Towards fast multidimensional separations in microfluidic devices
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Chapter 4

Methacrylate Monolithic Capillary Columns for Gradient Peptide Separations
4.1 Introduction

The interest in micro total-analysis systems (µTAS) or lab-on-a-chip technology is still growing. This is mainly due to the expected superior performance of micro-fabricated devices in terms of speed and sample throughput compared to analytical equipment of conventional size. Also, micromachining technologies provide improved possibilities for the creation of integrated complex systems, for example for two-dimensional separations (in time or in space) [1 - 6]. These microfluidic devices may be employed for the separation of complex mixtures, such as peptides in a tryptic digest, when a one-dimensional separation does not provide the necessary resolving power (peak capacity). Comprehensive multidimensional separation systems are increasingly applied in new application areas of analytical chemistry (proteomics, metabolomics), preferably interfaced with high-resolution mass spectrometers for detection and identification.

The peak capacity of a two-dimensional separation system is ideally the product of the peak capacities of the individual dimensions. However, the entire peak capacity can only be used if we are able to realize orthogonal dimensions, i.e. when the mechanisms by which the analytes are separated are uncorrelated. Completely orthogonal two-dimensional separations are hardly ever encountered in practice, but we should pursue the highest possible degree of orthogonality by carefully selecting both dimensions, in such a way that they reflect the variation in different physico-chemical properties of the sample components as much as possible. Good examples are the combinations of strong cation-exchange chromatography and reversed-phase liquid chromatography (SCX×RPLC) for separating peptides [7] and of RPLC and size-exclusion chromatography (RPLC×SEC) for separating synthetic polymers [8].

In chip-based separations many different separation mechanisms have been applied, viz. electro-driven separations, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and isoelectric focusing (IEF) [9 - 11], separations based on the topography in nano-structured channels [12 - 19], and various modes of liquid chromatography in channels packed with particles [20, 21] or monolithic stationary phases [22 – 25]. For application in microfluidic separation devices methacrylate monoliths are of special interest, for several reasons. Their main advantage is the possibility of in-situ
production of methacrylate-based stationary phases by means of a single-step UV-initiated polymerization reaction, which makes it possible to precisely determine the position of the retentive phase in the microfluidic device. The reaction mixture can be introduced into the channel as a liquid, which is much more convenient than packing particles. A second advantage concerns the high permeability of methacrylate monolithic columns, resulting in low back-pressures. Most microfluidic devices cannot stand high pressures.

Methacrylate monolithic stationary phases have been introduced by Svec and Frechet over a decade ago [26] and since that time much effort has been put into the study and development of these materials. This has resulted in a wide range of available materials with different monomers incorporated in the polymer to perform various modes of liquid chromatography [27 - 33] and in an increased understanding of the factors determining the analytical performance [34 - 37].

Previous work in our group has led to the conclusion that in isocratic separations the efficiency of methacrylate monolithic capillary columns is high for unretained compounds, but that it decreases with retention. This was observed for different classes of compounds, albeit to varying extents. In contrast, Eeltink et al. [32] and Le Gac et al. [24] showed fast and efficient separations of peptide mixtures under gradient conditions, using a lauryl methacrylate monolith. An explanation for this difference in behaviour of a methacrylate column in isocratic or gradient separations may be found in the type of application. The peptides present in tryptic digests show a very strong dependence of their retention factors ($k$) on the volume fraction of organic modifier in the mobile phase. This means that minor changes in the composition of the mobile phase can cause a peptide to be either almost indefinitely retained or virtually unretained. This behavior, referred to as an on/off mechanism, may provide an explanation for the good results presented for gradient separations. In the study described in the present paper we have used a novel approach to investigate the retention and efficiency characteristics of a butyl methacrylate monolithic column in the isocratic and gradient modes. Moreover, we have compared the results obtained with that of a commercially available capillary column packed with C18-modified silica particles, which is the most common stationary phase for peptide separations, to put the performance of the methacrylate monolith in perspective. Also we optimized the conditional peak capacity in relation to the gradient duration for both types of columns, for a specific mixture of peptides that is thought to be representative for the components of a
typical tryptic digest [40]. For this optimization we evaluated the retention behavior of the peptides on both columns, which determines the size of the retention window, and the relation between retention and peak width in gradient separations.

