A nanoLC-MS-based platform for peptide analysis

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Fast, High-Efficiency Peptide Separations on a 50-μm Reversed-Phase Silica Monolith in a nanoLC-MS Set-Up

4.1 Introduction

Highly efficient separations are the cornerstone of many analytical methods that deal with extremely complex mixtures. Examples are the analysis of the proteome of a cell, tissue or body fluid, which are currently the focus of many research activities in academic groups and pharmaceutical companies alike. Proteomic studies continue to foster the development of novel technologies able to comprehensively analyse large sets of proteins or peptides. These new analytical tools need to be fast and of sufficient resolving power to detect minute qualitative and quantitative changes in a protein profile. Monitoring such changes could lead to interesting new drug targets or diagnostic tools.

Until recently, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [1] has been the method of choice for the analysis of complex protein mixtures. However, 2D-PAGE shows limitations when it comes to analysing proteins with extreme size and pI. Moreover, the dynamic range is often not sufficient to detect low-abundance proteins [2]. Attempts to overcome these limitations have led to the development of two-dimensional liquid chromatography (2D-LC) for protein and peptide analysis. 2D-LC requires less sample handling and can be more easily

automated. In addition, it can be coupled to mass spectrometry either on-line via electrospray ionisation (ESI) [3–5] or off-line after spotting onto a target plate for matrix-assisted laser desorption ionization (MALDI) [6, 7]. In order to increase peak capacity, the separation mechanisms in 2D-LC must be orthogonal, meaning that they must be based on different interaction principles [8]. Whereas many chromatographic retention mechanisms have been used in the first dimension, the favourite mode of separation for the second LC dimension is reversed-phase (RP). In order to make full use of RPLC coupled to MS/MS for peptide sequencing, the so-called shotgun method was developed by Yates and coworkers, which involves digesting a protein mixture with trypsin prior to separation [3, 9, 10]. Especially in its comprehensive form, complex peptide mixtures generated by shotgun proteomics have to be separated by 2D-LC, which often requires a full day of analysis time per sample [4, 11]. Consequently, great efforts continue to be made to optimise the separation efficiency of LC columns to reduce the overall analysis times. Options to improve separation efficiency include porous or non-porous particles of smaller diameter (i.e. 1 μm) [12], which come at the expense of an elevated backpressure. To circumvent the general problems resulting from the need for very high pressures for μm-sized particles and the low capacity of non-porous media, perfusion chromatography, was introduced [13]. Shortly thereafter, a new support for membrane chromatography was developed to achieve the same goal [14]. In these so-called organic monolithic materials, separation takes place on a very short and wide macroporous polymeric membrane characterised by low backpressures. However, organic polymers are often subject to swelling or shrinking in organic solvents and exhibit micropores [15], drastically deteriorating the performance of a column. A silica-based monolith designed for HPLC was synthesised [16] based on the hydrolysis and poly-condensation of alkoxysilanes [17]. It exhibited a bimodal pore structure with μm-sized throughpores and nm-sized mesopores. This structure allowed high-efficiency separations at very low backpressure. First synthesised in 9.0-mm-I.D. polycarbonate molds [16,18,19], silica monoliths have also been prepared in the capillary format [20, 21] and coupled to mass spectrometry for application in proteomics research [21–23]. The study of the proteome often requires high-sensitivity measurements and has thus stimulated further miniaturisation of monolithic chromatography columns. In this study, we present the evaluation of 50-μm-ID reversed-phase silica monoliths which are compatible with nanoelectrospray ionisation, using tryptic digests of standard proteins as well as a real-life sample. The influence of various chromatographic parameters on efficiency, resolution and analysis time was assessed. To demonstrate the capability of such an analytical system for high-resolution, high-speed separations, a tryp-

tic digest of depleted serum from a cervical cancer patient was analysed. The throughput time of this analysis was 30 min per sample, with peak widths of approximately 10 s at half height. High repeatability of retention times (0.5 - 1.6% relative standard deviation (RSD) between columns) and peak heights (<20% RSD without the use of an internal standard) in a nanoLC-MS set-up allowed comparative analyses of complex samples using these reversed-phase silica-based monoliths, as required in biomarker discovery projects.

4.2 Experimental

4.2.1 Chemicals

Formic acid (FA) and hexylbenzene (98-100% pure) were purchased from Merck (Darmstadt, Germany), and acetonitrile (HPLC Supra-Gradient grade) from Biosolve (Valkenswaard, The Netherlands). Horse-heart cytochrome c (CC) (>95% pure), dibutylaniline (DBA) (97% pure), ammonium bicarbonate and ammonium acetate (>98% pure) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Modified trypsin (sequencing grade) was purchased from Promega (Leiden, The Netherlands).

4.2.2 Equipment

The nanoHPLC system was based on a Series 1100 set-up, consisting of a dynamic nanoflow splitter with flow meter, micro vacuum degasser, thermostated microwell-plate autosampler with a six-port micro switching valve combined with a thermostated column compartment, six-port micro switching valve and ion trap SL mass spectrometer (Agilent Technologies, Waldbronn, Germany) [5]. The nanoelectrospray ion source was modified in-house to minimise post-column dead volumes (<20 nL). Nanoelectrospray tip and analytical column were butt-connected using a teflon sleeve of 360-μm ID, thereby reducing post-column dead volumes by approximately 80% as compared to the original design. The C_{18} trap column was loaded using an ISCO pump model 2350 from Teledyne ISCO, Inc. (Wierde, Belgium).

