Peptide profiling by capillary separation techniques coupled to mass spectrometry

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Chapter 4

Fabrication of narrow-bore packed capillary columns for high-efficiency nanoscale LC-MS analysis of peptide mixtures

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Downscaling of conventional 75 µm bore nanoscale chromatography presents several technical challenges, like column fabrication and large sample volume injection. An original packing procedure based on the concept of self-assembled particle frits (Ishihama et al. J. Chrom. A, 979 (2002), 233-239) is here described. The fabrication procedure could be applied to packing capillary columns down to 30 µm bore size. Thanks to a virtually zero post-column dead volume, high efficiency of separation could be obtained even at the lowest flow rates used (30-40 nL). A valveless trapping column/analytical column setup allowed injection of relatively large sample volumes. The analytical tool provided peptide detection at 1-2 femtomoles level using old-generation ion trap instrumentation.

Introduction

In the last ten years, an explosive increase of interest has characterized the field of nanoscale liquid chromatography. This expansion began with the first explorations of nanoelectrospray ionisation[1,2]. The apparent concentration-dependent behavior of the electrospray interface encouraged the development of infusion systems running at very low flow rates, thus facilitating MS analysis in sample-limited situations. Soon, these nanospray infusion interfaces were coupled at their front end with compatible nanoscale chromatographic separations running at sub-microliter/minute flow rates[3], which contributed to a substantial improvement in overall sensitivity because of their capability of concentrating the analytes of interest in sharp nanoliter-sized volumes prior to their infusion in the mass spectrometer. After years of development, the field of nanoscale LC-MS has now an extremely broad range of applications, among which a special place is held by protein and peptide analysis, surely catalyzing most of the research efforts in the area[4].

A n-fold downscaling of an LC-MS analytical method respect to flow rate, is expected to yield a corresponding n-fold increase in sensitivity. Commercially available nanoscale LC instrumentation usually relies on separations in 75 µm columns running at flow rates of 200-300 nL/min. This represents a good compromise between benefits, i.e. increase of sensitivity, and costs/efforts needed to adapt conventional HPLC systems to performing nanoscale separations. Further downscaling of the chromatography below 200 nL/min requires dedicated, custom-made setups.

Surely one of the most important differences between conventional LC-MS and nanoscale LC-MS lies in the amount of the allowed post-column dead volume. The

importance of minimising post-column dead volume has been already addressed in the literature, and it is estimated to be roughly half of the average peak volume produced by the nanoscale separation[5]. This means that, in case of separations running at or below 200 nL/min, a post-column dead volume of 50 nL or less can be tolerated if separation efficiency has to be preserved. An effective solution to this problem has been proposed by several groups, and consists in packing the electrospray tip directly[6-8] , in order to have the analytes introduced in the mass spectrometer as soon as they leave the chromatographic column. These high efficiency columns were applied to shotgun proteomics studies, requiring separation and identification of large numbers of peptides using multidimensional biphasic columns (MudPIT approach)[9] or used for the analysis of peptides and protein digests for protein identification[8].

Because of the fact that the challenge of minimising post-column dead volume greatly increases with reducing column dimensions, packed ESI emitters represent an attractive solution for separations running at flow rates below 200 nL/min. Despite this fact, very few reports based on packed ESI emitters have explored the possibility of further scaling the analytical chromatographic separation down to columns of 50 µm ID or less.

This work describes an original packing procedure for obtaining high-performing capillary columns down to at least 30 µm bore size. Fabricated columns were used for peptide separations using a valveless trapping column/analytical column setup capable of achieving sub-femtomolar sensitivity using conventional ion trap MS instrumentation. The analytical system was also connected to a new-generation high-resolution FTMS mass spectrometer for the analysis of a complex tryptic digest of cerebrospinal fluid proteins.

Experimental

Materials

Fused silica capillaries (30-75 µm ID, 360 µm OD) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Reversed phase particles (3 µm, C_{18}, 300 Å) were from Grom. Nanofittings unions and tees were from Upchurch Scientific (Oak Harbor, WA, USA).

All chemicals were from Sigma (Zwijndrecht, The Netherlands), unless otherwise indicated. Acetonitrile was from Biosolve B.V. (Valkenswaard, The Netherlands); HPLC water was from JT Baker (Deventer, The Netherlands); formic acid and trifluoro acetic acid (TFA) were from Merck (Darmstadt, Germany).

De-ionised water used for preparing buffers was from a Milli-Q system (Millipore, Bedford, MA, USA).

Sample digestion

Bovine serum albumin: 1 mg of protein was dissolved in 250 μL of 6M urea buffered at pH 8.0 with 50 mM tris. First, disulfide bonds were reduced by adding 25 μL of a 100 mM solution of DTT and incubating 1 h at 37° C. Subsequently, cysteines were alkylated by adding 30 μL of a 200 mM solution of iodoacetamide (1 h incubation at 37° C). Finally 5 μL of 100 mM DTT were added to remove iodoacetamide excess (20 min incubation at 37° C). The sample was then diluted by adding 680 μL of 1mM $CaCl_2$ to diminish urea concentration before digestion. Sequencing grade modified trypsin from Promega (Madison, WI, USA) was added in two steps using in both cases an enzyme/substrate ratio of 1:100; overnight and 4 h incubations were allowed respectively. The digested protein was stored at -20° C before analysis.