4.2 Experimental

4.2.1 Materials and reagents

Fused-silica capillaries with a UV-transparent coating were purchased from Polymicro Technologies (Phoenix, AZ, USA). The peptides used as model compounds (Table 1), as well as the ingredients of the polymerization mixture, butyl methacrylate (99%, BMA), ethylene dimethacrylate (98%, EDMA), lauryl methacrylate (99%, LMA), 1,4-butanediol (99%), azobisisobutyronitrile (98% AIBN) and 3-(trimethoxysilyl)propyl methacrylate (99%, γ-MAPS), were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Recombinant chlorite dismutase (a gift from Karlstad University Department of Chemistry) was treated with trypsin for 20 h at 37°C. The laboratory-prepared BMA monolithic columns were compared with an Agilent capillary column of 15 cm length and an I.D. of 75 μm, packed with 3.5-μm Zorbax 300SB-C18 particles. Other chemicals were obtained from standard suppliers and used as received.

Table 1. Compound name, molecular weight and m/z value used for MS detection of model peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$M_r$</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Phe</td>
<td>222.24</td>
<td>223.24 (1+)</td>
</tr>
<tr>
<td>Neurotensin fragment 1-8</td>
<td>1030.13</td>
<td>516.06 (2+)</td>
</tr>
<tr>
<td>Phe-Phe</td>
<td>312.36</td>
<td>313.36 (1+)</td>
</tr>
<tr>
<td>Luteinizing hormone releasing hormone</td>
<td>1182.29</td>
<td>592.15 (2+)</td>
</tr>
<tr>
<td>Angiotensin 2</td>
<td>1046.18</td>
<td>524.09 (2+)</td>
</tr>
<tr>
<td>[val$^5$]-angiotensin 1</td>
<td>1282.45</td>
<td>642.23 (2+)</td>
</tr>
<tr>
<td>Substance P</td>
<td>1347.63</td>
<td>674.82 (2+)</td>
</tr>
<tr>
<td>Renin substrate</td>
<td>1759.01</td>
<td>880.51 (2+)</td>
</tr>
<tr>
<td>Momany peptide</td>
<td>770.88</td>
<td>771.88 (1+)</td>
</tr>
<tr>
<td>Insulin chain B oxidized</td>
<td>3495.89</td>
<td>874.97 (4+)</td>
</tr>
<tr>
<td>Melittin</td>
<td>2846.46</td>
<td>712.62 (4+)</td>
</tr>
</tbody>
</table>
4.2.2 Preparation of monolithic columns

Butyl methacrylate monolithic columns were prepared in fused-silica capillaries of 75 μm internal diameter after vinylization of the inner wall to ensure covalent anchoring of the monolith to the capillary wall. Following the procedure described by Yu et al. [38], capillaries were rinsed with acetone, flushed with 0.2 M sodium hydroxide for 30 min and washed with deionized water, again flushed for 30 min with 0.2 M hydrochloric acid and rinsed with ethanol. Next, a 20% solution of 3-(trimethoxysilyl)propyl methacrylate in ethanol at pH 5 was flushed through the capillary at a rate of 0.25 μL/min for 1 h using a syringe pump (KdScientific, New Hope, PA, USA). After this, the capillary was flushed with acetone and dried overnight by a stream of compressed air. The polymerization mixture consisted of 24% BMA or LMA, 16% EDMA, 33.8% 1-propanol, 25.8% 1,4-butanediol and 0.4% AIBN (all w/w) [39]. After injecting the mixture into the capillaries, the ends were sealed with pieces of septum. A UV-Crosslinker (Spectroline, Westbury, NY, USA) was used to irradiate the capillaries with an intensity of approximately 3 mW/cm² for 50 min at 254 nm. Before use, the monolith-filled capillaries were flushed extensively with acetonitrile (ACN) and cut to a length of 25 cm.