The loading pump was directly connected to the microwell-plate autosampler, so that the flow passed through the micro switching valve, sample loop and needle. The sample plug was concentrated on a C_{18} trap column, which was mounted between two ports of the switching valve in the thermostated column compartment. The flow was further directed to waste during sample loading. The nanopump

was also connected to this second micro switching valve, in order to direct the flow through the trap column and elute the analytes onto the monolithic (analytical) column in the backflush mode. The analytical column was butt-connected to the gold-coated nanoelectrospray emitter (50-μm fused-silica capillary tapered to 10 μm ID, Nanoseparations, Nieuwkoop, The Netherlands) using a 360-μm-ID Teflon sleeve, and subsequently mounted in front of the nanoelectrospray source entrance.

4.2.3   Liquid chromatography

The following conditions were used for the analysis of tryptic peptides throughout this study, unless otherwise stated:

Analytical column: silica-based reversed-phase (C_{18}) monolith, 560 x 0.05 mm, fabricated for research purposes using a method similar to that reported in [20] (Merck, Darmstadt, Germany). Capillaries prepared following this procedure generally exhibit throughpores of 2-3 μm, a skeleton of 1.0-1.5 μm and mesopores of 18 nm.

Trap column: Zorbax 300 SB C_{18}, 5 μm particle diameter (d_p), 5 x 0.3 mm (Agilent, Waldbronn, Germany)

The loading pump delivered a solution of 0.1% FA in water: acetonitrile (ACN) (97.5:2.5) at 50 μL/min. The trap column was switched on-line with the analytical column after a 5-min loading time.

Gradient elution was performed with 0.1% FA in water as mobile phase A and 0.1% fa in ACN as mobile phase B. Gradient elution, unless otherwise stated, was performed according to the following scheme: 2.5% B from 0 to 5 min, linear increase to 40% B in 25 min, linear increase to 80% B in 5 min, column wash at 80% B for 4 min, return to initial conditions (2.5% B) in 6 min. Equilibration of the column at 2.5% B for 10 min. The flow rate was set as described in the Results section.

4.2.4   Mass spectrometry

The following settings were used throughout this study. Ionisation parameters for nanoelectrospray experiments: drying gas (N_2), 6.0 L/min; temperature, 285˚C; capillary exit voltage, 115.0 V (CC experiments) and 106.5 V (DBA experiments); skimmer voltage, 40.0 V. The capillary voltage was set between 1550 and 2650 V, depending on the flow rate and the slope of the gradient. Ionisation parameters for experiments using the standard ESI source: nebulising gas (N_2), 16.0 psi; drying gas (N_2), 6.0 L/min; temperature, 285˚C. The capillary voltage was set

at 3200 V, below which no stable signal could be obtained. For peptide analysis, the mass analyser parameters were: octapole 1 DC, 12.0 V; octapole 2 DC, 2.24 V; octapole RF, 150 Vpp; lens 1, -5.0 V; lens 2, -60.0 V; trap drive, 78.6 V. For DBA, the mass analyser parameters were: octapole 1 DC, 12.0 V; octapole 2 DC, 1.70 V; octapole RF, 132.8 Vpp; lens 1, -5.0 V; lens 2, -60.0 V; trap drive 29.5 V. For both types of experiments, the following parameters were kept constant: dampening gas, He; target, 30 000 ions; maximum accumulation time, 30.0 ms; number of averaged scans: 4; rolling average 'ON', number: 2.

4.2.5 Sample preparation

4.2.5.1 Tryptic digest of cytochrome c

0.5 mg CC was dissolved in 50 μL of freshly made 100 mM ammonium bicarbonate (pH $\approx$ 8.0). 50 μL of trypsin (0.4 mg/mL trypsin in 100 mM acetic acid in water), 25 μL CaCl$_2$ (100 mM) and 425 μL NH$_4$HCO$_3$ (100 mM) were added to the vial containing the CC solution. The mixture was incubated overnight at 37°C and 350 rpm in an Eppendorf thermomixer. After about 16 h, the solution was diluted 100 times with 0.1% FA in water: ACN (97.5:2.5) to a concentration of 833 fmol/μL. The sequence, molecular weight, m/z and assigned number of the studied peptides are shown in Table 4.1. For a second batch of CC tryptic digest, prepared using the same protocol, peptide 6 was not observed. The more hydrophilic peptide 7 was studied in place of peptide 6.

Peptide Number	Sequence	Molecular Weight	m/z
1	KTGQAPGFSYTDANK	1584.7	529.8 and 793.2
2	TGQAPGFSYTDANK	1456.8	729.3
3	EDLIAY	723.6	723.6
4	MIFAGIK	779.8	390.8 and 779.5
5	TGPNLHGLF	955.5	478.8
6	GITWGEETLMEYLENPK	2010.0	671.0 and 1005.5
7	IFVQKCAQCHTVEK	1633.8	545.7

Table 4.1: Assigned number, amino acid sequence, molecular weight and m/z of selected tryptic peptides of cytochrome c. Peptides 3 & 5 resulted from the digestion of CC by residual chymotryptic activity present in the trypsin stock solution.

4.2.5.2 Preparation of a tryptic digest of human serum

Serum was obtained from the Department of Gynecological Oncology (University Medical Center Groningen, The Netherlands) and depleted of albumin and γ-

globulins as previously described [24]. 1.26 pmol/μL CC were added to the original
serum for internal calibration. The depleted serum was subsequently digested with
trypsin as described above at an enzyme-to-total protein ratio of 1:20.

4.2.5.3   Preparation of dibutylaniline and hexylbenzene solutions

A solution of 4.4 μM DBA was prepared by making 6 consecutive dilutions of
1 volume of DBA solution in 9 volumes of 10 mM ammonium acetate (pH =
6.86): ACN (1:9), starting from pure liquid DBA (molecular weight = 205.35
g/mol, density = 0.906, m/z = 206.4). A solution of 1.06 mM hexylbenzene
was prepared by diluting 20 μL of pure hexylbenzene (molecular weight = 162.28
g/mol, density = 0.861) solution to 100 mL using methanol : water (80:20).