Cerebrospinal fluid: 100 μL of a human cerebrospinal fluid (CSF) sample were precipitated using a three-fold volume excess of cold acetone, allowing precipitation to proceed overnight at -20° C. After recovery of the protein pellet, the sample was reconstituted in 25 μL of 6M urea buffered at pH 8.0 with 100 mM tris. Total protein amount (40 μg) was determined using a protein concentration assay (Bradford). Sample reduction/alkylation/digestion proceeded as the BSA protocol described above, but using volumes smaller by a factor of ten.

Column fabrication

The procedure for making a tapered capillary was essentially according to Meiring *et al.*[5]. Briefly, a small piece (1-2 cm) of polyimide coating was removed from the centre of a fused silica capillary 40-50 cm long, by burning with a butane torch followed by ethanol cleaning. The exposed silica was subsequently heated using the same torch, while firmly pulling both ends of the capillary from opposite sides until the single fused silica capillary piece was divided in two tapered emitters. The tip of the emitter was then adjusted to the correct ID, and finally etched in 40% hydrofluoric acid for 30 min while a gentle flow of water was passed through the capillary to prevent acid from etching the inner side of it.

As illustrated in Figure 1, the prepared pulled tips were inserted for 1-2 minutes in a concentrated slurry of packing particles (3 µm, C_{18}, 300 Å) in isopropanol (100-200 mg/ml) by paying attention not to damage the delicate tip end. After letting the self-assembled frit sediment at the tip end by gravity (30 min), the capillary was inserted

into a packing bomb and packed with the same reversed particle slurry (10-20 mg/ml) by initially limiting the backpressure upper value to 20-30 BAR, and subsequently applying a constant packing pressure of 150-180 BAR after the first 0.5-1 cm of packing bed were visible at the tip of the column. Maximum column length for efficient electrospray was found to be 10 cm, probably because of the fact that electrospray voltage was applied at the column inlet, causing problems of excessive voltage drop in case of long columns.

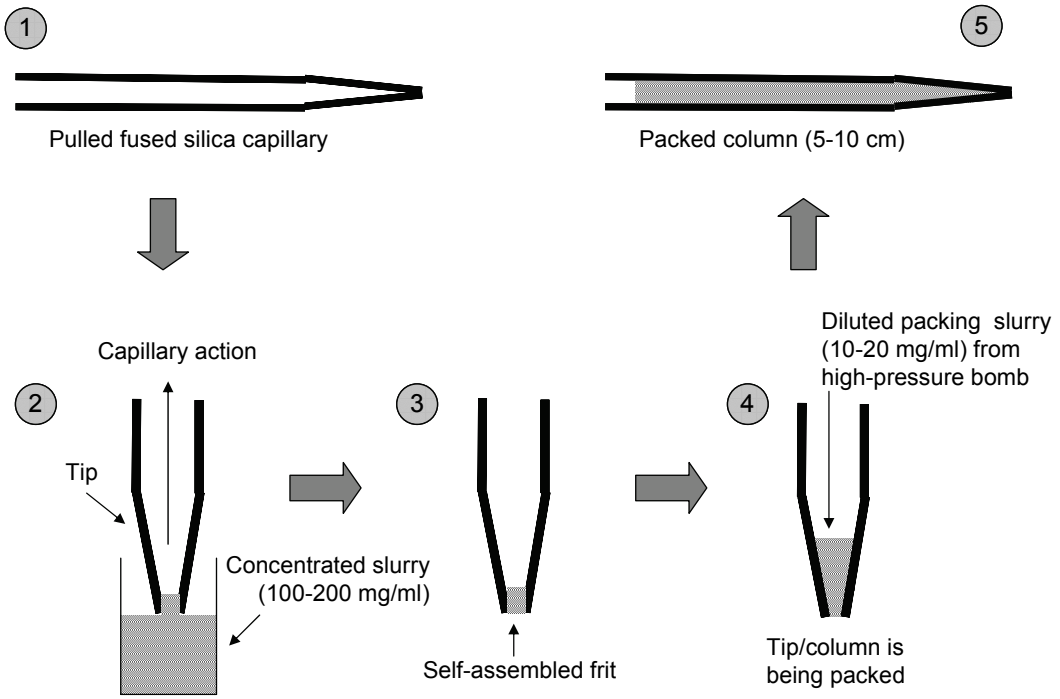

Figure 1 Schematic of the steps comprising the packing procedure used to fabricate the nanocolumns. 1) A fused silica tip is prepared; 2) the tip is immersed in a concentrated slurry of stationary phase; 3) A self-assembled frit is obtained at the tip end without sintering the silica particles; 4-5) the column is being packed with a diluted slurry, allowing packing of very narrow-bore capillaries.

