High aspect ratio pillar array columns for deep proteome profiling at moderate LC pump pressures

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ABSTRACT

The performance that can be achieved with LC columns depends to a large extent on the flow rate range they can handle and their length. By increasing the aspect ratio of GEN2 pillar array column formats, separation beds with higher operational flexibility and increased resolving power have been manufactured. Peak capacity values up to 1736 (FWHM based) were achieved with a 110 cm length prototype in both standalone nanoHPLC analysis as well as hyphenated to mass spectrometry. Compared to the current state-of-the-art in packed bed column technology, increases in proteome coverage up to 10% on the protein group level and 47% on the peptide group level could be achieved when coupling this column to an Orbitrap Fusion Lumos system equipped with a FAIMS pro interface. Near comprehensive proteome coverage (8603 protein group ID's) could be obtained by injecting high sample loads and performing 4 hour gradient analyses.

INTRODUCTION

The quality of LC-MS based proteomics research relies to a large extent on the resolving power, scanning speed and sensitivity that HRAM mass spectrometers can provide to identify and quantify proteins with high confidence. The impact of the resolving power achieved with LC separation of enzymatically digested proteins must however not be underestimated. In search of increased separation power, LC column technology has been continuously evolving towards using smaller packing materials to present a continuous feed of peptides to the mass spectrometer. In this contribution, we report the evaluation of a novel type of pillar array column where the combination of reduced inter pillar distance and increased etching aspect ratio result in improved separation performance at moderate operating pressures.

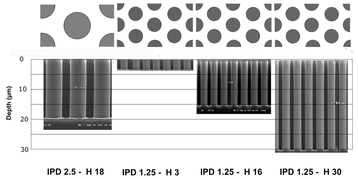

Figure 1. Schematic overview of different micro pillar array chromatography beds. IPD = interpillar distance, µm – H = pillar height, µm - AR = Aspect ratio, H/IPD, / - GEN1 = 2,5 µm IPD. GEN2 = 1,25 µm IPD

	Pillar diameter [µm]	Inter pillar distance [µm]	Channel width [µm]	Channel depth [µm]	Aspect Ratio (/)	Circular eq diameter [µm]	Column volume [µL]	Max Flow rate [nL/min]
200 cm GEN1	5	2.5	315	18	7.2	85	9	1000
50 cm GEN2 - 2021	2.5	1.25	1000	3	2.4	60	1.5	300
50 cm GEN2 - 2022	2.5	1.25	180	16	12.8	60	1.5	750
110 cm GEN2	2.5	1.25	180	30	24	85	4.5	750

Table 1. micro pillar array bed specifications GEN1 and GEN2.

MATERIALS AND METHODS

Sample Preparation – All samples that were used throughout the study were reference standards. Thermo Scientific™ Pierce™ HeLa Protein Digest Standard was dissolved in 0.1% FA to respective concentrations of 100, 200, 500 and 1000 ng/µL. Pierce retention time calibration peptide mixture was added to a final concentration of 50 fmol/µL. Cytochrome C digest standard was dissolved in 0.1%TFA and diluted to a concentration of 250 fmol/µL

Experimental set-up – LC columns were characterized using a Thermo Scientific™ Ultimate™ 3000 nanoRSLC system, either as a standalone unit with 3 nL volume flow cell UV detection or coupled to MS. A Thermo Scientific™ TSQ™ vantage triple quad MS was used for chromatographic performance evaluation, a Thermo Scientific™ Orbitrap Fusion™ Lumos™ instrument equipped with a FAIMS pro interface and Thermo Scientific™ EASY-Spray™ source were used to evaluate proteome coverage.

Data Analysis – UV data were processed using Thermo Scientific™ Chromeleon™ (v7.3), SRM data from the triple quad were analyzed using Skyline software. For HR DDA MS data, standard database search was performed using Sequest HT in Proteome Discoverer v2.5.0.402 and human SwissProt database (Homo sapiens; release 2020_12). Identified spectra were rescored using Percolator as implemented in PD and filtered for 1% FDR at the peptide spectrum match and peptide level.

RESULTS

Chromatographic performance evaluation

Analogous to the trend in LC column technology where particle sizes have been consistently reduced to improve chromatographic performance, pillar array performance can be improved by reducing pillar and inter pillar dimensions. Even though this theoretically results in a net gain in separation resolution at reduced analysis times, some implications need to be considered [1].