4.2.6   Determination of column and system characteristics

4.2.6.1   Optimum plate height for dibutylaniline and hexylbenzene

For independent determination of the optimum height equivalent of a theoretical
plate (HETP) value for the 50-μm-ID monolithic column, a chromatographic sys-
tem where both the flow and the sample are split before the column was used.
Post-column band broadening was minimised using a capillary electrochromatog-
raphy (CEC) UV detection cell directly connected to the column. The CEC cell
was a Knauer K-2501 model (Knauer, Berlin, Germany) fitted with a 100-μm ID/
360-μm OD capillary with a window for detection. A split ratio between 1:600
and 1:1000 was obtained, depending on the pressure produced by the monolithic
column. Column flow rates between 0.1 and 0.2 μL/min were obtained (0.81 to
1.62 mm/s, respectively). A 1.0 μL sample was injected and split before the col-
umn to bring a 1.0-to-2.0 nL sample plug onto the column. Separation efficiency
was determined under isocratic conditions with 10 mM ammonium acetate (pH
6.86) and ACN, at a ratio of 1:9 (v/v) for a sample containing 4.4 μM DBA in
10 mM ammonium acetate (pH = 6.86): ACN (1:9). For hexylbenzene, isocratic
elution conditions were methanol/water at a ratio of 80:20 (v/v). Hexylbenzene
was injected as a solution prepared in mobile phase.

4.2.6.2   Van Deemter curve for cytochrome c digest

1 μL of digested 833 nM CC in 0.1% formic acid in water: ACN (97.5:2.5) was
injected onto the nanoLC system (trap column + monolithic column) at varying
flow rates, from 0.25 to 1.95 μL/min (linear velocities between 2.4 and 18.4 mm/s)
at a constant mobile phase composition of 25% ACN in 0.1% aqueous formic

acid. Five injections were made at each flow rate, and the average HETP was determined.

The Van Deemter curve for the monolithic column without the trap column was determined in a similar fashion, by injecting 1.0 μL of a solution of 833 nM CC tryptic digest in 0.1% formic acid in water: ACN (97.5:2.5) directly onto the column under the same chromatographic conditions used above, to focus the peptides at the beginning of the column. This methodology was also applied to assess the linear flow rate dependence for the HETP obtained for DBA. Eluent conditions as described in section "Optimum plate height for dibutylaniline and hexylbenzene".

4.2.6.3 Loadability and capacity for cytochrome c digest

The loadability of the system and the capacity of the monolithic column were evaluated by injecting increasing amounts of a CC tryptic digest ranging from 10 ng (833 fmol) to 8.0 μg (666 pmol).

4.2.6.4 Optimisation of chromatographic parameters for rapid separations

The influence of flow rate on resolution under gradient elution conditions was investigated by injecting 833 fmol of CC tryptic digest 5 times each at flow rates ranging from 0.25 to 1.95 μL/min and a gradient slope of 1.5 %. Similarly, the effect of gradient slope on the separation was examined by injecting 833 fmol of CC tryptic digest 5 times consecutively at each gradient slope, ranging from 0.25 to 9.0 %, at a flow rate of 1.0 μL/min.

To shorten the analysis time of depleted, digested serum from a cervical cancer patient, we used a reduced loading time for the trap column (1 min at 50 μL/min) and increased the flow through the monolithic column to 1.5 μL/min. Gradient slope was 3.0 % between 10 and 40% and 4.0 % up to 80%, a composition that was maintained for 4 min. The column required an equilibration time of 5 min at 1.75 μL/min at the end of the cycle reducing the total cycle time to 30 min.

4.3   Results and Discussion

4.3.1   Chromatographic efficiency of 50 μm reversed-phase silica-based monoliths

Traditionally, low-molecular-weight compounds such as benzene derivatives have been used to characterise silica-based reversed-phase monoliths [25] and RPLC columns in general. Chromatographic performance of the 50 μm silica-based monolith was thus first evaluated by studying the relationship between HETP and the linear flow rate for the analytes DBA and hexylbenzene. To avoid extracolumn contributions to band broadening, a split-flow system was used, and detection was performed by UV absorbance in a CEC detector cell. Isocratic elution gave minimum HETPs between 7 and 9 μm at linear flows between 1 and 2 mm/s for DBA, and 11 μm at 1 mm/s for hexylbenzene. Similar results are reported for other monoliths [19], including a 50-μm-ID-sized silica-based monolith [20].

Monolithic columns have high column permeability and small-sized skeletons that decrease the diffusion pathlength of molecules in the stationary phase, resulting in a reduced contribution of both the A- and C-terms to band broadening. This characteristic is of special interest for molecules having low diffusion coefficients, such as proteins and peptides. Taking this into account, the system efficiency was also evaluated for selected tryptic peptides of CC, mimicking the situation encountered in proteomics applications.

A tryptic digest of CC in 0.1% aqueous FA : ACN (97.5:2.5) was analysed under isocratic conditions of 25% ACN in 0.1% aq. FA over a linear flow range of 2 to 18 mm/s (0.25 to 1.95 μL/min). Direct injections were made onto the monolithic column (no trap column), and detection was by nanoelectrospray MS. Figure 4.1 shows that minimum HETP of 5 to 10 μm were obtained at a linear flow velocity of 2.4 mm/s. HETP increased to approximately 140 μm at the upper end of the linear flow range, a region that cannot be studied in conventional packed columns. It should be noted that even the use of more than 4000 bars in UltraHigh Pressure Liquid Chromatography (UHPLC) [26] does not allow linear flows of this magnitude. Thus, HETP values for a low-molecular-weight analyte (DBA) and tryptic peptides from CC were comparable under similar flow conditions.

