Pillar-structured microchannels for liquid chromatography

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Chapter 2

Topographic structures and chromatographic supports in microfluidic separation devices

Abstract

A review is given of the literature on the design, development and use of micromachined devices for separations in the liquid phase. The emphasis is on those devices that offer more than just an empty channel for, e.g., electrophoretic separation. Topographic structures have been incorporated in the channels during their microfabrication, offering a variety of possibilities for the separation of (mainly) DNA molecules based on different principles. Supports for a stationary phase for chromatographic separations have been introduced in the channels in different ways: by packing of the channels with stationary phase particles, by polymerization of monolithic structures, or by lithographic machining of pillars in the channels. It is shown that the latter strategy gives the highest potential for increasing the separation power of the devices. Still, more conventional approaches are closer to a routine application.
2.1 Introduction

The separation of the components of the sample is often an essential step in analytical procedures for the identification and quantification of analytes. In some cases the separation serves only as a pretreatment step with the aim to reduce the sample complexity by isolating a single component or a group of compounds from a complex mixture. More often the purpose of the separation is also the identification or characterization of the components. In the developments towards micro-total analysis systems (μTAS) or lab-on-a-chip devices of the past decades, the design and optimization of separation units in a miniaturized format has therefore received much attention. The driving forces for the miniaturization of analytical instrumentation, reducing the size of separation devices from mL-sized columns via μL-volume capillaries to nL channels on chips, have been discussed extensively in the literature. In the life sciences the limited available sample volumes can be the main motivation. More general, however, is the argument that scaling down improves the speed and efficiency of separation systems.
Many reviews on chip-based separation systems, methods, and applications can be found in the recent literature (see e.g. refs. [1-8]). The separations can be carried out in open micro-fabricated channels. Free-flow or zone electrophoresis and isoelectric focusing are the most common separation modes in open microchannels. Alternatively, the microconduit can be structured with micrometer- or submicrometer-sized solid features. The focus of this review will be on these structured microchannels.

It is convenient to divide the microstructured channels into two main categories. In the first category, the sample and the solid support have a direct physical interaction, and the geometry of the solid features is essential and sufficient for the separation to occur. We refer to this category as topographic structures. The second type of structures is the chromatographic support. In this case the main purpose of the solid features is to hold a stationary phase in place. A concomitant and equally important objective is to reduce the diffusion distances, thereby accelerating the establishment of the distribution equilibria of the sample compounds between the mobile and the stationary phase. For these second types of structures the exact topology is of less importance.

Different strategies have been followed to fabricate structured channels. The solid features, for instance pillars, can be created during the microfabrication process. This is usually the way to make topographic structures. In other cases the material was created inside the microchip after the chip was fabricated. Examples of methods for in-situ structuring are polymerization, or by precipitation of porous silica. The third possibility is to use externally prepared solid material and pack (force) this into the microchannel, as is done with standard stationary phase particles.

### 2.2 Topographic structures

Gel electrophoresis, with polyacrylamide or agarose gels, is the standard technique in genomics for the separation of DNA and DNA fragments. The gel acts as sieving matrix for the biopolymers which are then separated according to size, with the smaller molecules migrating with higher velocities. However, the technique has several drawbacks and limitations. First of all, considering its integration in micro-analysis systems, it is clear that filling microchannels with highly viscous gels can be
problematic, so that developing microfluidic systems for routine application may be technically difficult. A more basic limitation is that, at least with a constant electrical field, a separation can only be accomplished for molecules smaller than the characteristic pore size of the gel. Classical gel electrophoresis can therefore be successful only for DNA fragments with a size of up to 20 kilobase pairs (kbp). Above this limit, sizing can still be accomplished, but only by applying pulsed electric fields. Pulsed-field separations are extremely time-consuming; they can require several hours when the DNA size is in the order of millions of base pairs.