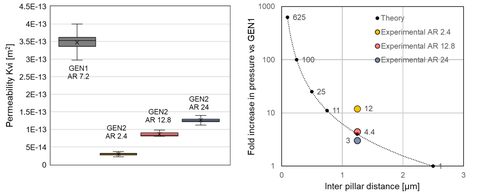

Figure 2. LEFT: Column permeability (K_{vt}) values for different micro pillar array chromatography beds. RIGHT: Effect of reducing inter pillar distance on operational pressure, assuming constant flow rate and column cross section. Theoretical calculations versus observed values for GEN2 pillar array prototypes

For columns with an identical cross section and length, the pressure drop across the separation bed scales inversely with the n^{th} power (n=2) of the average flow through pore (typically particle diameter/3 for a packed bed of spherical particles, inter pillar distance for the pillar array format). Reducing the inter pillar distance by a factor of 2 will hence result in an increase in operational pressure by a factor of 4. To rule out any effects of cross section or length, column permeability values (K_{vt} = (u x η x L)/(ΔP.ε.A) have been determined for a range of pillar array column formats. The initially developed GEN2 format (GEN2 AR 2.4) suffered from excessive back pressures, which can probably be attributed to 'bottleneck' pressure buildup in confined connecting areas. As can be seen from Figure 2A and B, these issues have been mitigated by increasing the pillar bed aspect ratio. For these high aspect ratio GEN2 pillar beds, pressure behavior is much more in line with theoretical expectations. This is of great interest as this allows for faster separation, column equilibration, sample loading and in general reduces the stress on the entire LC/MS setup.

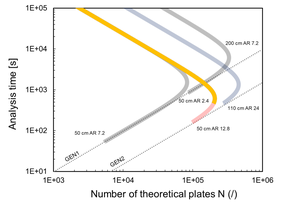

Figure 3. Constrained kinetic plot showing the performance that can be achieved in isocratic separation mode with different micro pillar array chromatography beds. Pressure is limited to 400 bar. Grey = GEN1 50 cm and 200 cm, orange = GEN2 50 cm AR 2.4, red = GEN2 50 cm AR 12.8, blue = GEN2 110 cm AR 24.

From a kinetic point of view, this opens up possibilities to perform faster separations on one hand and design longer columns with increased resolving power on the other hand [2]. Based on experimentally determined LC performance, kinetic performance metrics for GEN2 pillar array formats with different aspect ratios and lengths have been determined (Figure 3). By increasing the aspect ratio from 2.4 to 12.8, significantly faster chromatography can be achieved without compromising separation resolution (Number of theoretical plates, N). Even though the overall cross section of both 50 cm GEN2 column types is identical (Table 1), the high aspect ratio column will operate at pressures 3 times lower than the low aspect ratio column. Taking advantage of the dimensional freedom in which pillar array beds can be designed, a column with a length of 110 cm and similar pressure to flow ratio was developed to provide increased separation resolution for comprehensive characterization of complex samples.

Chromatographic resolution in gradient separation mode is typically quantified in terms of peak capacity (n_C = 1+(T_G/FWHM)) [3]. This value takes both the separation window as well as the average peak width into account. The maximum peak capacity that can be achieved depends on intrinsic performance (particle diameter/packing quality/connectivity) and column length. To validate chromatographic performance, a set of experiments (both UV and MS) have been conducted to determine the maximum peak capacity that can be achieved with GEN2 pillar array column formats.

Results obtained with true 'zero dead volume' connections (UV) as well as those obtained in LC-MS configuration (with ES993 10 µm ID emitter and post column grounding point) have been pooled and were used to determine the resolving power of GEN2 pillar array columns (Figure 4). Experimental results show fair accordance to theory, where an increase in maximum peak capacity by a factor of 1.48 is expected.

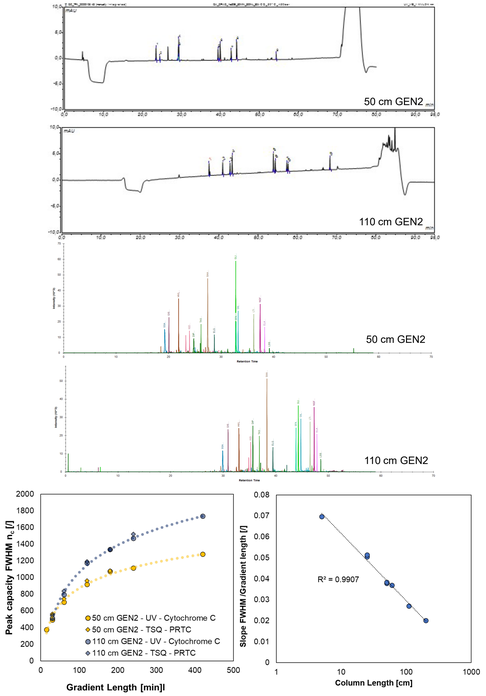

Figure 4. TOP: representative UV chromatograms obtained for the separation of 125 fmol Cytochrome c digest, direct injection 1 µL, 60 min gradient, µPAC GEN2 50 and 110 cm. MIDDLE: representative EICs showing PRTC peptide separation and detection using a TSQ vantage triple Quad, cycle time = 0.5s. BOTTOM LEFT: Peak capacity versus gradient time obtained for the GEN2 µPAC columns. BOTTOM RIGHT: Peak broadening slope as a function of column length, µPAC n=4, packed bed n=3.

By combining these results with a large set of previously determined dispersion measurements (column length ranging from 5 to 200 cm) a good correlation between column length and the rate at which chromatographic peaks become broader in gradient elution mode was revealed (Figure 4). As the data set included both pillar array column formats as well as packed bed formats, this correlation can be used to estimate the peak dispersion that can be expected for a given column length and gradient time and can help determine the best column length for a given separation duration.