In order to evaluate performance under conditions usually employed for proteomics samples, the peptide mixture was injected onto a nanoLC-MS system containing a trap column. The analyses showed that the contribution of the trap

column to the overall efficiency was negligible. Omission of a trap column in the system led to slightly reduced performance for the most hydrophilic peptides. For example, peptide 2 (see Table 4.1) had a peak width at half height ($w_{0.5}^-$) of 5.2s (RSD = 22.7%) without a trap column, while $w_{0.5}^-$ was 4.8s (RSD = 15.3%) with the trap column (based on extracted-ion chromatograms). This was likely due to band broadening induced by the rather large injection volume. Since omission of a trap column is not practical for proteomics applications, we pursued evaluation of the complete system, including the trap column.
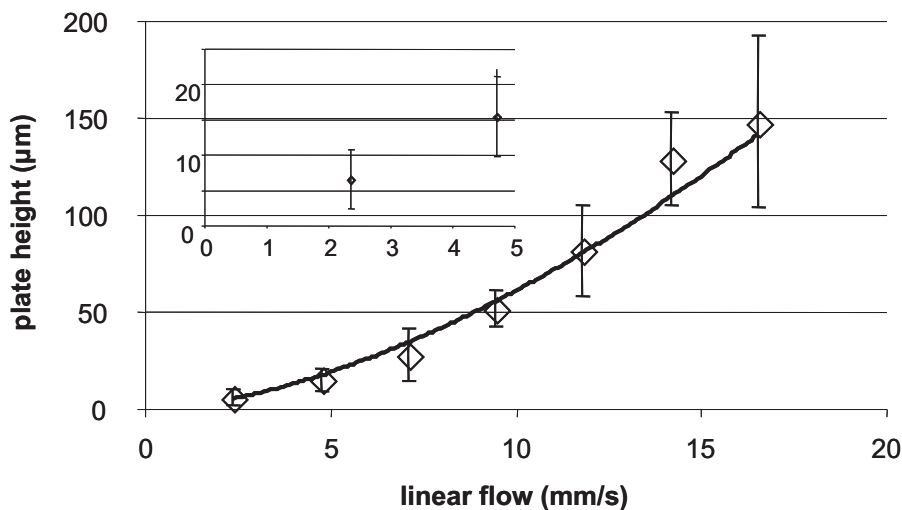

Figure 4.1: Van Deemter curves for a 50-μm-ID silica-based reversed-phase monolith for a tryptic digest of CC (diamonds) (isocratic in 0.1% FA in water: ACN (3:1)). Each point is the average of 5 determinations for the 6 different CC peptides and standard deviations are shown as error bars. Flow rate was varied between 0.25μL/min and 1.75 μL/min (average plate height of 6 peptides) corresponding to a linear flow range of 2.0 to16.5 mm/s.

4.3.2 Repeatability

Comparative proteomics analyses require that separations be highly reproducible, notably with respect to retention times. To evaluate the repeatability of separations on 50-μm silica-based reversed-phase monoliths, a tryptic digest of CC was injected 24 consecutive times onto two different monolithic columns on different days and analysed by nanoLC-MS. The separations were performed using gradient elution at 1.5 %. Separation showed an intra-column repeatability of <0.5% relative standard deviation (RSD) for retention times and <15% for peak widths

at half height. The MS signal intensity showed a RSD of <35%. Inter-column reproducibility proved to be very high, with RSDs of the corrected retention times between 0.5 and 1.6%. Inter-column reproducibility for peak width at half height showed RSDs between 13 and 30%. This relatively large variation in peak width originates primarily from spray instability rather than from differences in separation efficiency between columns. The results obtained for reproducibility of retention time agree well with the findings of Kele et al., who reported RSD values inferior to 0.2% for retention times and between 1 and 4% for peak width at half height in a long-term reproducibility experiment [27]. Instability of the nanoelectrospray signal was the main cause for the lower repeatability in peak width or intensity in the nanoLC-MS system as compared to UV detection (Figure 4.2 and Table 4.2). Even though repeatability is negatively affected by some instability of the nanoelectrospray, it was our intention to evaluate the system under conditions normally required for high-sensitivity proteomics work. The retention time repeatability and stability over time for the evaluated silica monoliths is promising with respect to their future use in comparative biomarker studies and for proteomics in general.

	Retention times (min)		Peak width at half height (min)		Peak height (counts)	
	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
Peptide 1	18.96	0.49	0.16	6.89	591445	10.59
Peptide 2	19.94	0.48	0.16	8.34	7451711	9.74
Peptide 3	22.77	0.35	0.19	8.28	3152229	17.51
Peptide 4	22.97	0.36	0.20	13.97	4577364	19.71
Peptide 5	24.69	0.28	0.20	9.87	5065457	12.95
Peptide 7	27.83	0.10	0.14	9.39	1789507	35.43

