Pillar-structured microchannels for liquid chromatography

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Chapter 5

Normal Phase Separations in Pillar Structured Microchannels

Abstract

The study of the use of Pillar-Structured Micro Channels (PSMC) for separations by HPLC is still in the beginning stage. As a contribution we investigated the separation of some fluorescent model compounds exploiting the bare surface of the oxidized pillars as retentive phase. For DNS-derivatized amino acids the retention appeared to be too low to obtain a separation. A very fast separation of a number of Rhodamine compounds could be achieved; the separation of the mixture occurred in the first millimetre of the channel. However, the retention factor for the analytes, already limited by the low surface to volume ratio for the structured channel compared to packed columns, was further reduced by the low surface density of silanol groups. The band dispersion observed for the retained compounds was comparable to that for the unretained peak at moderate and low flow rates. However, under certain conditions peak tailing was observed. A possible explanation for the peak asymmetry could be the large fraction of isolated silanol groups on the surface.
5.1 Introduction

One of the bottlenecks in the study of the use of a PSMC for separations are the difficulties encountered in preparing a homogeneous and stable coating of stationary phase on the pillar surface. This problem was already mentioned in the first experimental study on the preparation of PSMC devices for reversed phase chromatography of De Malsche et al. [1].

At present, the material of choice for the fabrication of PSMC chromatographic devices is silicon. The suitability of silicon for the fabrication of highly accurate and still affordable microstructures is so far unsurpassed. Materials such as quartz and PDMS have also been used for the fabrication of PSMCs. Quartz could be machined with Electron Beam lithography to fabricate DNA sizing devices [2]. However, the costs involved in this technique are very high, and the technology is not available in most clean-rooms nowadays. PDMS was used to fabricate PSMCs for capillary electrochromatography [3]. However, this material is too soft, and too much prone to deformation to become a serious candidate for ultra-high efficiency pressure driven chromatography devices.

In addition to the advantages related to the microfabrication, silicon has another favourable property: when its surface is oxidized by exposure to air [4], its surface chemistry becomes similar to the surface of silica, which is by far the most widely used...
material in the preparation of particulate for packed columns. Similar to silica, silanols group are present on the surface of oxidized silicon. The silanols are the locations where the stationary phases can be bonded. The same reactions as used for the surface modification of silica particles could be applied for PSMCs.

The expected experimental complications in modifying the surfaces of PSMCs are much more severe than with silica particles, when the modification can be performed in a batch process before packing of the column. Extreme conditions (e.g., high temperatures) can not be used to avoid the risk of damaging the chip device. Another important risk is channel fouling, and partial or total clogging of the separation or connecting channels. This is a serious problem which can occur during a multi-step surface modification. The experimentator will face the risk of having to discard the chip without even having tested it. The limitations for the reaction conditions will increase the possibility that patches of the pillar surface are not covered by the stationary phase, or that in a part of the microchannel the surface modification does not succeed at all. Even with conventional particles after the modification of the silica surface not all the silanols moieties are bonded with the stationary phase. In reversed phase chromatography the presence of unreacted silanol groups is a source of peak asymmetry (tailing) and band dispersion due to the lower desorption kinetics of the polar compound from the silanol groups [5]. The adsorption of a compound on a silanol group happens through hydrogen-bonds or through electrostatic interaction in the case that the species is positively charged. Isolated silanols interact much more strongly with the sample compounds than non-isolated ones [6]. It is expected that unreacted silanol groups, both isolated and not-isolated, are present also after the surface modification is performed on a PSMC. Therefore, for a preliminary study of the performance of PSMCs under retentive conditions, the possibility of exploiting the bare surface of the silicon pillars as retentive phase would be extremely appealing, as it would alleviate the researcher from time consuming and risky coating steps.

As already mentioned, a layer of silicon dioxide is formed on the silicon surface when it is exposed to air. The surface becomes therefore hydrophilic, and the chromatography which can be performed in such microchannels is of the normal phase type. The sample compounds to be selected for the testing of the PSCM must be retained under such
conditions. Also the detection possibilities will influence the choice of the sample compounds. In our set-up the best detection method for the PSMCs is still fluorescence microscopy. This detection mode is sensitive and allows the visualisation of the sample behaviour. This is important, e.g., to control the injection or to check the presence and importance of unwanted side-wall effects [1,7]. Therefore, the sample compounds to be analyzed must be fluorescent. When performing fluorescent microscopy detection, the excitation light from an emitter (for example a mercury lamp) has to be filtered, in order to select the wavelength or the band that excites the target molecule. Similarly the fluorescent light emitted by the target must be filtered in order to block the light at all unwanted wavelengths, above all the intensely scattered excitation light. It is clear that when dealing with a mixture of compounds, the emission and excitation spectra of the different components must be at least partially overlapping to allow the use of a unique set of filters. One more requirement for the sample mixture is that one of the fluorescent compounds is unretained under the experimental conditions chosen. This is essential for the estimation of the retention factors k and the velocity of the mobile phase. In the set-up used in our experiments and with the multi-channel devices that were available it is not possible to work at a constant, known flow rate.