Proteome coverage

Using a well defined complex peptide sample (Hela cell lysate digest), we then evaluated the impact of increased LC peak capacity on proteome coverage that can be achieved in a typical bottom-up proteomics workflow. Exploratory experiments were first conducted to optimize MS instrument settings and setup configuration.

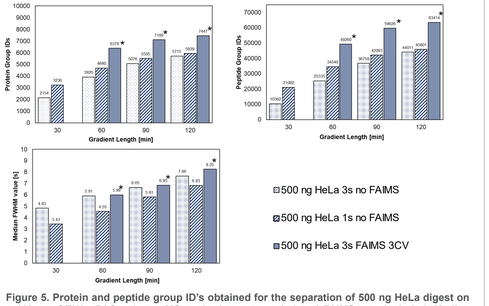

Figure 5. Protein and peptide group ID's obtained for the separation of 500 ng HeLa digest on a 110 cm GEN2 µPAC column, MS cycle time was varied and FAIMS was included. Median FWHM values obtained with the apQuant node in PD 2.5 are also compared. * gradient times for runs with FAIMS were slightly longer (10 min).

When injecting 500 ng of HeLa digest and performing separations ranging from 30 to 120 min gradient time, we found a striking difference in proteome coverage when operating the instrument at different scanning speed settings (Figure 4). By decreasing the instrument cycle time from 3 to 1s, up to 50% more protein group ID's could be achieved for 30 min gradient separations. As expected, the effect of cycle time on proteome coverage diminished with increasing gradient length as broader eluting peaks do not require fastest scanning methods. Peak width determination did also vary with MS scan speed settings, resulting in broader observed peak widths when fewer datapoints per peak were sampled. Even though gradient times were slightly larger when the setup was complemented with a FAIMS Pro interface, clear benefits of additional ion mobility filtering is demonstrated by the increase in proteome coverage (up to 25-35% on the protein group level, 40% on the peptide group level) [4].

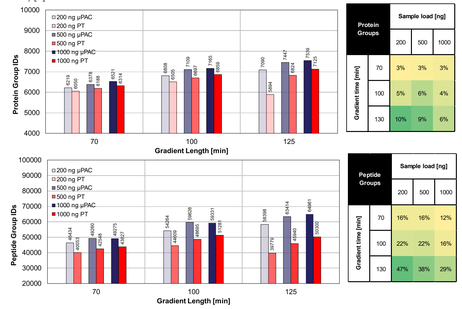

Figure 6. Protein and peptide group ID's obtained for the separation of 200-1000 ng HeLa digest on a 110 cm GEN2 µPAC column and a 25 cm pulled tip packed bed column. Relative increase/decrease is visualized to the right.

Using the FAIMS Pro interface equipped LC-MS setup, a series of benchmarking experiments was performed to investigate the potential benefits compared to a pulled tip emitter column LC/MS setup (1.6 µm particles, 25 cm length).

Improved proteome coverage was observed over the entire range of conditions tested (gradient time 70-125 min, sample load 200-1000 ng). However, relative gain in coverage became larger with increasing gradient length and decreasing sample load. Whereas the latter can be probably be explained by the superficially porous nature of the pillar array format, improved coverage for longer gradients is expected as a result of the increased separation length.

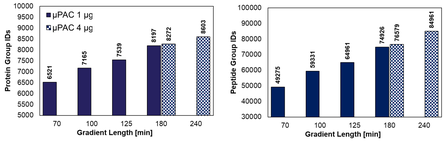

Figure 7. Protein and peptide group ID's obtained for the separation of 1000-4000 ng HeLa digest on a 110 cm GEN2 µPAC column. Gradient times were extended to 240 min.

In search of comprehensive proteome coverage, gradient times and sample loads were increased even further, up to a point where 4 µg of HeLa sample was separated using a 4 hour gradient. This resulted in consistent identification of 8603 protein groups in a single shot DDA experiment.

CONCLUSIONS

Redesigning GEN2 pillar array column formats has opened up several opportunities to increase LC separation power and at the same time increase operational flexibility.

- Columns can be operated at higher flow rates, reducing the impact of sample loading and equilibration on total analysis time.
- Column length can be increased with limited impact on operational backpressure, further extending resolving power for complex analyses.
- A 50 cm GEN2 pillar array column was designed to serve high to medium throughput nanoLC applications (15-60 min gradients) and relatively low sample loads (10-500 ng protein digest).
- A 110 cm GEN2 pillar array column delivers improved separation performance and increased proteome coverage for gradient lengths longer than 60 min.

The microfabricated nature of pillar array column technology provides clear benefits over packed bed column technology when aiming for comprehensive analysis of complex peptide mixtures

- The superficially porous nature of µPAC technology reduces column related carry over.
- Perfectly ordered separation beds provide improved chromatography, even when a replaceable ESI emitter tip is used.

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TRADEMARKS/LICENSING

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