Table 4.2: Repeatability of retention times, peak widths at half height and peak heights for a silica-based monolith (50 μm ID) as assessed by 24 consecutive injections of a cytochrome c tryptic digest in the course of one day. Peptide 6 was not observed due to a missed cleavage, peptide 7 was the most hydrophobic peptide in this case (see Table 1 for peptide identities).
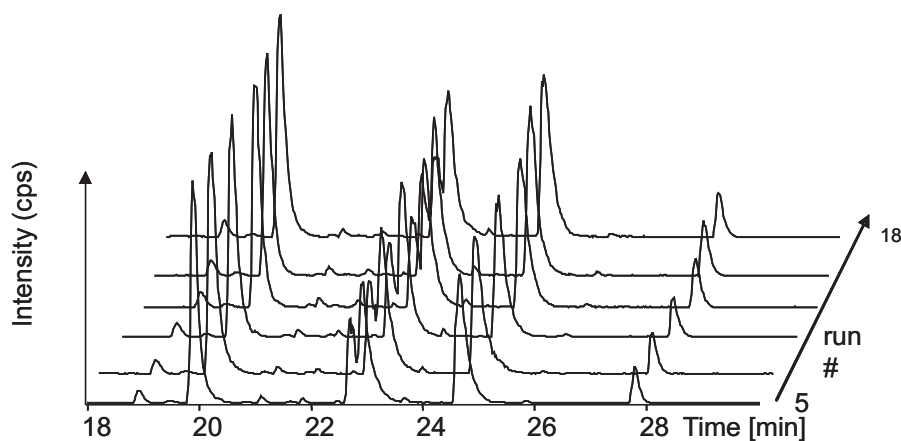
Figure 4.2: Repeatability of the separation of selected tryptic peptides (peptides 1 to 7 except peptide 6, cf. Table 4.1) of CC with 1.5% gradient slope and a flow rate of 1.0 μL/min. Runs were performed over 24 h. Not all chromatograms are shown. The lowest chromatogram corresponds to run number 5, while the highest chromatogram corresponds to run number 18. Combined extracted ion chromatograms corresponding to the m/z values reported in Table 4.1.

4.3.3   System loadability

Monolithic stationary phases attempt to combine high efficiencies at relatively low pressures with sample loadabilities comparable to porous silica particles. This is achieved through a bimodal pore size distribution [28]. Sample loadability is of great importance in proteomics studies, since many peptides of interest occur at low relative abundances in the sample.

Loadability of a column can be determined by either injection of increasing amounts of sample (elution chromatography) or by continuous infusion of sample until it appears in the breakthrough (frontal chromatography) [29]. In a nanoLC system consisting of a trap and an analytical column, it is important to evaluate the loadability for both columns. The trap column serves to quantitatively retain analytes, and can be almost saturated with peptides, as long as the monolithic (analytical) column has sufficient loading capacity and a stronger hydrophobicity than the trap column to refocus the analytes. The loadability of the trap column was first determined by applying increasing amounts of a CC tryptic digest ranging from 10 ng (833 fmol) to 8.0 μg (666 pmol). Elution was performed with a gradient slope of 1.5 % and a flow rate of 1.0 μL/min. The peak area for each peptide was chosen as an indicator of overloading of the trap column, due to loss

of peptides to waste as a result of breakthrough during loading and washing. Injections of increasing amounts of a tryptic CC digest resulted in an increase in peak area up to approximately 1.0 μg (83.3 pmol). For larger amounts, the peak area of the early eluting peptides started to decrease while the peak area kept on increasing for the more hydrophobic peptides (Figure 4.3). This shows that competition for the stationary phase occurred on the trap column, and that the most hydrophilic peptides started breaking through due to displacement by the more hydrophobic peptides. There was no indication of peptides in the flow-through of the monolithic column at a loaded amount of 1.0 μg, showing that overloading occurred on the trap column and not on the monolith. Therefore, it is important that the amount of sample injected onto this system remains smaller than 1.0 μg for a trap column of 5 $\times$ 0.3 mm (void volume $\approx$ 1.0 μL) in order not to perturb the original composition of the sample by selectively losing the more hydrophilic peptides to waste.
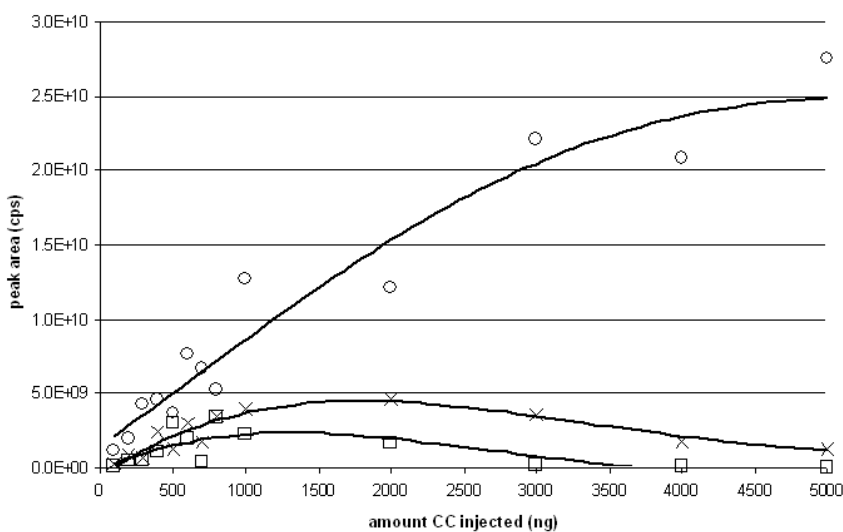