Column testing

Column fabrication quality was tested as described below. The packed capillary was connected to a tee piece. Another tee port was connected to a 2 X 150 mm analytical column (whose outlet flow was then going to waste), which was in fact used as flow restrictor. The third tee port was connected to an Alliance 2610 HPLC pump (Waters) provided with autosampler for sample injection and deliverying a mobile phase of 200

μL/min. Mobile phase A was H_2O/acetonitrile/formic acid 97.9:2:0.1 (v/v/v); mobile phase B was H_2O/acetonitrile/formic acid 19.9:80:0.1 (v/v/v). Since the injected sample too was splitted between nanocolumn and flow restrictor (i.e. the standard analytical column, going to waste), this system was not meaningful for real sample analysis, but it was very useful to test efficiency and resolution of the fabricated columns.
Injected amounts on-column were calculated by estimating the splitting ratio value.

Valveless trapping column/analytical column setup
Trapping columns were made by packing 50 μm ID fritted fused silica capillaries (Integra frits from New Objective, Cambridge, MA, USA). Trapping columns were packed to a length of 1 cm with the same C_{18} stationary phase used for column fabrication. The analytical column used was a 50 μm ID X 10 cm long column packed with C_{18} stationary phase as described in the previous paragraph.
Chromatography was performed on an Ultimate nano LC system from LC Packings (Amsterdam, The Netherlands). Mobile phase A was H_2O/acetonitrile/formic acid 97.9:2:0.1 (v/v/v); mobile phase B was H_2O/acetonitrile/formic acid 19.9:80:0.1 (v/v/v). The setup which was used is illustrated in Figure 2. Microliter sample amounts (1-10 μL) were injected by the Famos autosampler and delivered to the trapping column using the Switchos isocratic pump delivering mobile phase A at 5 μL/min. Such high flow was allowed because the vent between the trapping column and the analytical column was open. Thus, injection solution and subsequent trapping column washes were delivered to waste. After a 5 min wash of the trapping column, the analytical column was switched on-line with the trapping column, while simultaneously switching the ESI voltage on (1.3 kV applied through a Pt wire) and opening the vent placed at the microcross. A 25 μL/min gradient was then delivered by the Ultimate pump and splitted at the microcross using a 15 cm X 800 μm microbore column connected on-line with the gradient flow and used as flow restrictor. A gradient flow of approx. 100 nL/min was thus delivered to the trapping/analytical column in order to elute peptides towards the mass spectrometer. MS detection was achieved by an LCQ classic ion trap MS (Thermo Electron, San Jose, CA, USA) using a nanoelectrospray interface from Proxeon (Odense, Denmark). Ions were introduced in the mass spectrometer through a heated capillary kept at 180° C. The ion trap was operated in full MS mode, scanning from 400 to 2000 *m/z* at three microscans average.