As was pointed out in early papers by Volkmuth et al., arrays of micro- or nano-pillars fabricated in microchannels represent a possible approach to tackle the limitations of gel electrophoresis [9,10]. The pillar structure can replace the gel as sieving matrix, with a characteristic dimension that can be chosen freely. Moreover, the separation mechanism in such structured microchannels can be studied in detail with the aid of microscopy imaging. The observations can – in principle – give accurate indications for the manufacturing of different pillared beds, with features (i.e. pillar size and spacing) tailored and optimized for specific separation problems.

In the work of Volkmuth et al. pillar structures with 2-μm inter-pillar spacing were fabricated by optical lithography on a silica substrate. Electrodiffusion of long DNA strands in the two-dimensional pillar arrays was studied by fluorescence imaging and theoretical models were compared with experimental observations. The “hooking” and “unhooking” of the long DNA strands around the posts appeared to play a crucial role in the sieving mechanism. The unhooking process could be characterized by a time constant (in the order of seconds) that increased linearly with the length of the DNA molecules.

The use of polydimethylsiloxane (PDMS) as a substrate for micro-pillar structures has also been proposed [11]. Pillars with a height of 10 μm, a diameter of 15 μm and 1 μm spacing could be molded in PDMS. DNA strands of different length (λ DNA with 48.5 kbp and T4 DNA with 166 kbp) were reported to have different residence times in the channel with the same continuous electric field. Observations of the moving biopolymer fragments by microscopy showed that the DNA tended to keep a globular morphology and that it elongated and accelerated only when it flowed through the
narrow space between two micro-pillars. The change of morphology is needed, because the gap is smaller (1 µm) than the radius of gyration of the DNA.

For pillar structures with characteristic dimensions smaller than 1 µm conventional lithographic techniques are not adequate; alternative techniques, such as electron-beam (EB) lithography, have to be used instead. The first real separations of DNA using pillar structures were shown with microfluidic devices fabricated with EB lithography by Baba et al. [12]. Channels with a depth of only 360 nm containing pillars with 250-nm spacing were created. With a separation length of 0.5 mm, double-stranded DNA molecules of 2, 5 and 10 kbp could be baseline separated within 100 s, as is shown in Figure 1. High-aspect-ratio (ratio between the height and the diameter of the pillar) nano-pillars have been fabricated in quartz substrates by Kaji et al., also using EB lithography (see Figure 2) [13]. DNA fragments in the range of 1 to 38 kbp could be separated with such a nano-pillar device with a continuous field, when the pillar diameter and the inter-pillar spacing were 500 nm, comparable to the average pore-size of the agarose gel commonly used for relatively small-size-DNA separations [14]. Also, fast separations (~ 10 seconds) of λ DNA (48 kbp) and T4 DNA (166 kbp) were shown. A difference in behavior of these DNA fragments was observed by fluorescence microscopy. The larger T4 molecules experienced frequent conformational changes (from globular to elongated), whereas the λ DNA exhibited a compact globular shape most of the time. Apparently, the λ DNA molecules are too short to get hooked around the 500-nm pillars. Separation of DNA molecules smaller than 1 kbp or larger than 200 kbp could not be realized with this geometry.

High-throughput projection contact photolithography has been proposed as an alternative for EB lithography for the fabrication of sub-micron pillar arrays with a very high aspect ratio [15]. EB lithography requires very expensive hardware and is extremely time-consuming. Therefore, only small channel areas have typically been fabricated. With the proposed technique, the fabrication time for a certain area was reduced from 7 to 12 h for EB lithography to a few seconds. High-quality structures smaller than 100 nm could be produced. DNA size separations were shown on an array of 150 x 8000 µm.

Pillar arrays have been used as separation media in combination with pulsed-field electrophoresis by Bakajin et al. [16]. In this work, hexagonal arrays of 2-µm pillars
were used and the electric field was forced to change orientation with the separation axis. A first pulse was oriented 60° clockwise and the next one 60° counterclockwise relative to the main direction of motion. The separation is based on the relaxation time of the polymers. Each time the electric field changes orientation the DNA strands must align with the new field direction before moving along a new path. Longer molecules, which are confined by the pillars, spend most of the pulse time reorienting, whereas shorter ones align more quickly and move in a straight trajectory during most of the period of the pulse. The difference in average velocity in the net direction of migration was also demonstrated when using a slightly wider angle (135°) between the fields applied during the two pulses [17,18]. For DNA molecules between 10 and 180 kbp an approximately linear decrease of velocity with size was observed, with the consequence that DNA above a certain size was virtually trapped in the array. The application of electric field with periodically switching direction for DNA separation was also reported by Yi and coworkers [19]. They applied the trapping principle described above for the selective extraction of smaller DNA strands from a mixture.