Figure 4.3: Capacity of the nanoLC-MS system with respect to a tryptic digest of CC. Increasing amounts of a CC digest were loaded at 50 μL/min onto a trap column with 0.1% FA in water: ACN (97.5:2.5). Separation was performed with a 1.5% gradient slope at a flow rate of 1.0 μL/min. (peptide 2, squares; peptide 4, crosses; peptide 6, circles). For peptide identities, see Table 4.1.
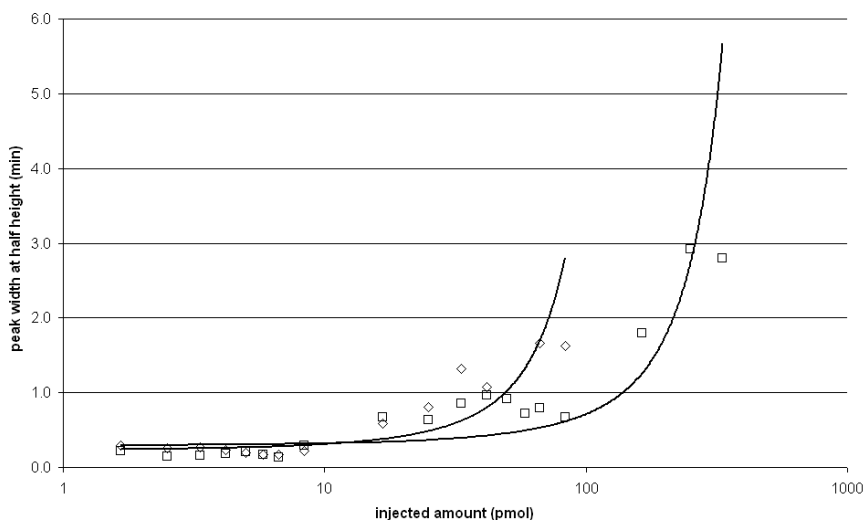
Figure 4.4: Evaluation of the capacity of the 50 μm monolithic reversed-phase column by measuring the peak width at half height versus the amount of loaded tryptic CC digest. Increasing amounts of the CC digest were loaded in 1 μL of 0.1% FA in water: ACN (97.5:2.5) and the separation was performed with a 1.5% gradient slope at a flow rate of 1.0 μL/min. The column is considered to be overloaded when the peak width increased by 20% or more compared to its original value. Points above the breakthrough point on the trap were not included. (peptide 3, diamonds (early eluting); peptide 6, squares (late eluting)). See Table 4.1 for peptide identities.

To evaluate the loading capacity of the monolithic column in a nanoLC-MS set-up, increasing amounts of a CC digest were loaded. An analytical column is considered overloaded with respect to a given component when the retention time of the analyte diminishes, the resolution between two compounds in a mixture decreases (increasing peak widths) and peak fronting appears, leading to an increase in the HETP. We chose to measure the peak width at half height as a sensitive indicator of overloading. The column was considered to be overloaded when the peak width increased by 20 % [26]. Overloading became apparent above 8.3 pmol (100 ng) CC digest (Figure 4.4), although this amount is well below the total loading capacity of the monolith. Larger amounts of tryptic digest resulted in excessive peak broadening, dramatically lower plate numbers and thus a much reduced resolution. For amounts greater than 1.0 μg of proteolytic digest, displacement effects on the monolith became visible, as exemplified for peptide 4

(Figure 4.5). A marked peak sharpening due to displacement of peptide 4 by the more hydrophobic peptide 6 on the analytical column was observed.
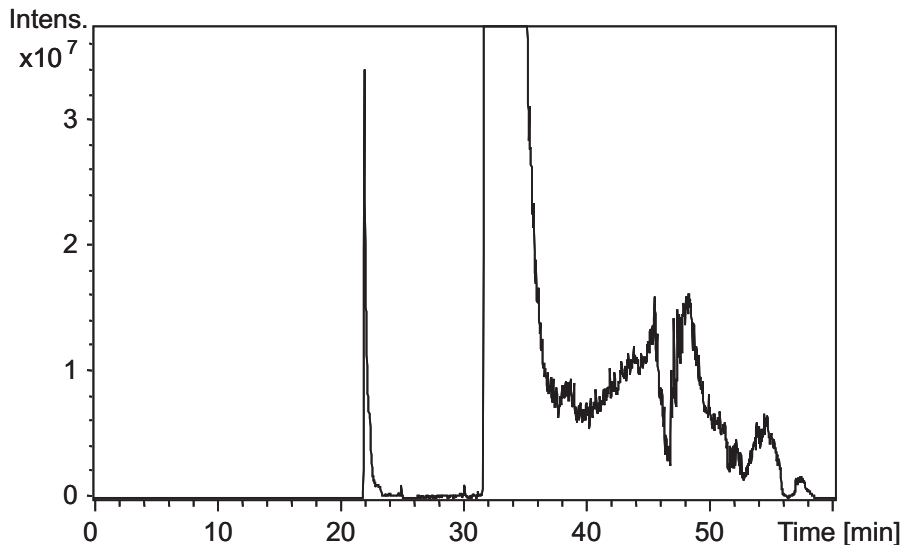

Figure 4.5: Example of displacement of peptide 4 (appr. 22 min) by a larger amount of the more hydrophobic peptide 6 (appr. 32 min) at a sample load of 3.0 μg (appr. 250 pmol). Separation with a 1.5% gradient slope at a flow rate of 1.0 μL/min. Combined extracted ion chromatograms of tryptic peptides 4 and 6 of CC (cf. Table 4.1).

The capacity of a column is related to its accessible surface area. Leinweber et al. [30] showed that silica monoliths have a specific surface area comparable to that of a capillary of similar dimensions packed with 5-μm porous silica particles. 1.5-μm ethyl-bridged hybrid silica porous particles [26] exhibit a specific surface area comparable to that of both monolith and conventional porous silica particles. To compare the capacity of columns of different dimensions filled with different materials, it is easier to refer to the capacity (in mol or g) divided by the capillary volume (in μL). From the data reported by Jorgenson and coworkers [26], ratios of 0.45 to 0.9 pmol/μL and 14 to 19 pmol/μL were calculated for 1.0-μm non-porous silica particles and 1.5-μm ethyl-bridged hybrid silica porous particles, respectively. Smith et al. [31] reported a capacity between 0.15 and 1.5 μg for a complex peptide mixture, depending on the experimental conditions for a 150-μm-ID capillary packed with 5-μm porous silica particles. This yielded capacity-to-

column volume ratios of 8.5 to 85 ng/μL. The monolith used in this study showed good resolution up to about 10 pmol/μL or 100 ng/μL and therefore exhibits a capacity comparable to porous silica particles and highly superior to non-porous particles.