Finally, two different sets of analytes were selected that fulfilled all a priori requirements, a set of dansylated amino acids and a set of rhodamine dyes. The separation of the mixtures of fluorescent compounds was first studied with a conventional stainless-steel column packed with non-porous silica beads of 1.5 μm. This was done to reduce the risks of clogging the chip, but also to allow an easier change of the mobile-phase. With the set of compounds and the chromatographic conditions selected, the separation was attempted in the PSMC device.

5.2 Experimental.

The instrumental hardware and the PSMC device have been described in the Chapters 3 and 4. For the data acquisition a Peltier-cooled CCD camera, a Colorview 12 (Olympus,
Zoetermeer, The Netherlands) was used. Data were collected both as video-files and as pictures. The video data were used for the calculation of the retention factors. The procedure for the measurement of retention factors was as follows. Before the actual experimental run began, the video-acquisition software was started, so that the complete experiment including the injection step was recorded. After the injection the chip was moved by the translation stage with step motor to a predefined position, with the observation point moved towards the end of the channel. In preliminary experiments the optimal detection position had been determined. This position was selected with the requirement of a base-line separation of the compounds on one hand, and at the same time of avoiding an unnecessarily long distance between the inlet and the detection point. The second requirement was imposed by the strong decrease of the signal intensity of the most retained compound with the distance from the injection. This decrease was due not only to the spreading of the sample but also to the low sensitivity of the camera for low light intensities. The data acquisition at the new detection point continued until all the compounds were detected. The chromatogram was afterwards generated from the video file by running a home-made routine with the image processing software. Briefly, the processing routine was as follows. First the video frames related to the final detection position were selected. Then a virtual detection window was set. This detection window was a rectangular area of the video-frame with a width equal to that of the structured channel, but a length of only a few micrometers. Then for each of the selected frames, the sum of the light intensity of the pixels inside the detection area was calculated. The total intensity for each frame was afterwards plotted against the frame number, and a frame-resolved chromatogram could be obtained. Since the rate of frames per second was known it was possible to convert the frame-resolved chromatogram into a time-resolved one.

Conventional HPLC experiments were performed with a 150 x 4.6 mm column home-packed with 1.5 μm non-porous silica particles and a standard instrument composed of a HPLC pump, a manual injection valve with a 20 μL loop and a UV detector.
5.3 Results.

5.3.1 Separation of derivatized amino acids

LIF and lamp induced fluorescence are techniques that allow the detection of samples at trace level. Induced fluorescence methods are popular in capillary electrophoresis where the small volumes of sample involved require ultrasensitive detection. For on-chip analysis fluorescence is by far the most widely used detection method. The use of UV adsorption detection, which is undoubtedly the most often used technique in liquid phase separations, is made difficult by the short light path available in microfluidic systems. Moreover, in some cases only the top part of the microfluidic device is transparent to light and consequently any light adsorption measurement becomes impossible. This last scenario is exemplified by the PSMC used in our work, which where machined in silicon and top-enclosed by a glass wafer [7,21]. Amino acids are one class of biological compounds which is detected by induced fluorescence methods. Although only tryptophan and phenyalanine are native fluorescent molecules, amino acids can be successfully labelled with fluorescent tags such as for instance FITC or Dansyl-chloride. Labelling of amino acids, peptides, and proteins with fluorescent groups is a common practice in biological analysis. Dansyl derivatives of amino acids (Dns-AAs) are commercially available. The Dns-AAs can be excited by electromagnetic radiation with a wavelength around 350 nm; the fluorescent light is emitted at about 450 nm. Amino acids have been tagged with the dansyl group to allow detection in CE, but Dns-AAs have also been extensively used to test the performances of chiral stationary phases ([8,9,10,11,12,13]). In a particular mode of chiral chromatography, i.e. enantioselective ligand exchange chromatography, the dansyl group bonded to the amino acids moiety enhances the selectivity of the enantiomeric separation.