Figure 1  Separation of DNA molecules with a quartz-pillar structured chip. (Reprinted with permission from Ref. 12.)
An original and interesting alternative for micromachining to create topographic structures for sieving was suggested by Viovy et al. [20,21]. Suspensions of superparamagnetic particles inside a microchannel will self-organize in arrays of columns when a magnetic field is applied across the channel. The submicrometer particles were organized in pillars of approximately 10 μm tall (the height of the channels) and used as a sieving matrix in DNA electrophoresis. Single-molecule visualization showed the “hooking” and “unhooking” of the DNA strands around the self-assembled structures as the basic mechanism for the separation.

Figure 2  SEM picture of the pillar chip from Ref. 13. (Reprinted with permission.)

In DNA separations, pillar arrays can be used for other purposes than as a sieving matrix. For example, the technique named entropic recoil separation [22,23] is based on the difference in free energy, inside a microchannel, between an open space and the region structured with nano-pillars. When a DNA strand in the open space is forced to enter the pillar region by an electric field pulse, the extent of the penetration depends on the length of the strand and on the duration and intensity of the electric pulse. If at the end of the pulse the DNA is straddling the interface, it will be extracted back by entropy effects to the open region, where it recoils to its natural form. The entropy of the long strand in the open space is more favorable than in the pillars region, where it is forced to maintain an elongated configuration. The entropy gradient is only present at the abrupt interface between pillars and open region. Therefore, if the DNA is already fully inserted it will not be extracted back into the open space. The principle is illustrated in Figure 3. Mixtures of DNA fragments of different length can be separated by applying
increasingly longer pulses. With a longer pulse, longer strands will be able to fully penetrate the pillar region and be separated from the mixture.

**Figure 3** Schematic explanation of the “entropic-recoil” DNA separation mechanism. (Reprinted with permission from Ref. 22.)

The free-energy difference of DNA when confined in dissimilar environments is also used in so-called entropic-trap separation devices [24,25,26]. An entropic trap consists of an open micro-fabricated channel with varying depth, in a way such that deep and shallow regions alternate (Figure 4). The channel depth is a few μm in the deep regions, and 100 nm or less in the shallow zones. DNA molecules are transported through this conduit by an applied electric field. Since the channel thickness in the nano-fluidic part is smaller than the radius of gyration of the DNA targets, the DNA molecules must stretch out in order to enter this region. The entropy decrease related to the elongation is a thermodynamic barrier for the molecules, which has to be overcome in order to pass a shallow region. When an electric field is applied across the channel, a mixture of differently sized DNA will be focused at the interface between the micro- and nano-regions. Because of Brownian motion and the electric field, part of a DNA strand can...
penetrate the shallow region. When a sufficiently large fraction of the DNA molecule has progressed into the slit, the entropic barrier can be overcome by the electrostatic interaction and the DNA molecule can continue its migration. The probability of such a successful passage is proportional to the polymer length. Hence, longer DNA molecules will be trapped at each interface for a shorter average time and travel through the channel faster than the shorter ones. With an entropic trap device a 5-kbp DNA ladder, with species from 5 to 40 kbp, could be separated [27]. It appeared that the applied voltage should be kept relatively low; at higher voltages the separation efficiency deteriorated, especially for the longer DNA fragments.

Figure 4  Entropic-trap separation device (Reprinted with permission from Ref. 24.)