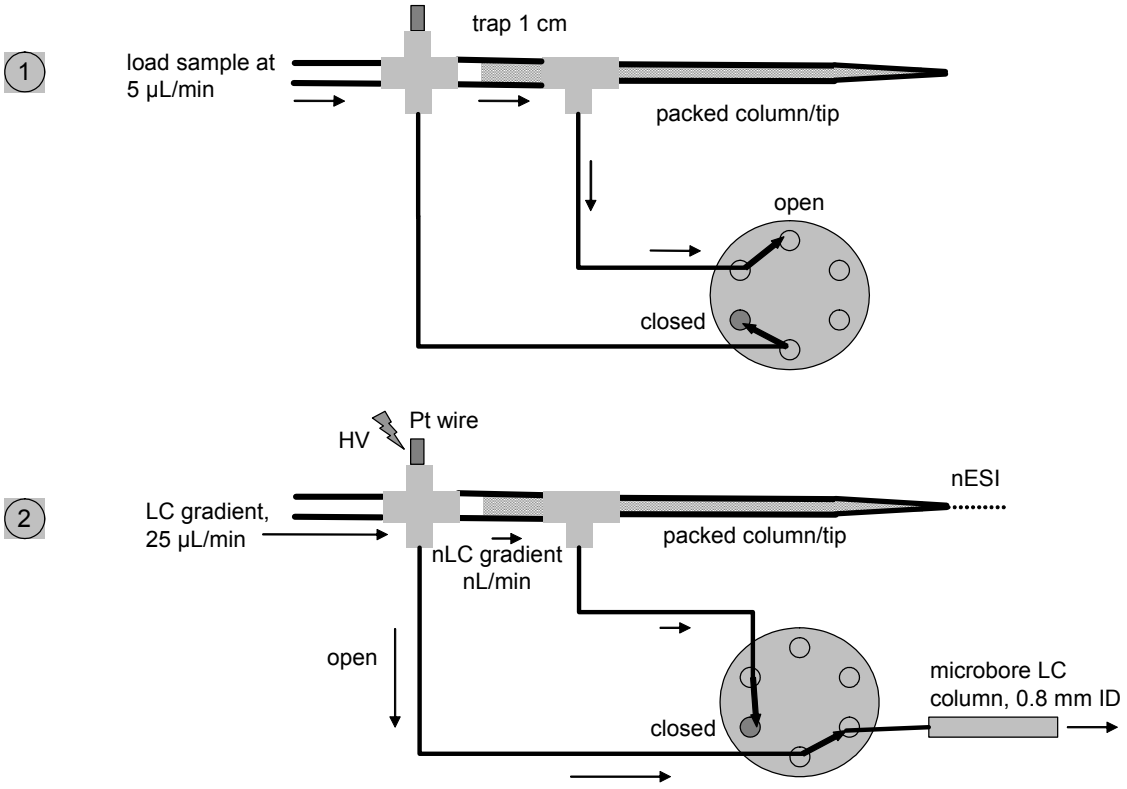

Figure 2 Valveless setup for nanoscale LC-MS. Trapping column: 1 cm X 50 µm ID, packed with C_{18} stationary phase; analytical column: 10 cm X 50 µm ID pulled tip, packed with C_{18} stationary phase. 1) Sample is loaded onto the trapping column at µL/min flow rates by opening the vent at the microtee; after loading, the trapping column is being washed with mobile phase A. 2) The vent at the microtee is closed, connecting the analytical column to the trapping column; a 25 µL/min gradient is delivered by the HPLC pump and splitted at the microcross; peptides are being eluted at approx. 100 nL/min.

For CSF analysis, a hybrid linear ion trap/Fourier transform ion cyclotron resonance mass spectrometer was used (LTQ-FTMS, Thermo Electron, San Jose, CA, USA). The mass spectrometer was used in data dependent mode by combining a single full scan in high resolution mode (resolution 25 000, scan time 1 sec., 200-2000 *m/z* scan) with three MS/MS events (1 microscan, about 0.33 sec each) performed by the linear trap in data dependent mode on highest three ion signals present in the recorded full scan spectrum.

MS/MS spectra were searched against the MSDB database (accessed on August 2004) using MS/MS ion search of the Mascot search engine (www.matrixscience.com). Search parameters used were as follows: peptide mass tolerance: 5 ppm; MS/MS

tolerance: 1 Da; allowed missed cleavages: 1; enzyme: trypsin; taxonomy: homo sapiens; fixed modification: carbamidomethyl (C); variable modifications: oxidation (M), deamidation (NQ), pyro (camC/E/Q).

Results and discussion

The observation that column frits might not be needed in order to pack fused silica capillaries, even if their tip ID is fairly larger than the packing particle diameter, was first reported by Ishihama *et al.*[8]. They stated that tapered fused silica capillaries would retain packing bed without the need for a frit when the ratio between tip ID and packing particle size was in the range from 2 to 5. A drawback of the method of Ishihama *et al.* is that the column is packed using a highly concentrated slurry of packing particles (100-300 mg/ml). At this slurry concentration, there's an increased risk of the packing process starting at some random point in the capillary, following the formation of a self-assembled frit of particles far from the tip end. This risk clearly increases as the bore of the column to be packed decreases, as observed by Kennedy and Jorgenson[10] in the first place. Nevertheless, also thanks to applying a continuous shaking during the packing process, the method of Ishihama *et al.* proved to be very successful in packing 75 μm capillaries. No attempt of fabricating smaller bore columns was reported by the group.

The procedure here reported makes use of the same self-assembled particle frit concept, but in a modified procedure which allows fabrication of columns having very narrow bore ID. The pulled capillary ready for packing is first immersed in a concentrated slurry solution, which is aspirated by capillary action. After 1-2 minutes, the particles are let tightly assemble at the tip of the column by the action of gravity. After formation of the self-assembled particle frit, the capillary is placed in the packing bomb and packed using stationary phase slurry of standard concentration (10-20 mg/ml). This procedure for obtaining the self-assembled frit makes it possible to down-scale column fabrication.

The packing method described was first tested using 75 μm capillaries. Figure 3 illustrates the separation of selected BSA tryptic peptides using two different columns fabricated with the procedure described above. No single fabricated column failed to produce good performance concerning resolution, even though no systematic study on reproducibility of column fabrication concerning retention time values in gradient elution mode was undertaken.

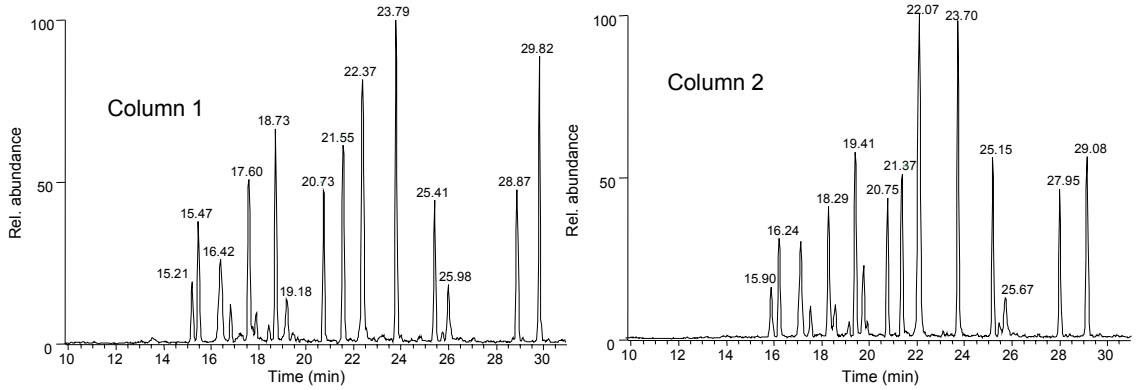

Figure 3 Selected ion chromatogram of several BSA tryptic peptides, specifically chosen to monitor column resolution. The setup was as described in paragraph "Column testing", experimental part. LC gradient was from 0 to 70% B in 35 min.