4.3.4 Optimisation of chromatographic parameters for rapid separations

An important aspect of high-performance separations is the reduction of separation time to a minimum, especially in proteomics studies, where large numbers of samples need to be compared. In the following, we have investigated how the overall analysis time can be shortened for complex biological samples by exploiting the unique properties of silica-based monoliths.

4.3.4.1 Influence of flow rate and gradient slope on chromatographic resolution

Except for peptides 3 and 4, which were very difficult to separate, resolution decreased marginally up to a maximum flow rate of 1.95 μL/min under gradient elution conditions (data not shown). This flow rate is 10 times higher than for columns of similar internal diameter packed with 5-μm beads, and makes it possible to regenerate and re-equilibrate the monolith-based capillary column much faster than regular columns. These flow rates are also much larger than those achieved in UltraHigh-Pressure Liquid Chromatography (UHPLC), but are generated at a fraction of the column pressure [26].

In order to shorten analysis time, we investigated the effect of increased gradient slope on resolution in the 50 μm monolith. Gradient slopes ranging from 0.25 to 9.0 % were investigated for the separation of a tryptic digest of CC (Figure 4.6). All peptides, except the closely eluting peptides 3 and 4, were adequately separated (resolution >1.25) within a time window of 3 min at 9% and a flowrate of 1 μL/min (Figure 4.6, lower trace). The use of a gradient as steep as 15% was investigated, but the time saved on the analysis was not large enough to compensate for the long time required by the pump to generate a stable flow.

In practice, sample complexity and the required sensitivity in MS detection dictate the maximum gradient slope and flow rate that can be employed. A relatively simple separation could be performed at 2.0 μL/min and a gradient of 9%. More complex mixtures may have to be analysed at lower flow rates with flatter gradients to increase resolution and especially to facilitate high-sensitivity nanoelectrospray.
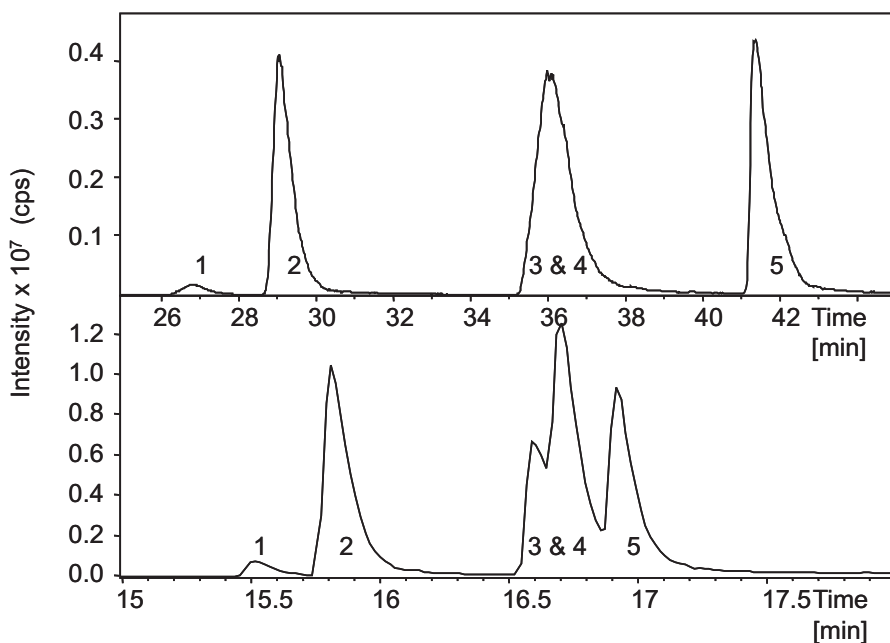
Figure 4.6: Effect of gradient slope on resolution between selected tryptic peptides of CC
(cf. Table 4.1) at 0.25 (upper trace) and 9.0 % (lower trace) at a flow rate of
1.0 μL/min. Combined extracted ion chromatogram of peptides 1 through 5 (see
Table 4.1 for m/z ratios).

4.3.4.2   Application of high-speed separations to a tryptic digest of serum from a cervical cancer patient

The study of diseased states by monitoring biomarkers is an increasingly impor-
tant field in proteomics research. Serum is a highly complex biofluid often used in
clinical studies for this purpose. In order to enhance the capability of analytical
methods to detect proteins of lower abundance, it may be advantageous to first
deplete the serum of high-abundance proteins [24, 32, 33] followed by tryptic di-
gestion of the remaining proteins. This generates extremely complex mixtures of
peptides. Analytical methods have been developed to study the serum proteome
of cervical cancer patients by LC-MS with chromatographic separation times on
the order of 3 h per run (unpublished results). In a field where samples from
many patients have to be analysed, such run times contribute significantly to the
overall analysis time and make larger clinical studies extremely time-consuming.
Using the 50-μm-ID monolithic capillary column described, we attempted to in-