A size-exclusion-chromatography (SEC) type separation of DNA can be realized in the nano-structured device proposed by Baba et al. [12]. In a channel fence-like posts were created perpendicular to the main direction of flow, with narrow (0.4 µm) gaps between the posts themselves and wider gaps (1.1 µm) between the rows of posts in the
flow direction. The DNA molecules were transported through the channel by an electrical field. Large DNA coils are sterically excluded from the narrow spaces between the pillars and migrate with the field between the rows. Smaller molecules, however, can enter the narrow gaps between the 'fence-posts' by Brownian motion, where they are shielded from the field and, therefore, retarded. Figure 5 illustrates the principle. With this device DNA molecules of 2, 5, and 10 kbp could be separated.

Figure 5 Electrodriven SEC in a microfabricated pillar array: schematic view of the separation mechanism (a); microphotograph of the nano-obstacles (b); separation of three DNA fragments of different size (c). (Reprinted with permission from Ref. 12.)
The same group also introduced a system for continuous separation of DNA, based on the same principle, with so-called anisotropic arrays of nano-pillars [28]. The device is a bi-forking microchannel structured with nano-pillars in the junction region (Figure 6). The sample is continuously transported through the microchannel by an electric field, with the voltage across the main channel being higher than that applied to the side branch. The gaps between the pillars are wide enough to let the small-size molecules pass, but the entrance for the larger ones is blocked and they can only enter into the side branch. The greater electric field across the main channel forces all the small molecules to take this direction.

![Figure 6 Anisotropic nano-pillar array. (Reprinted with permission from Ref. 28.)](image)

Two-dimensional periodic and asymmetrical arrays of obstacles or Brownian “ratchets” can be used for separations of macromolecules based on differences in diffusion coefficients [29,30]. Sample molecules that are driven in one direction by a potential gradient (electric or pressure) will also drift in the orthogonal direction due to Brownian motion. The probability of deviating from the main pathway at each row of obstacles – and with that the average direction of migration will depend on the diffusion coefficient of the molecule. The mechanism and a possible device design are illustrated in Figure 7. The ratchet arrays have been used for the continuous separation of charged, fluorescent-labeled lipids [31]. The phospholipids migrated in a lipid bi-layer, which behaves like a fluid, supported by a silica surface, patterned with titanium-oxide nano-
structures (the diffusion barriers). Other authors showed the (continuous) separation of DNA species in the 100 kbp range on structures created in silicon with obstacles of 3 by 12 μm [32,33]. A special layout for a Brownian-ratchet device intended for working with an alternating electric field was proposed by Derenyi et al. [34]. To our knowledge this particular type of Brownian ratchet was never experimentally realized.

A related strategy for continuous separations of particles or DNA using asymmetrical arrays of micro-obstacles was proposed by Huang et al. [35]. The particles flow through rows of micro-obstacles, with each row horizontally shifted with respect to the previous one by a fixed distance. Under laminar-flow conditions point-like particles will not be displaced in the perpendicular direction when crossing a series of obstacle rows. However, the centers of mass of larger particles cannot collect all flow streams and these are, therefore, displaced horizontally (toward the right in the case of Figure 8. This deterministic lateral displacement can be regarded as an on/off process, which occurs above a critical particle size [36]. By using arrays with pillars that vary in size along the direction of flow, differential separation systems can be obtained. Separations of latex particles and of bacterial artificial chromosomes have been shown.

Facile and fast self assembling of short (1 cm) micro-channels with (sub-) micron-sized spheres of polystyrene and silica has been reported [37]. The microfluidic system layout consisted of a typical cross, with four reservoirs for buffer and sample inlet and for waste. The chip was fabricated in PDMS and irreversibly sealed by a glass slide.
The packing procedure was as follows. All reservoirs, except the buffer-waste reservoir at the bottom of the cross, were filled with an aqueous dispersion of the colloidal spheres; the filled reservoirs were sealed to prevent evaporation. The dispersion moved toward the waste reservoir by capillary force. The water was allowed to evaporate at the end of the micro-channel and the particles started to aggregate at the liquid-air interface. The aggregation then proceeded spontaneously toward the top of the microchannel, while the water kept flowing towards the end of the channel, where it evaporated. The packed beds thus formed were used as sieving matrices for the electrophoretic separation of DNA (0.05-50 kbp) and proteins (20-200 kDa). The effective size-separation range depends on the size of the self-assembling colloidal particles.