We have investigated the possibility to use Dns-AAs as model compounds for the evaluation of our PSMC system, because of their suitable fluorescent properties, and the availability of a large set of compounds with different hydrophobicity. Although the Dns-AAs are commonly separated with reversed phase LC, we have studied their separation in a normal phase system. The separation was performed with the column packed with non-porous silica particles already mentioned above. The mobile phase used was a 95:5 mixture
of ACN and phosphate buffer (10^{-3} M). This type of normal phase chromatography is also known as Hydrophilic Interaction Liquid Chromatography (HILIC) [14]. In HILIC the retention of polar analyte occurs by the partition equilibrium between the eluent (a mixture of organic solvent and water), and the water layer adsorbed onto the packing particles. In addition, a cation exchange equilibrium with the silanols group (in the case that silica is the packing material) can enhance the retention, provided that the analyte is positively charged.

We found that Dns-Asparagine is slightly retained on the bare silica column and that it can be separated from other Dns derivatives of polar neutral amino acids, which are essentially unretained. For instance, Dns-Asparagine and Dns-Tyrosine could be base-line separated at neutral pH. The retention factor for Dns-Asparagine was 0.14 on the packed column. However attempts to transfer the method for the separation of the amino acids pair to the PSMC system were not successful. With none of the mobile phase compositions tested a separation was obtained. One obvious explanation is that the retention of all tested Dns-AAs in the PSMC is too low to obtain a significant separation, because of the unfavourable surface to volume ratio (i.e., phase ratio).

5.3.2 Separation of fluorescent dyes.

The retention of a set of highly fluorescent dyes was screened under normal phase conditions. Most of the commercially available fluorescent dyes are water soluble, and consequently a HILIC type of separation was expected to be more suitable then a classical normal phase system with an apolar, alkane rich mobile phase. A set of 3 model compounds was selected consisting of two phenantrenes, namely Rhodamine B (RhB), Rhodamine 6G (Rh6G), and the oxazine Nile Red (NR). Although the excitation and emission spectra of the three components do not exactly match, all of them are efficiently excited by light of 514.5 nm and their fluorescence can be detected when a 550 nm cut-off filter is used. In preliminary tests with the packed column a suitable mobile phase mixture and separation protocol was found. The mobile phase for the separation of the phenantrenes and the oxazine was a 1:9 (v/v) mixture of water and acetonitrile. NR is a hydrophobic dye which is expected not to be retained under the aforementioned conditions. This was confirmed by the preliminary tests with the packed column. The retention factors of the
retained compounds decreased when the amount water in the mobile phase was increased, which shows that a hydrophilic type of interaction is the base for the separation. When the 1:9 mixture of water and acetonitrile was used as mobile phase, the retention factors were 0.28 and 3.20 for RhB and Rh6G, respectively. The strong difference in retention between the rhodamines can be explained by their different acidity. As it can be seen from Figure 1, both molecules have a permanently positively charged moiety. However RhB also has a carboxylic group that has a pKa value of 3.2 in water. Therefore it is expected that, in the neutral, not buffered mobile phase this group will be ionized, and the overall molecule charge will be zero. The exact retention mechanism probably includes hydrogen-bond interactions. Rh6G preserves its positive charge. Therefore, beside other more general hydrophilic interaction with the stationary phase, a cation exchange mechanism with the negatively charged silanols has to be regarded as the main source of retention.

Figure 1  Molecular structure of Rhodamine 6G (left) and Rhodamine B (right)

The separation of the three components was successively performed in a PSMC. In Figure 2 snapshots of the channel after injection of a mixture of NR and RhB are shown. Figure 3 shows a chromatogram of the separation of NR and RhB as constructed from pictures taken at some distance along the channel from the injection slit. The observed retention factor for RhB was 0.15, roughly half of the value for the packed column. Despite the low k value, the base-line separation from the unretained compound was achieved in 1 second, and approximately already in the first millimetre of the channel. Separations in such short length, and low retention, are difficult to achieve with a packed column. The
The retention factor for Rh6G was 0.45. The decrease of the retention from the packed column to the PSMC device was much stronger for Rh6G than for RhB. The chromatographic retention factor \( k \) depends on the partition constant for the sample between the phases and the phase ratio:

\[
k = \frac{t_R - t_0}{t_0} = \phi \cdot K_{eq}
\]