A following step to the construction of a highly performing nanoscale LC-MS system was to allow injection of relatively large sample volumes (5-20 μL) without compromising analysis time or separation efficiency. The use of trapping columns in-line with the corresponding analytical column has been already reported in the literature[5,11]. Here, a similar valveless setup was adopted, as illustrated in Figure 2. Samples could be injected at relatively high flow rate (2-5 μL/min) during trapping column loading, while subsequent valve switching allowed the nanoscale LC gradient to elute peptides from the trapping column, through the analytical column, to the MS detector. A larger bore packed column was used as a flow restrictor instead of a capillary to generate the required flow splitting (approximately 1:250). While a virtually zero post-column dead volume was present after separation, the valveless setup needed just a minimal dead volume also between trapping column and analytical column (about 30 nL). Such small volume proved to have a little influence on peak broadening. The chromatographic performance of this setup can be appreciated in Figure 4, where the separation of a relatively high amount (100 fmol) of BSA tryptic peptides is displayed.

Commercially available nano LC-MS solutions have recently proven to be robust and amenable to automation. Nevertheless, if maximum sensitivity has to be achieved, further downscaling of the analytical system while maintaining (or improving) chromatographic efficiency is mandatory. The setup described in Figure 2 relied on: (i) reduced flow rate (100 nL/min), (ii) minimisation of post-column dead volume, (iii) the use of a valveless trapping column/analytical column arrangement to deliver excellent sensitivity even with the use of old-generation MS instrumentation (LCQ

Classic). Figure 5 illustrates a peptide analysis close to the limit of detection (2 fmol of BSA digest). Selected ion chromatograms for several BSA tryptic peptides are shown. Signal intensities suggest sub-femtomolar LODs for some of the tryptic peptides illustrated.