![Deterministic-lateral-displacement separation device](image)

**Figure 8** Deterministic-lateral-displacement separation device. The row of obstacles is horizontally shifted by a distance \( \frac{\lambda}{3} \) (A). Three flow streams under laminar conditions are illustrated by way of example. The three flow paths are separated by the second and the third rows of obstacles and then rejoin at the fourth row. Small particles will follow the stream-lines and therefore will not be displaced horizontally (B). The centre of mass of bigger particles is excluded from approaching the obstacles and these will sample only the central flow stream. Therefore, they will be displaced toward the right side each time they hit an obstacle (C). (Reprinted with permission from Ref. 35.)

Fast electrophoretic separations of DNA in the range of 1 to 15 kbp have been achieved in a suspension of hydrophobic nanospheres (around 30 nm) [38]. The concentration of the beads in the medium proved critical for the quality of the separation. The system is competitive to standard separation techniques used for DNA sizing. One of the advantages compared to separation with agarose-gel matrix is that the migration rate of the DNA was size-dependent up to 15 kbp, unlike for agarose-gel electrophoresis where a saturation of migration velocity is reached for bigger DNA...
molecules. The low viscosity of the nanoparticles suspension is also an important advantage compared to agarose gel, especially for chip-based separation devices.

Electrodriven SEC of DNA strands was demonstrated also by Sano et al. [39]. In this case the pores (about 80 nm in diameter) of anodic alumina acted as size-selective narrow spaces. Even though only the separation of two DNA molecules with a large size difference (0.3 and 3.2 kbp) was reported, the authors stated that the size of the pores could be precisely controlled within the range of 5 nm to 450 nm. Thus, the SEC separation is conceivable for biomolecules of widely different sizes, for example proteins, if adsorption effects on the alumina surface can be avoided.

2.3 Stationary phase supports

Different possibilities have been investigated to create a support for the stationary phase in microfluidic devices for chromatographic separations. The first task of a support is to firmly retain the stationary phase inside the separation channel while the mobile phase carrying the sample flows through. An additional purpose of the support is to break the main flow generated by the pumping device into many small flow streams frequently merging and splitting. This so-called cross-channel mixing is the most practical way to minimize the distances that sample molecules have to bridge by diffusion in the mobile phase in order to reach the stationary phase. When the diffusion distances are too large, the band dispersion increases and the separation deteriorates. An alternative way to overcome this so-called mass-transfer contribution to band dispersion, without the need for cross-channel mixing, is to perform chromatography in very narrow channels. The characteristic dimensions (channel diameters) should be of the order of a few µm and the inner wall should be coated with the stationary phase. Of course, this limits the amount of sample that can be injected in the column and therefore the detection capability. Breaking a bulk flow in small flow streams can be seen as a way to mimic the more-efficient narrow channel, creating a multitude of short and narrow pathways. The channels need to split and merge (“cross”) frequently in order to effectively average the flow differences between them and to minimize band spreading [40].
2.3.1 Packed beds

Porous particles still are the most widely used support for liquid chromatography. Columns packed with porous silica particles are in most of cases still the best choice to ensure good separations. The homogeneity of the particles in terms of size and shape has become more than satisfactory for most of applications. Packed columns are robust and well characterized. Thanks to the porosity of the particles their loadability (sample capacity) is high. Still, beads packed inside a fluidic system are not the ideal stationary phase support. The flow inside a packed bed is not homogeneous, due to the random arrangement of the particles, and this feature yields additional band dispersion [41]. The homogeneity of the flow depends on the quality of the packing. To achieve a good quality packing with conventional columns – using the standard slurry-packing technique – a high pressure is required. This is not possible with a micro-fabricated channel. Leak-proof connections are needed and the device may break during packing. Short microchannels can be manually packed with a syringe and/or by applying a vacuum at the channel outlet [42,43].