crease sample throughput for nanoLC-MS. This was done by using short loading times (1 min) for the trap column at 50 μL/min, as well as a high flow rate and a steep gradient on the analytical column. Using this approach, the total cycle time was reduced by a factor of 6 to a total of 30 min while retaining good chromatographic resolution. Peak widths at half height were on the order of 10 s, as shown for the extracted ion chromatogram of an endogenous peptide (Figure 4.7).
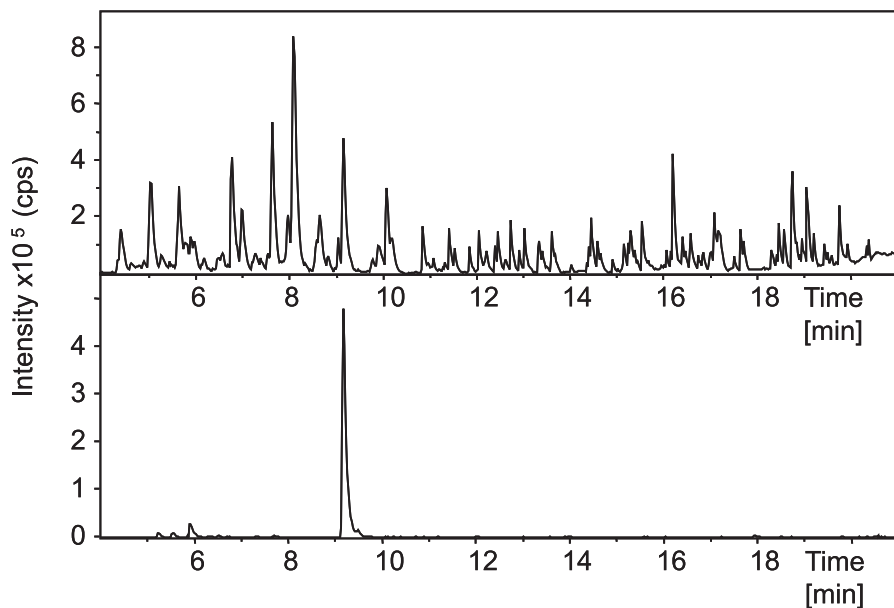

Figure 4.7: Analysis of a tryptic digest of depleted serum from a cervical cancer patient showing short analysis time and narrow peaks. (upper trace: base peak chromatogram; lower trace: extracted ion chromatogram of a peptide fragment at m/z = 694.4). 1.0 μL of depleted serum spiked with CC for internal standardisation ($\approx$1.25 pmol serum calculated on the basis of an average molecular weight of 50000 Da and 1.26 pmol of CC) was digested with trypsin. Separation was performed with a 3.0% gradient slope at a flow rate of 1.5 μL/min.

The use of small (1.5 μm dp), non-porous particles in 150-μm-ID capillaries has resulted in comparable run times as described here for the analysis of proteolytic digests, but at the expense of a sharply decreased loading capacity. Long (up to 80 cm) 150-μm-ID columns packed with 3.0-μm-dp porous particles were also able to resolve very complex peptide mixtures in about 30 min but at significantly higher column pressures [34, 35]. Recently, Jorgenson [26] investigated the use of even

smaller, hybrid porous silica particles (1.5-μm-dp ethyl-bridged silica) that can
withstand pressures up to about 5000 bars in 30-μm-ID and 15-cm-long capillaries.
It proved possible to separate small compounds very efficiently (h$_{min}$ = 2.5-3.0 μm
at 1.5 mm/s). However, the use of small particles in conjunction with relatively
long capillaries requires the purchase of special UHPLC systems that can operate
at pressures greater than 1000 bars [26]. Monolithic columns are a compromise
in this area, in that they allow very fast separations at pressures that can be
handled by any conventional HPLC system, while maintaining acceptable loading
capacities. Our investigation of a 50-μm silica-based reversed-phase monolith
for nanoLC-MS of peptide mixtures shows that very efficient separations can be
achieved in a total analysis time of less than 30 min without requiring more than
300 bars pressure. When sample complexity can be reduced, for example by
introducing a first-dimension pre-fractionation, even shorter throughput times ($\approx$
4 min) can be obtained using very short monoliths at high flow rates and gradient
slopes [36].

4.4   Conclusion

A 50-μm reversed-phase silica-based monolith was chromatographically charac-
terised by nanoLC-MS with special emphasis on proteomics applications. The
high efficiency of this material and its low resistance to flow allowed separation of
highly complex mixtures in a fraction of the analysis time usually achieved with
porous particle-packed columns. Peak widths at half height were on the order of
10 s. A wide range of flow rates, up to 1.95 μL/min, and gradient slopes, from a
conventional 0.5 % to an unusually fast 9%, could be efficiently used. Depending
on the complexity of the separation, a large increase in flow rate and/or gradient
slope resulted in only a limited loss in resolution. Separations showed very high
repeatability over time, which will be a great asset for future comparative bio-
marker studies and for proteomics in general. Two measures for loadability were
determined during the evaluation of the nanoLC-MS system. The loadability of
the trap column was large, with up to 1.0 μg sample retained. The capacity of
the monolith, which accounts for the amount of analyte that can be loaded onto
the column without deteriorating the quality of the separation, was equal to $\approx$
10 pmole (or 100 ng in the case of a tryptic digest of CC).

Use of monoliths to their full extent is limited by nanoLC instrumentation, with
which it is difficult to generate very steep gradients (15 %). In addition, nano-
electrospray interfaces function best at flow rates below 1 μL/min [37] and the
relatively large peak volume at high flow rates does not favour high sensitivity

analyses.

For fast, high-resolution separations of complex samples, the 50-μm-ID reversed-phase monolith investigated in this study appears to be a viable alternative to the recently introduced UHPLC systems We expect that rapid separations by nanoLC-MS on columns based on silica monoliths will extend the possibilities for proteomics, notably for the comparative analysis of larger sets of samples. The good repeatability in terms of retention times is encouraging in this respect.

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