where \( K_{eq} \) is the partition equilibrium constant for the solute between the stationary and mobile phase, and \( \phi \) the phase ratio of the column, that in this case should be defined as the surface area of the stationary phase over the mobile phase volume. When the only responsible factor for the differences of the \( k \) values would be the different phase ratio, the retention factors for RhB and Rh6G should change in the same proportion. In order to compare the \( \phi \) value for the packed column and the PSMC, the surface of the solid material per volume of mobile phase has to be considered. For the PSMC used in this work, the \( \phi \) value is approximately 2 m\(^2\)/mL, as estimated from the design geometry and the voidance of the PSMC. For a column packed with 1.5 µm non-porous particles, with an assumed porosity of 0.29, the surface to volume ratio is approximately 10 m\(^2\)/mL. The difference in retention for the weakly retained RhB is smaller, and that for the more strongly retained Rh6G larger than the difference in the phase ratio. It is possible that the surface density of silanols in the PSMC is different than the 8 µmole/m\(^2\) typical for silica stationary phases [15, 16]. According to Zhuravlev [17] the surface concentration of silanols on fully hydroxylated amorphous silica has the characteristics of a physicochemical constant, independent from the process used to produce the silica; 8 µmole/m\(^2\) was also in this case the measured value. Zhuravlev’s work suggests that the layer of silicon dioxide which forms on the silicon wafers surface should have the same silanols concentration, and indeed other authors based their study on this value [18]. However smaller surface concentration of silanols have been found when the silica surface was not fully hydroxylated [19, 20]. In spite of the fact that the PSMC was flushed with a NaOH solution before use, it is possible
that the hydroxylation was not complete. This can explain why the difference of the retention factors between column and PSMC is not the same for the two rhodamines.

5.3.3 Band dispersion for retained compounds.

In Figure 4 the Van Deemter curves for RhB and NR are shown that have been constructed from the measured band spreading. For flow velocities below 1000 μm/s there is no significant difference between the efficiency of the retained and the unretained compound. The minimum plate height for (unretained) fluorescein observed in the same PSMC was previously reported to be 3.1 μm [21]. The minimum plate height for both NR...
and RhB approach this value. For higher flow rates the plot suggests a stronger band spreading for the retained RhB. The peak symmetry of RhB quickly deteriorates when the flow velocity increases and strong tailing can then be seen (Figure 5). The tailing effect is not present for lower flow rates, and this suggests a kinetic effect rather than a thermodynamic (overloading) effect.

![Graph](image)

**Figure 3** Peaks of the unretained NR (on the right) and RhB (on the left). RhB peak is asymmetrical.

The band dispersion for Rh6G was strong in the whole velocity range. Also in this case the peak asymmetry appears to be of fundamental importance in the generation of the band spreading; however the peaks of Rh6G (not shown in this paper) are fronting.

It appears from the results described above that a different surface to volume ratio is not the only factor determining the lower retention in the PSMC in comparison with the packed column. The strong tailing of RhB at higher flow rates could be directly related to a lower surface density of silanols. Most of authors agree on the fact that isolated silanols are unwanted sites of strong adsorption [19,22,23,24]. When the silanols are not isolated they can form hydrogen bonds with each other; the electron pair of the oxygen atom is no longer free to interact with the sample, and the silanol hydrogen becomes less acidic. Some doubts still remain about the case when the silanols are paired rather than grouped: within this scenario the acidity of the silanol hydrogen is even enhanced by the hydrogen bonding with
the neighbour silanol [25]. It has been stated that when the silica has a lower surface concentration of silanols, the concentration of isolated silanols becomes relatively high. [22]. A relatively high surface concentration of isolated silanols in the PSMC could explain the strong tailing observed for RhB.

![Van Deemter curves of Nile Red (filled squares) and RhB (open triangles).](image1)

**Figure 4** Van Deemter curves of Nile Red (filled squares) and RhB (open triangles).

![Peak of RhB showing strong tailing](image2)

**Figure 5** Peak of RhB showing strong tailing
5.4 Conclusions.

In the preliminary study described in this chapter it has been shown that the high separation efficiency of a PSMC can also be realized for retained compounds. With a normal phase type of separation the performance of a PSMC in pressure driven chromatography could be tested, even when it was not straightforward to find a set of compounds that met all requirements. Different sets of fluorescent compounds that were found separable on a packed silica column, showed lower retention with the PSMC. The lower retention is likely due to both the lower surface to volume ratio of the PSMC, and to a lower surface density of silanols of the micromachined chromatographic device. This last issue is important not only for separations performed on the untreated PSMC, but also when a retentive phase is to be bound to the silica surface since the silanol moieties are usually the anchoring place for the stationary phase. Therefore, special attention should be paid to the hydroxylation of the oxidized silicon surface before any other step is carried out.
References