4.2.3 Instrumentation and chromatographic conditions

Isocratic and gradient experiments were performed using an Agilent 1100 series NanoLC system interfaced to an ion-trap mass spectrometer via an Orthogonal Nanospray ion source (Agilent Technologies, Waldbronn, Germany) with a picotip emitter needle of 3 cm length and 8 μm i.d. (New Objective, Woburn, MA, USA). Electrospray ionization was performed at a temperature of 230°C with a dry-gas flow rate of 6 L/min. The capillary voltage was 1500 V. Data were acquired in the m/z range of 200 to 900 at a rate of 20 scans/s.

During all experiments mobile phase A was 0.1% trifluoracetic acid (TFA) in water, mobile phase B was 0.1% TFA in ACN. All sample concentrations were 10 μg/mL in solvent A and volumes of 0.05 μL were injected.
4.3 Results and Discussion

4.3.1 Separation of a tryptic digest

The morphology of a methacrylate monolithic column – and with that its performance as a stationary phase – can be influenced through the composition of the reaction mixture used for polymerization. BMA columns that were produced from a polymerization mixture containing 30 or 35% monomers showed a high permeability, but lacked the large surface area necessary to achieve sufficient retention for the relatively polar peptides. Therefore a reaction mixture was used consisting of 40% monomers and 60% porogens, which had been shown to give excellent separation for proteins [32]. Initial experiments for the evaluation of the capillary columns were performed based on the gradient separation of a tryptic digest of recombinant chlorite dismutase, a key protein involved in the microbial degradation of toxic chlorite to molecular oxygen and chloride ion. Figure 1A shows the separation of the tryptic peptides on the packed C18 column, with a gradient of 2 – 40\% ACN in 60 min. Figure 1B shows the separation of the same sample with a gradient of 2 – 30\% ACN on the BMA column.

Figure 1. Tryptic digest of recombinant chlorite dismutase on the packed column (A) gradient: 2 – 40\% ACN in 60 min. \( F = 0.5 \, \mu\text{L/min} \) and on the BMA column (B) gradient: 2 – 30\% ACN in 60 min. \( F = 1 \, \mu\text{L/min} \).
The gradients have been optimized to fill the separation space as much as possible. It appears that the packed column gives a better separation, but this is difficult to quantify because of the large number of peaks, many of which exhibit some degree of overlap. Therefore, to evaluate the column performances we chose to separate a standard mixture of 11 peptides. Wang et al. [40] suggested that this mixture resembles the elution behavior of a tryptic digest. Names, molar masses and m/z values used for detection of the model compounds used in this study are given in Table 1. Retention times and peak widths were determined from the extracted-ion chromatograms. Occasionally some of the peptides were co-eluting, but this did not cause problems, since we could use known m/z values. Separations of these peptides were optimized with the objective of maximizing the available separation window and minimizing the average peak width in order to achieve the highest possible peak capacities. The retention behavior of the peptides was first studied under isocratic conditions, so that an appropriate gradient program could be chosen in order to fill the available separation space as much as possible. Next the relationships between peak width and retention were studied in gradient analysis in order to explain differences in peak capacities for the different columns.

4. 3.2 Retention

For conventional reversed-phase (C18) columns it is well established that peptides, proteins and other macromolecules exhibit a very strong dependence of the retention factor \(k\) on the volume fraction of ACN in the mobile phase.
Figure 2 shows a plot of $\ln k$ versus volume fraction ACN ($\phi$), illustrating that this is also the case for certain peptides on the BMA monolithic column. Two of the peptides (Gly-Phe and neurotensin fragment 1-8) of the standard mixture are not included in this plot because they were not retained significantly on the BMA monolith, not even when 100% water was used as eluent. In reversed-phase liquid chromatography the relationship between retention and composition is generally described by the following equation [41 - 44]:

$$\ln k = \ln k_0 - S\phi$$  \hspace{1cm} (1)

where $k_0$ is the retention factor in pure water. The slope ($S$) generally varies with the size of analyte molecules. Thus, while for many peptides it is of similar magnitude, it is larger for very large and smaller for very small peptides. In Figure 3 the slopes of the $\ln k$ versus $\phi$ curves of the eight peptides that can be retained on the BMA column are plotted against the values obtained on the packed column.
There is a general trend on both columns towards steeper slopes for larger peptides. On the BMA column the $S$ values are on average slightly higher. However, the $S$ values of the individual peptides often differ strongly for both columns. This implies that the differences in retention are not only caused by differences in the phase ratio of the two columns. Differences in the type and strength of interactions of the peptides with the two stationary-phase materials also play a role.

The retention properties of the two column types for the peptides were compared by calculating the volume fraction of ACN ($\phi_0$) needed to obtain a retention factor of 1. This volume fraction was calculated by interpolation from the experimental data for the eight peptides that showed significant retention (a retention factor of at least 1 on the BMA column with pure water as the eluent). The value of $\phi_0$ is used as a general measure for the retention of a peptide on each individual stationary phase. The values obtained for the two different columns are plotted against each other (methacrylate monoliths versus the packed column) in Figure 4.
Figure 4. Plot of volume fraction ACN needed to elute peptides with \( k = 1 \) (\( \varphi_0 \)) from the BMA column (◊) and LMA column (■) versus the packed column.

The eight data points in this plot follow approximately a straight line with a slope close to unity. The plot shows that on average the C18 column requires approximately 15% more ACN to elute a peptide with \( k = 1 \) than the BMA column. Since the slopes of the \( \ln k \) versus \( \varphi \) curves are similar for both strongly (e.g., melittin) and weakly (e.g., Phe-Phe) retained peptides, this difference is approximately the same for a different value of the retention factor. The lower retention observed for the BMA column will lead to a smaller separation window in gradient elution, since obviously the starting concentration of ACN in the mobile phase cannot be lower than 0%. Peptides with low retention will be eluted before the onset of the gradient and they will not be resolved.

The gradient conditions (initial and final composition of the eluent) have been adjusted based on the isocratic retention data. On the methacrylate columns gradients of 0 – 40% ACN have been run, while for the packed column gradients of up to 50% ACN were needed to elute all sample components within the gradient. When the C18 column was used gradients were started at 2% ACN to prevent collapse of the stationary-phase packing.
4.3.3 Peak widths

The peak capacity of a separation is determined by the retention window and by the average peak width. As was already shown in a previous study [37], under isocratic conditions the efficiency of methacrylate monolithic columns appears to decrease with retention. For well-packed particulate columns, on the other hand, it is generally observed that the efficiency is almost independent of the retention factor.

![Figure 5](image-url)

*Figure 5. Plot of peak width in a 15 (♦), 30 (□) and 60 min (●) gradient versus volume fraction to elute a compound at $k = 1$ on the BMA column (A) and on the packed column (B).*
In order to compare the column efficiencies in gradient elution, we plotted in Figure 5 the peak standard deviations of the peptides that elute within the gradient against the retention measure $\varphi_0$ as introduced in the previous section. In the plot data for different gradient durations are shown. Figure 5B shows that the packed column behaves as expected: the peak widths of all compounds eluting in the same gradient are similar, and the average peak width increases with gradient duration. In the plot for the BMA column (Figure 5A) on the other hand, we see that early eluting compounds give much wider peaks than the late eluting peptides. This is especially the case for shallow (60 min) gradients.

