HPLC columns

Efficient and sensitive peptide mapping approach by µPAC columns with ultralow sample loading

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Abstract

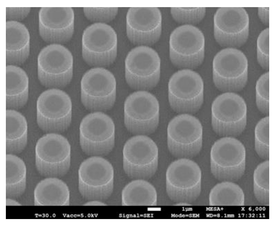
Peptide mapping is an important approach to analyze monoclonal antibodies for the identification of sequences, post-translational modifications and mutations. Traditional packed columns are usually used for peptide mapping at analytical flow rate with large sample loading. For improved separation and sensitivity, low flow chromatography has become the preferred LC method. Microfabricated pillar array columns (Thermo Scientific™ µPAC™ columns) were introduced as an innovative technology for low flow. Here, peptide mapping is conducted using Thermo Scientific™ µPAC™ Neo 50 cm columns and Thermo Scientific™ µPAC™ Neo 5.5 cm High Throughput columns. With only 20 ng NISTmAb tryptic digest, 96.4% and 98.6% are achieved for heavy chain (HC) and light chain (LC) in 15 mins elution time using 50 cm µPAC Neo column. The same sequence coverages are achieved in a 5 mins elution time using 5.5 cm High Throughput µPAC Neo column.

Introduction

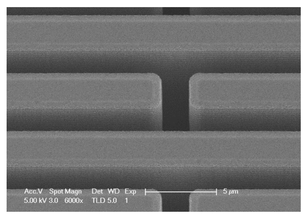
Compared with packed bed (and monolithic) column technology microfabricated pillar array columns (µPAC) are an innovative technology that enables high peak capacity separations at moderate LC pump pressures. Through the implementation of lithographic pattern transfer and deep reactive ion etching (DRIE) into silicon wafers, separation channels can be manufactured that contain micrometer sized silicon features that are perfectly positioned according to a pre-defined design. The introduction of perfectly-ordered separation beds eliminates any Eddy dispersion originating from heterogenous flow paths through the column and increases column permeability. It also provides high peak capacity separations at low flow rate with enhanced ionization sensitivity.

Table 1. µPAC Column properties

Parameter	50 cm µPAC Neo	5.5cm High Throughput µPAC Neo
Pillar shape	Cylindrical	Rectangular
Pillar Diameter (µm)	2.5	75 x 3
Interpillar distance (µm)	1.25	2
Channel Width (µm)	180	1850
Channel depth (µm)	16	25
Length (cm)	50	5.5
Surface morphology	Core-shell	
Porous layer thickness (nm)	400	500
Pore size range (Å)	100-300	
Surface functionalization	C18 + HMDS	
Flow rate range (nL/min)	100-750	250-2500
Void volume (uL)	1.5	
Maximum Pressure (bar)	450	
Maximum Temperature (°C)	60	



SEM photo of 50 cm µPAC Neo column



SEM photo of 5.5 cm High Throughput µPAC Neo column

Materials and methods

Standard NISTmAb tryptic digest was dissolved in water with 0.1% formic acid. Ultra-low sample amounts of respectively 1.6, 4, 10, 20 and 40 ng were loaded on 50 cm µPAC Neo and 5.5 cm µPAC Neo High Throughput columns. In search of optimal performance, different flow rate and gradient time were investigated. Digested peptides mixtures were separated by the Thermo Scientific™ Vanquish™ Neo UHPLC system then directly analyzed by MS/MS on Thermo Scientific™ Orbitrap™ Exploris 480 Mass Spectrometers. Raw data were analyzed by Thermo Scientific™ BioPharma Finder™ 5.1 with automatic parameter values. Peptides with |ppm error| ≤ 10ppm, identified only by MS2, MS Area ≥ 1E5, and Miss cleavage ≤ 2 are selected to calculated sequence coverage. Glycopeptides were manually confirmed by MS2 spectrum.

Solvent A is water with 0.1% formic acid (FA), and solvent B is 80% acetonitrile with 0.1% FA. 15 mins method and mass spectrometry parameters are listed as an example.