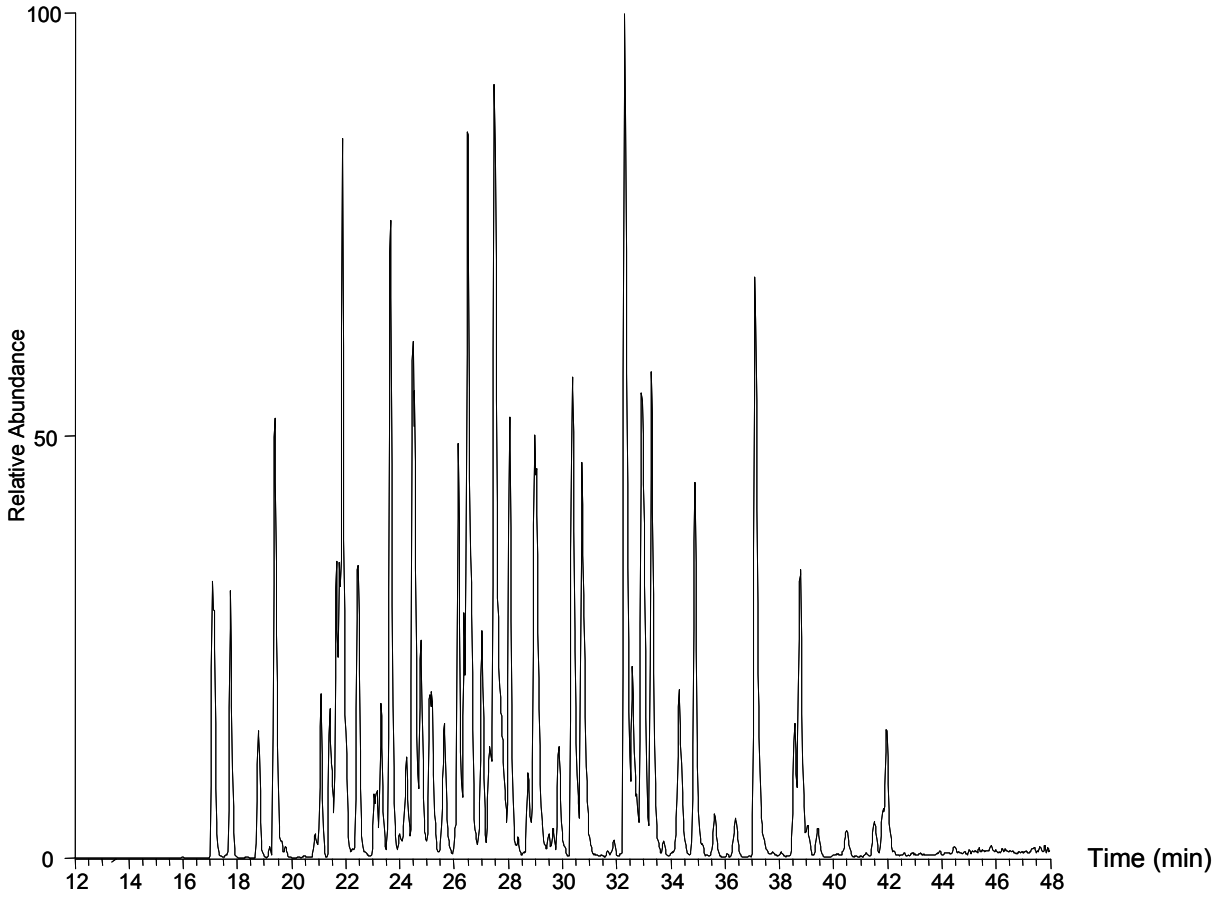

Figure 4 Base peak chromatogram of a separation of 100 fmol BSA digest. Setup as described in Figure 2. LC gradient was from 0 to 60 % B in 40 min, starting at 5 min after sample injection.

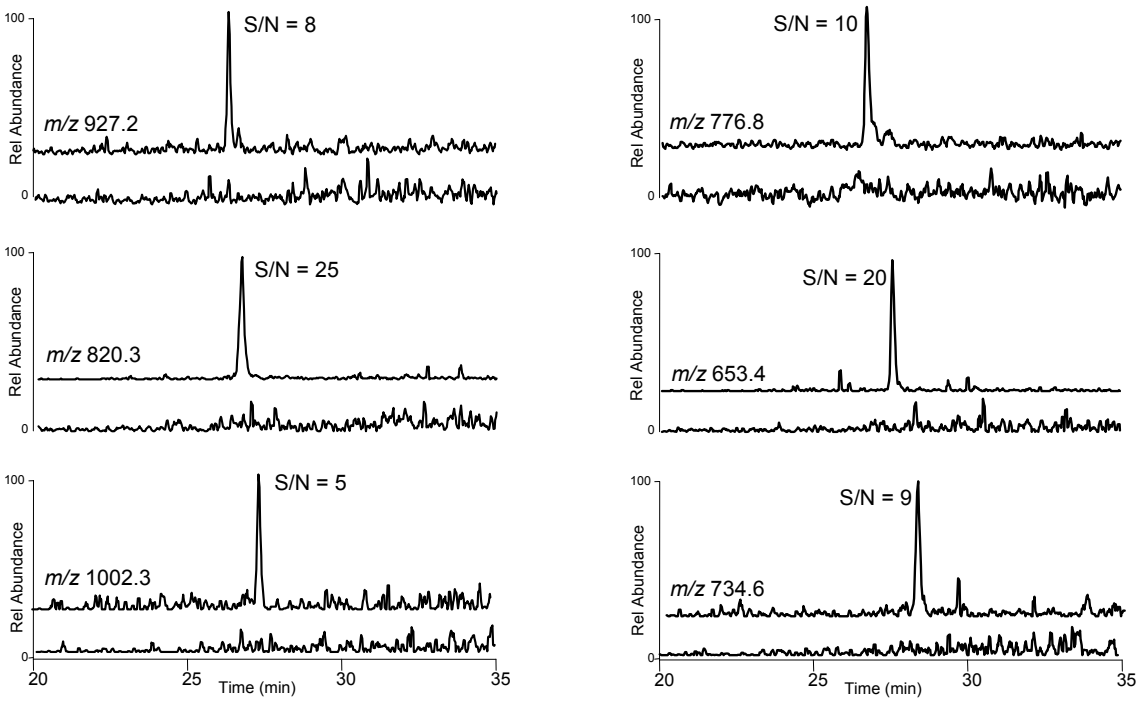

Figure 5 Selected ion chromatograms (SIC) for six BSA tryptic peptides. 1 µL of a 2 fmol/µL digest solution was injected. Conditions as in Figure 5 . Below each SIC, the corresponding chromatogram from a blank injection can be appreciated.

This same nanoscale LC setup was used in combination with state-of-the-art MS instrumentation (Fourier transform ion cyclotron resonance LTQ FTMS) for the analysis of a complex biofluid proteome. Approximately 100 nanograms of a whole digest of human cerebrospinal fluid (CSF) prepared as described in the experimental section were loaded onto the C_{18} trapping column and analysed by gradient elution as described for the BSA standard digest. Tryptic peptides were analysed by the mass spectrometer in data-dependent mode. The profile obtained by such analysis (base peak chromatogram) is displayed in Figure 6. Thanks to the high mass accuracy achieved by the FTMS in full scan mode, and to the high MS/MS throughput achieved by the linear ion trap, over 1500 sequencing attempts were performed in a single, 60 min run, leading to the identification of 200 unique peptides, ultimately assigned to 45 distinct gene products. A full list of identified gene products is reported in Table 1.