For a better understanding of this phenomenon we have studied the relation between retention in gradient elution and the column efficiency. For this, we followed the approach of Neue [45] to calculate plate heights from gradient measurements. First, from the retention time of a peak, taking the dwell time of the system and the dead time of the column into account, the volume fraction of ACN prevailing at the moment of elution of the peak was calculated. Next, using the data for $k_0$ and $S$ as determined in isocratic measurements, the retention factor at the moment of elution ($k_e$) for every single peak was calculated. With the values of $k_e$ determined in this way, peak standard deviations could be transformed into plate heights $H$. The combined results for $H$ from the 15 and 60 minute gradients are plotted against the retention factors at the moment of elution ($k_e$) in Figure 6, where they can be compared for the BMA and the packed column. Despite some scattering of the data points (caused by inaccuracies in the data used and the particular behavior of specific peptides) it is clear to see that for the C18 column the plate height is relatively constant, with values in the order of 10 - 20 $\mu$m for most peptides. The plate heights calculated for the BMA column on the other hand show a definite increase with the retention factor, just as it has been observed for isocratic separations on BMA columns [36, 37].
With the BMA column the $k_0$ and $S$ values of the peptides are strongly correlated (see Figure 2). Together with the relatively long dwell time of the system (2.0 min under the conditions employed), this causes weakly retained peptides to migrate through a large part of the column length at unfavorable conditions when a shallow gradient is applied. In general, for later eluting peptides the $S$ value on the BMA column is higher. This implies that their elution behavior approaches more an on/off mechanism. The result is that they migrate over almost the complete column length with a low $k$ value and elute in narrow bands similar in width to bands from the C18 column. In a steep gradient, all peaks migrate with a low retention factor because of the fast increase in mobile phase elution strength.

4.3.4 Conditional peak capacity

For the separation of the highly complex mixtures that are, for instance, encountered in metabolomics and proteomics, a very high separation power is required. Gradient-elution liquid chromatography is a powerful tool for such separations. The most important metric for the quality of a column under gradient conditions is the peak capacity. The peak capacity is defined as the maximum number of peaks that can be separated with a given
resolution [46, 47]. It is determined by the widths of the peaks and the available separation window. In this study we have used the conditional peak capacity \( n_c \), a parameter that also takes into account the fact that most samples only occupy a limited part of the available separation space. The conditional peak capacity can be defined as:

\[
\frac{t_{R,n} - t_{R,1}}{W}
\]

where \( t_{R,n} \) and \( t_{R,1} \) are the retention times of the last and the first compound that elute in the gradient in a specific application, and \( W \) is the average peak width at the base (4\( \sigma \)) of the compounds studied [48]. Peak capacities for one-dimensional separations of peptides can be improved using long columns and shallow gradients at elevated temperatures [40]. However, in the end two-dimensional (or multi-dimensional) separations, combining for instance liquid chromatography with isoelectric focusing [49] or SEC [50], will be needed to obtain the required resolving power for the most-demanding applications. In two-dimensional separations it is very important to run the second-dimension gradient as fast as possible, in order to maintain the resolution obtained in the first dimension and to restrict the total analysis time. A possible optimization goal for the second dimension-separation is therefore to acquire the highest peak capacity in the shortest possible analysis time. We calculated the conditional peak capacity from the observed retention window and the average peak width (Equation 2), for the packed and monolith columns as a function of the gradient duration. Gradients from 2 to 50% ACN were applied with the packed C18 column and from 0 to 40% with the BMA column. With the packed column all 11 peptides in the test mixture could be included in the calculation, because every peptide eluted within the gradient. This was different for the monolithic column because the least hydrophobic peptides (Gly-Phe, neurotensin fragment 1-8 and Phe-Phe) eluted before the start of the gradient and were therefore not included in the determination of the retention window. The results are shown in Figure 7.
Gradients of up to 15 minutes yield similar peak capacities of about 75 for both columns. Applying shallower gradients with the C18 column resulted in a further increase in peak capacity, up to approximately 150 for 60 min gradients. However, with longer gradient times the peak capacity of the BMA column increases only to a limited extent, reaching a plateau value of approximately 100. The increase in width of early eluting peaks is the limiting factor with the BMA column.

