptic Hela cell digest, roughly corresponding to the content of 50 cells, over 3000 protein groups can be identified in a single 120 minute gradient run.
- Whereas the traditional nano LC columns used to achieve high analytical performance are generally limited in terms of operation flexibility (very high backpressure, low flow rates only), 200 cm long µPAC™ columns can easily be operated in a wide range of flow rates hence enabling chromatographers to optimize separation parameters according to their needs.

REFERENCES

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