Table 2. 15 mins LC method

Time (min)	%B
0	1
0.10	4
10.10	22.5
13.85	35
15.00	45

Table 3. MS parameters

Column	50 cm µPAC Neo	5.5cm High Throughput µPAC Neo
Spray Voltage	2kV	2kV
Resolution	30K	30K
m/z range	200-1600	200-1600
RF lens %	50	50
AGC (%)	100	100
Max IT	40	5
Microscan	1	1
Intensity Threshold	5.00E+03	5.00E+03
Charge state	1-7	1-7
Dynamic Exclusion	10	3
Top N	8	8
Isolation Window	2	2
HCD	30	30
Resolution	15K	7.5K
First Mass	120	120
AGC (%)	100	200
Max IT	30	12
Microscan	2	1

Results

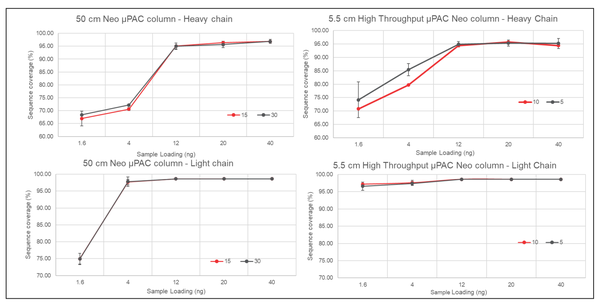


Figure 1. Sequence coverages vs. Sample Loading and Gradient Time

For both columns, sample loading increasing from 12 ng to 40 ng doesn't impact the sequence coverage and reaches the plateau with 20 ng sample loading. The impact of gradient time is also investigated, for 50 cm µPAC Neo, 15 min and 30 min gradient gave the same results, which significant increase the analysis efficiency. For 5.5 cm High Throughput µPAC Neo column, short gradient is better for low sample loading, like 1.6 ng of sample.

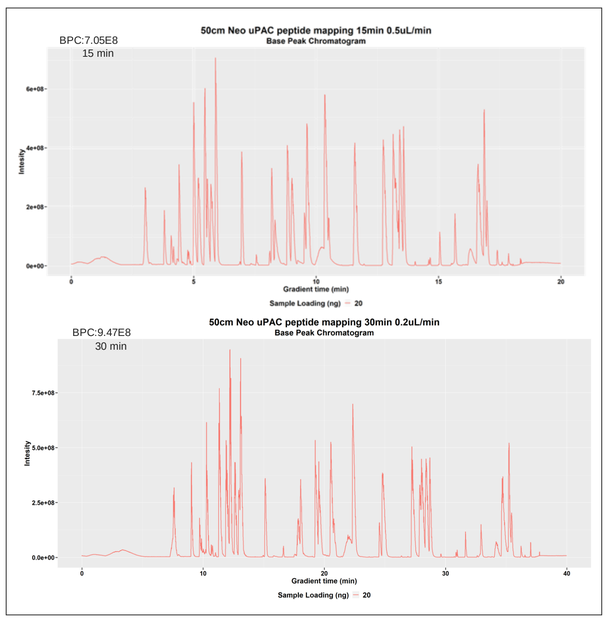


Figure 2. Base peak chromatography of 50 cm µPAC Neo column (15 min vs 30 min)

Base peak chromatography of 50cm µPAC Neo column shows that both 15 mins and 30 mins method can provide good separation for the NIST mAb digests.

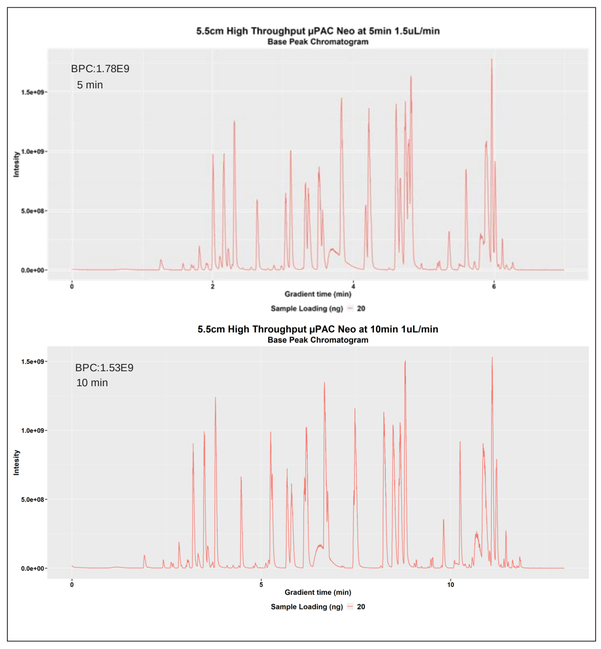
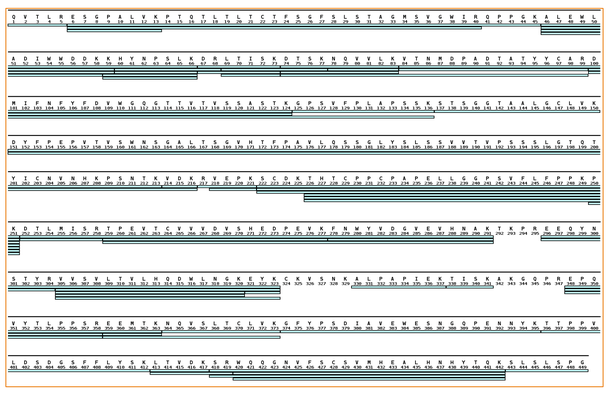


Figure 3. Chromatography of 5.5 cm High Throughput µPAC Neo column (5 min vs 10 min)

Base peak chromatography of 5.5 cm High Throughput µPAC Neo column shows that both 5 mins and 10 mins method can provide good separation for the NIST mAb digests.