### 4.3.5 Increasing the peak capacity of methacrylate monolithic columns

In the previous sections, we have seen that the performance of butyl methacrylate monolithic columns cannot match that of a well-packed C18 column. Especially for shallow gradients, the packed column performs much better. Two approaches have been considered to increase the peak capacity of methacrylate monoliths. The first approach was to replace butyl methacrylate with the more hydrophobic lauryl methacrylate to provide more retention for the relatively polar peptides. The resulting column was more retentive than the BMA column, with longer retention times in the same gradient than the BMA column. Again, $\phi_0$ was calculated for the peptides studied, and it appeared that on average...
10% ACN more was needed to elute a peptide with a retention factor of one from the LMA column than from the BMA column (see Figure 4). The increased retention can be attributed partly to the higher hydrophobicity of the polymeric material. However, a higher surface area of the LMA monolithic material could also be a factor. The morphology of the LMA column was clearly different from that of the BMA column. The BMA column showed a back pressure of 50 bar at a linear velocity of 1 mm/s, whereas the LMA column gave a back pressure of 100 bar when operated at the same velocity. This indicates that the LMA column has a smaller average pore size than the BMA column. Peak widths of the earliest eluting peptides were narrower on the LMA column than on the BMA monolith. The resulting peak capacity was approximately 125 in the 60 min gradient.

The second approach studied was to increase the length of the BMA column while keeping the column dead time constant. The high permeability of the BMA column made it possible to double the column length and at the same time to increase the flow rate to keep the column dead time constant. Increasing the column length has two advantages in gradient elution. First, assuming that the plate heights for the 250 and 500 mm long columns are equal, the peak capacity should increase since it is proportional to the square root of the number of plates. Moreover, the distance that a weakly retained peptide migrates through the column with an unfavorable high retention factor in the dwell time is relatively smaller. In a 60 min gradient we found a peak capacity of 137. The peak capacity of the 50 cm long BMA column approaches that of the 15 cm long packed column. The observed conditional peak capacities of the different columns tested can be compared in Figure 7.

4.4 Concluding remarks

The BMA monolithic columns tested in this study provided considerably less retention for peptides than conventional C18 packed columns. This gave a smaller retention window for the peptides in gradient elution, mainly because some of the more-polar peptides were not sufficiently retained on the monolithic column. At the same time, in the nano-flow system used in our work the large dwell volume resulted in migration of the weakly retained species under unfavorably high retention factors, so that their peak widths increased, especially when shallow gradients were used. An increased retentive power of the
monolithic material (i.e. by replacing BMA with LMA) helps to improve the performance of this type of columns in low-volume gradient chromatography.

In isocratic elution the monolithic columns are clearly inferior to the packed columns with respect to efficiency, because the peak widths are found to increase strongly with increasing retention. However, this drawback is less manifest in gradient elution with steep gradients. In that case the peptides elute with a low effective retention factor, giving rise to narrow peaks. This explains the good results presented in the literature for peptide separations on methacrylate monolithic columns under gradient-elution conditions and the often disappointing results obtained with isocratic separations. When relatively steep gradients are applied (gradient times up to 15 min), the conditional peak capacities obtained with the BMA monolith and the packed column are similar (i.e. of the order of 75).

When shallow gradients are used the C18 column is again clearly superior over the BMA column. The maximum peak capacity that was achieved with the packed column in the selected time frame was about 150. On the other hand, the higher permeability of the butyl methacrylate monolithic stationary phase allows one to use longer columns, and since the peak capacity is proportional to the square root of the column length this gives an increased peak capacity.

We have opted for methacrylate-based monolithic stationary phases, since they are very attractive for liquid chromatography in microfluidic devices, thanks to the possibility of in-situ UV-initiated polymerization and the high permeability. In this study we have demonstrated that the BMA columns are competitive for reasonably fast gradient separations, and that monoliths that provide more retention (such as an LMA monolith) approach the performance of a packed C18 column even with shallow gradients.

References