Figure 9 Electrosomotic packing in a separation chamber. The hook-like geometry of the particle-inlet channel is visible on the top left. The CEC inlet and outlet channels cannot be seen in this picture. (Reprinted with permission from Ref. 46.)
In order to avoid the problems related to high-pressure packing, various other techniques have been tested. A method for packing PDMS microcolumns by centrifugal forces was presented by Penrose and coworkers [44]. The molded PDMS device was placed on a conventional compact-disc, with the separation channels pointing outwards from the center of the disc. The unit was then attached to a centrifuge rotor through the centre hole of the compact-disc. The end of the microchannel was tapered in order to retain the particles. The first step of the packing protocol was to fill the entire separation channel with the slurry (Spherisorb-C8, 5.8-μm beads) by gravity. Next, the device was spun at 2700 rpm to ensure bed consolidation by centrifugal forces. The packed bed shrunk upon centrifugation, both by consolidation of the bed and by some loss of beads from the channel outlet. Eventually, a satisfactory packing with an external porosity of 0.46 was achieved across 22 mm of the 32-mm long microchannel. A centrifugal force was also used as the propulsive mechanism for the mobile phase in this work.

Electroosmotic flow was used to pack Spherisorb ODS-1 particles, with diameters in the range from 1.5 to 4 μm, in very short glass channels [45]. The separation chamber was only 200 μm long and 300 μm wide. The separation region was connected to inlet and outlet channels and to a side channel used as the slurry inlet during packing. The flow resistance of this side channel was much higher than that of the inlet and outlet channels, in order to prevent leakage of flow through it during the separation. In the packing procedure the channel was first filled with acetonitrile and the side inlet reservoir with the slurry. A positive voltage on the slurry reservoir was ramped from 200 to 800 V in 5 minutes, while the reservoirs of the mobile phase and the waste were grounded. In this way the slurry was electroosmotically forced into the separation chamber to form a packed bed. A problem encountered with this packing method was that the electroosmotic flow towards the separation chamber generated a backflow, as happens in closed electrophoretic systems. This backflow prohibited the complete filling of the chamber. The problem was solved by designing the feeding channel with a hook-like geometry as shown in Figure 9. When the packing was successfully accomplished, the feeding channel was closed with a methacrylate-monolith plug (formed by UV- or thermally initiated polymerization) [46]. In later work from the same group this electroosmotic packing method was also applied for 1-, 2-, and 5-mm long chambers [47]. With these longer channels electroosmosis alone did not suffice to
achieve an effective packing and vacuum suction from the waste reservoir was required in addition. The authors reported that the 1- and 2-mm long channels were successfully packed in most of the attempts and that they were stable for one month or more of daily use. The 5-mm long chambers, however, often showed poor packing quality, with many voids in the bed. The packed channels have been used for solid-phase extraction (SPE) and for separations by capillary electrochromatography (CEC).

During and after packing the particles must be held inside the conduit by some kind of channel restriction. Several possibilities for this have been proposed. Short channels with a diameter smaller than the beads have been fabricated at the end of the main channel [48,49]. Multichannel structures at the ends of the separation channel can act as micromachined frits. This approach was followed by Ro and coworkers for PDMS chips [50]. Trapping of beads in a microchannel by two 1-μm deep weirs (at the entrance and exit of the separation channel) has been proposed by Harrison et al. [45-47], as schematically shown in Figure 10. Simple tapering of the channel appears to be sufficient to retain the particles, even when the final channel cross section is wider than the particle diameter. A simpler mechanism to explain how particles can effective clog (tapered) channels is the “keystone” effect, where the particles are positioned such as to form a stable “bridge” across the channel [51]. Although the tapered-channel principle is simple, this method requires optimization of the slurry density in order to be effective. Also, an annealing step is recommended to stabilize the bed [51].

Despite all the techniques described above packing microfluidic channels with stationary phase particles is still so difficult that their chromatographic performance, in terms of the number of theoretical plates, is relatively poor. To obtain packed separation channels of a reasonable length, with efficiency comparable to that of standard packed
columns, high-pressure packing methods are apparently still indispensable. A successful application of high-pressure packing of microfluidic channels, with pressures up to 120 bar, has been described in a paper by Yin et al. [52] and in later work [53]. Microchannels of 4.5-cm length were packed with 3.5-μm beads. The material and the fabrication technique for the microfluidic system had been inspired by the inkjet-printhead manufacturing process. The microchannel was etched by laser ablation in a polyimide film along with other features, such as connection ports, an enrichment column, and a nano-electrospray tip for coupling to a mass spectrometer (see Figure 11). Different parts of the device were machined in three different polyimide films, which were joined together by hot lamination. The chip can be sandwiched between the stator and the rotor of a HPLC switching valve. The microfluidic device is now commercially available.