(a)



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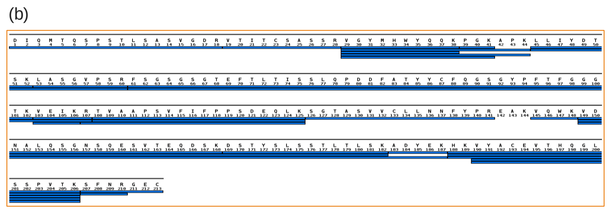
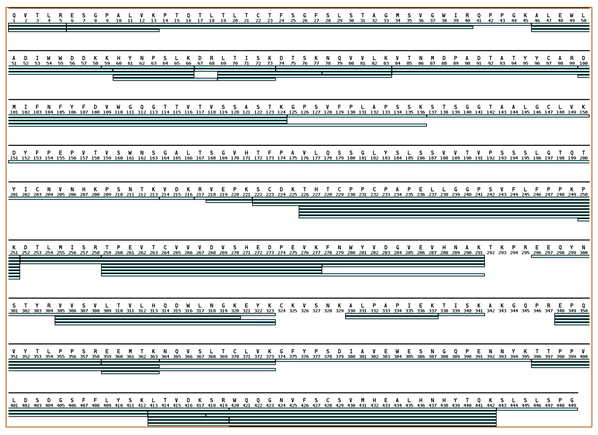


Figure 4. Sequence coverage for HC and LC for 50 cm µPAC Neo Column[1]

With only 20 ng NIST mAb tryptic digest, (a) 96.4% and (b) 98.6% are achieved for heavy chain (HC) and light chain (LC) in 15 mins using 50cm µPAC Neo column.

(a)



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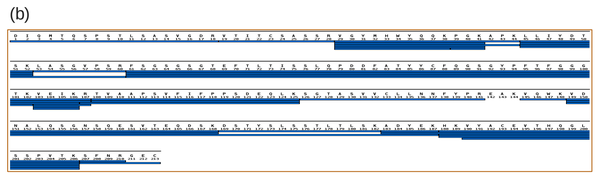
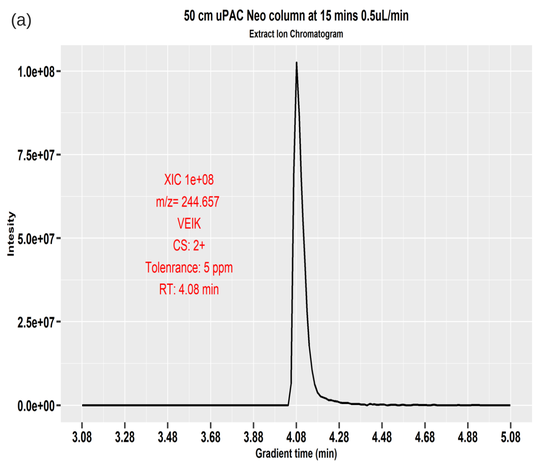


Figure 5. Sequence coverage for HC and LC for 5.5 cm High Throughput µPAC Neo column[1]

With only 20ng NIST mAb tryptic digest, (a) 96.4% and (b) 98.6% are achieved for heavy chain (HC) and light chain (LC) in 5 mins using 5.5 cm High Throughput µPAC Neo column. As the extreme short gradient time, EEQYNSTYR with G0F can't be identified by MS2.

(a)



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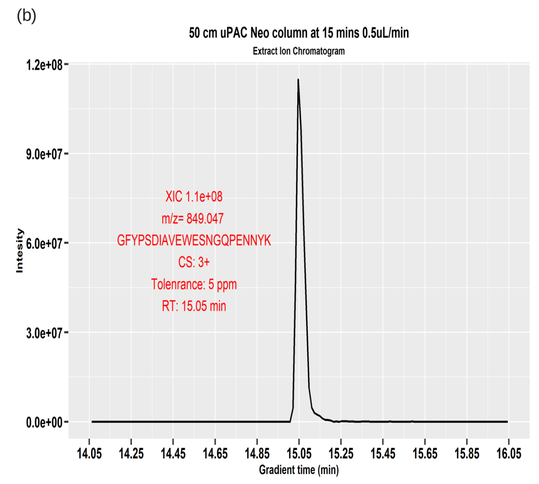