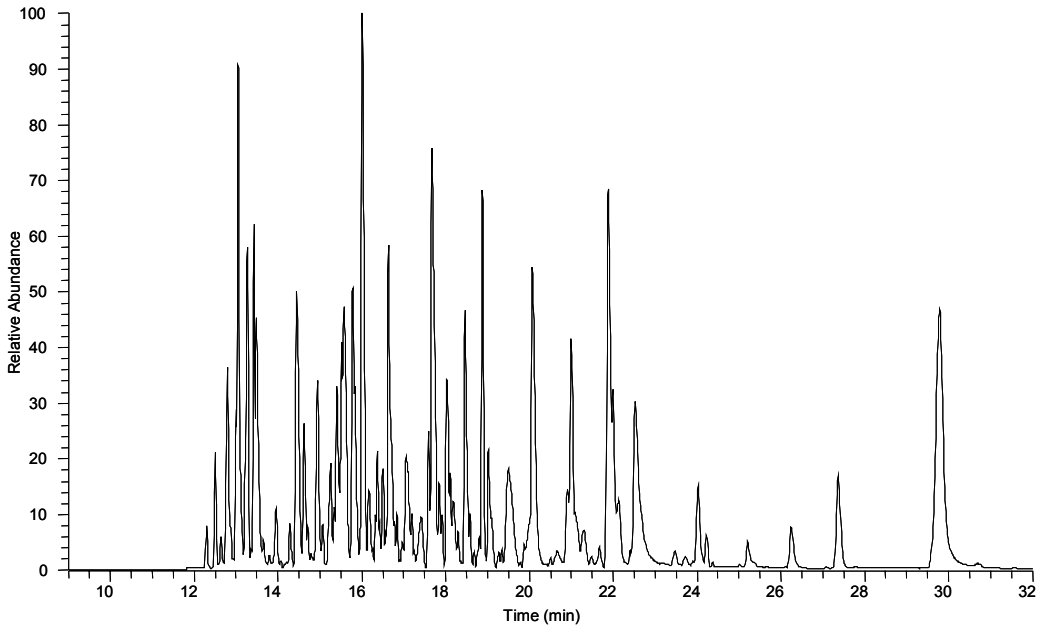

Figure 6 Base peak chromatogram of a nanoscale LC-MS analysis of 100 nanograms of a CSF digest. MS detection was achieved by a LTQ-FTMS mass spectrometer. LC gradient was from 0 to 50% B in 40 minutes.

Table 1 List of identified gene products from nanoscale LC-MS/MS analysis on 100 ng of CSF digest.

Entry	Protein description	N.pep. IDs
ABHUS	serum albumin precursor	45
TFHUP	transferrin precursor	29
LPHUA1	apolipoprotein A-I precursor	8
C3HU	complement C3 precursor	9
AAB59518	HUMAPOE NID	5
A41386	clusterin precursor	4
ANHU	angiotensin precursor	5
1B6DA	immunoglobulin, chain A	4
BAC05421	CDNA FLJ25951 fis, clone SYN00365, highly similar to Homo sapiens immunoglobulin lambda heavy	5
OMHU1	alpha-1-acid glycoprotein 1 precursor	4
BAC01805	Immunoglobulin lambda light chain VLJ region (Fragment)	3
UDHU	cystatin C precursor	5
CNHUB	chromogranin B precursor	4
OQHU	hemopexin precursor	7
1ZAGA	zinc-alpha-2-glycoprotein, chain A	3
C4HU	complement C4A precursor	3
LPHUD	apolipoprotein D precursor	3
BAC01678	Immunoglobulin kappa light chain VLJ region (Fragment)	3
ITHU	alpha-1-antitrypsin precursor	4
APA4_HUMAN	Apolipoprotein A-IV precursor (Apo-AIV)	2
BAA87044	AB045205S7 NID	2
Q9UDW8	WUGSC:H_DJ0747G18.3 protein	1
Q9UC25	Chromogranin B isoform (Fragment)	2
XHHU3	antithrombin III precursor	3
1IGAA	iga1 chains a and b, heavy, chains c and d, light, chain A	2
1TLMA	Transthyretin (also called prealbumin) complex with Milrinone, chain A	3
ITHUC	alpha-1-antichymotrypsin precursor	3
BAB18265	Anti HBs antibody light-chain Fab (Fragment)	2
Q96IZ1	Secreted phosphoprotein 1 (Osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	3
FAHUP	gelsolin precursor, plasma	3
A44455	prostaglandin-D synthase (EC 5.3.99.2) PTGDS, brain	2
Q8TBH8	Fibulin 1	2
BAC01819	Immunoglobulin lambda light chain VLJ region (Fragment)	3
Q9UHG2	PROSAAS precursor (Granin-like neuroendocrine peptide precursor)	1
HPHUR	haptoglobin-related protein precursor	1
WOHU	alpha-2-HS-glycoprotein precursor	1
OMHU1B	alpha-1-B-glycoprotein	1
MAHU	alpha-2-macroglobulin precursor	2
Q9UCD9	Beta-trace protein (Fragment)	1
FINC_HUMAN	Fibronectin precursor (FN) (Cold-insoluble globulin) (CIG)	2
KUHU	ferroxidase (EC 1.16.3.1) precursor	1
C1HUS	Complement subcomponent C 1SBAR.GIF (EC 3.4.21.42) precursor	1
A39842	insulin-like growth factor-binding protein 6 precursor	1
KHL3_HUMAN	Kelch-like protein 3	1
T09575	smoothelin	1