(c)

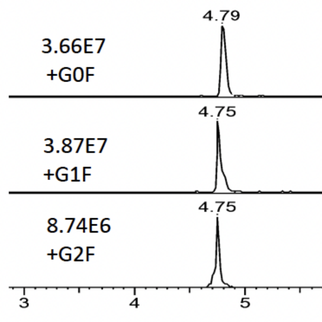
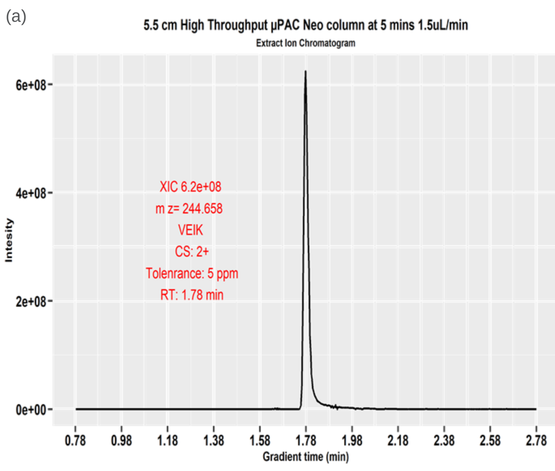


Figure 6. EIC of signature peptides using 50 cm Neo uPAC column

In a 15 min method (a)The FWHM for VEIK, a hydrophilic peptide is 2.94 sec. (b)The FWHM for PENNY peptide is 2.43 sec. (c) EIC of three different glycopeptides, and +G0F and other two glycoforms are separated by 2.4 sec.

(a)



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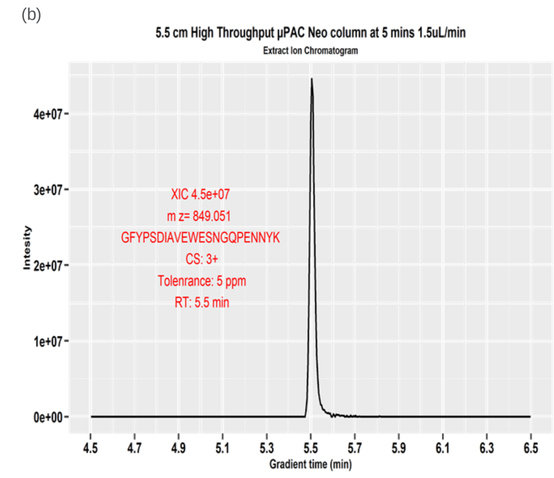

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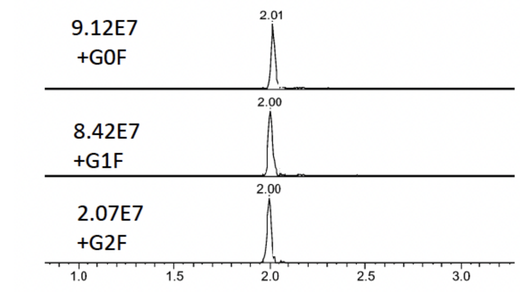

Figure 7. EIC of signature peptides using 5.5 cm high through Neo column

In a 5 min method. (a)The FWHM for VEIK, a hydrophilic peptide is 1.22 sec. (b)The FWHM for PENNY peptide is 1.38 sec. (c) EIC of three different glycopeptides and can barely be separated.

Conclusions

1. For 50 cm µPAC Neo column, in a **15 mins** method, 96.4% and 98.6% are achieved for heavy chain (HC) and light chain (LC) with only 20ng NIST mAb tryptic digest. It is better for analyzing peptides with PTMs (like glycopeptides) as it is suitable for longer elution time.
2. For 5.5 cm High Throughput µPAC Neo column, in a **5 mins** method, 96.4% and 98.6% are achieved for heavy chain (HC) and light chain (LC) with only 20ng NIST mAb tryptic digest. It is better for high throughput screening to get the sequence coverage in 5 mins.
3. Small tryptic peptides such as TKPR and VSNK in heavy chain are not covered because their MS1 signals are too low to be detected in a 20ng sample loading.
4. Compared with traditional microflow columns which usually need 5 ug of sample loading for peptide mapping, this ultralow sample loading of 20 ng using µPAC Neo columns will speed up the total analysis.

References

1. Kavan, D. and Man, P. "MSTools - Web based application for visualization and presentation of HXMS data" Int. J. Mass Spectrom. 2011, 302: 53-58. http://dx.doi.org/10.1016/j.ijms.2010.07.030.

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