In order to demonstrate the potential of the developed column fabrication procedure in packing very narrow bore capillaries, a 30 μm ID column was constructed and tested using the column testing procedure described in the experimental. Figure 7 shows the separation of ~ 1 fmol of BSA digest separated on the 10 cm long, 30 μm ID column using the standard column testing method (the amount loaded on-column was calculated by dividing the injected amount, 5 pmol, by the theoretical flow-splitting ratio, 4500). Though this test was mainly designed to asses the column packing efficacy, the ability of the nanoscale separation to obtain such high signals from a very low sample load certainly demonstrates the utility of aiming for additional miniaturisation of the technique. The use of the 30 μm ID fabricated columns into a trapping column/analytical column setup has not been implemented yet, but it would certainly represent a further sensitivity improvement of the system.

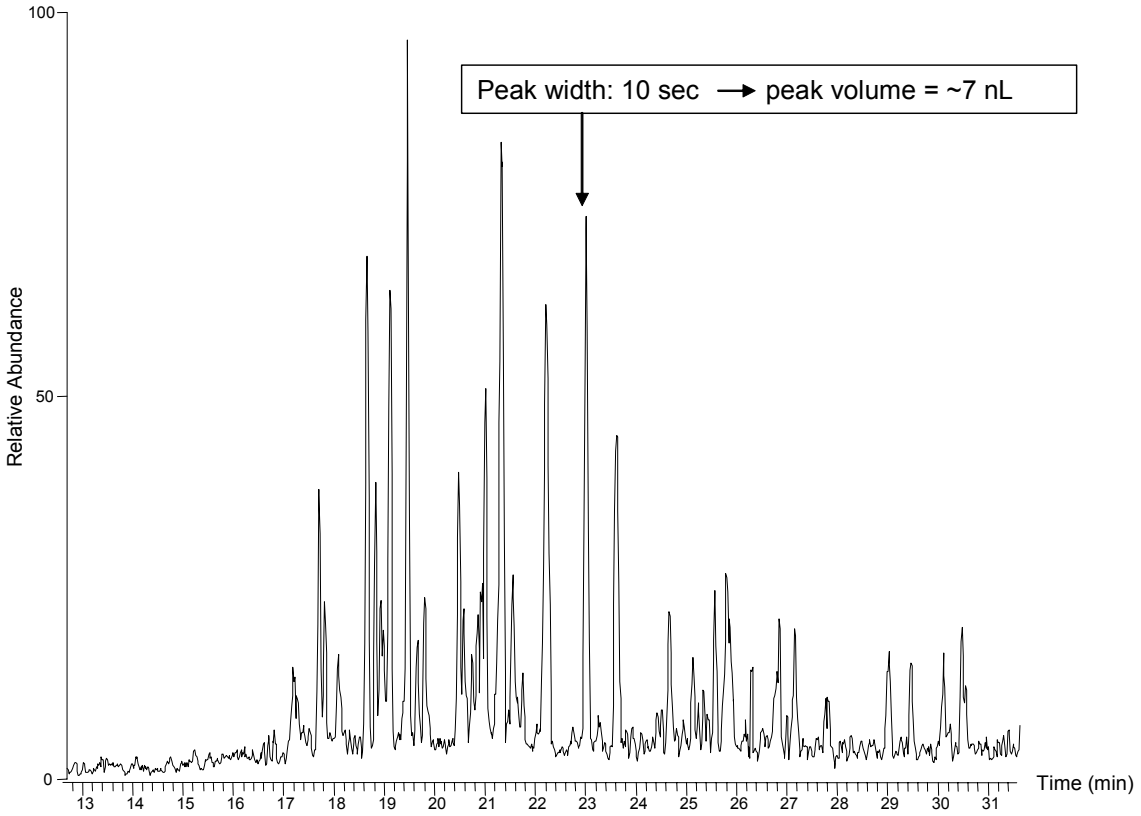

Figure 7 Base peak chromatogram (MS: LCQ classic) for the separation of a BSA typtic digest on a 30 µm ID X 10 cm column. Estimated injected amount was 1 fmol. The setup was as described in paragraph "Column testing", experimental part. LC gradient was from 0 to 70% B in 35 min.

Conclusions

Simple and effective fabrication of narrow-bore chromatographic columns down to 30 μm ID has been demonstrated. Packed pulled electrospray tips of 50 μm ID have been successfully coupled to trapping columns for fast sample loading, and used for high sensitivity peptide profiling. Chromatographic peak volumes in the low nanoliter range and sensitivity in the low- to sub- femtomolar range have been achieved.

Acknowledgements

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