Several applications of separation systems with the packed microfluidic devices described above have been published. A parylene microfabricated column [49] (100 μm wide, 20 μm deep, and 8 mm long) packed with 5-μm C18 particles was used for the separation of a mixture of amino acids with temperature gradient chromatography. The authors reported the separation of five derivatized amino acids by raising the temperature from 25°C to 65°C in 11 minutes. A similar chip, with possibilities to create mobile-phase gradients was applied for the separation of a tryptic digest of BSA
The on-chip LC system described by Lazar et al. [43] was used to separate a protein digest for proteomic applications. The analysis results with the chip-LC were compared to the results obtained with a bench-top HPLC instrument using a packed column with the same length as the microchannel and packed with the same particles. The number of peptides identified by the chip-LC system was slightly lower than with the standard column.

2.3.2 Monoliths

Monolithic stationary phases can be created in situ inside microfluidic channels. Synthetic-polymer monoliths are made by UV or thermally initiated polymerization of a monomer mixture containing a so-called porogenic solvent, which causes the formation of flow-through pores in the final monolith. Silica-based monoliths are created by sol-gel processes. Silica monoliths can be chemically modified after their creation in the separation channel. Polymer monoliths are generally used without any further modification. For microfluidic devices the main advantage of using monolithic materials is the ease of preparation, although the batch-to-batch repeatability remains an issue. Another advantage over packed-bed channels is that no frits are required to hold the stationary material in place. The monolith can be chemically bonded to the microchannel walls. Monoliths are normally more porous and more permeable than packed beds. As a consequence, lower pressures are required to perform chromatographic separations. For a microfluidic system this aspect is much more important than for instruments that use conventional columns and capillaries.

Ericson et al. [55] polymerized acrylates in quartz chips to obtain a continuous polymer bed. They manufactured different monolith-chips with different features to perform electrochromatography (in the reversed-phase mode) and liquid chromatography (in the anion-exchange mode). Channels with a semicircular cross section of 40 μm and lengths from 1.8 to 28 cm (in a serpentine geometry) were filled with monolith. To achieve this, the monomer solution containing the porogenic solvent was flushed into the channel by nitrogen pressure and left overnight to polymerize. The authors showed that the channels performed as well as capillary columns, with minimum plate heights of around 4 μm for an unretained compound. Separations of low-molecular-weight compounds, as well as of proteins were shown.
Early attempts to cast acrylate-based monoliths in glass chips were not very successful [56]. A separation of three naphthalene-2,3-dicarboxaldehyde (NDA) labeled peptides was achieved in the CEC mode in 8-cm long microchannels, but the efficiency for the modified peptides was low. The authors explained this by a possible electrostatic interaction of the peptides (with a residual positive charge) with the negatively charged surface of the monolith. In later work by the same group much-better performances (with up to 200 000 plates/m) were reported for the CEC separation of polyanaromatic hydrocarbons on butylacrylate monoliths in a fused-silica chip [57,58]. The efficiency was comparable to that of capillary-column systems. Also, for the CEC separation of (derivatized) peptides with acrylate-based monoliths a chip system can be comparable to or even better than capillaries, as was shown by Throckmorton et al. [59]. Comparable separations took 45 s in a 6-cm long microchannel and 9 minutes in a 23.5-cm capillary. The authors explained the remarkable improvement from the better Joule-heat dispersion in the shallow microchannels, which allowed the use of higher field strength.

Reichmuth et al. showed that fast pressure-driven separations of fluorescently labeled peptide and protein mixtures could be realized in a 1.7-cm long microchannel filled with a stearyl-acrylate monolith [60]. The channels had been created in fused-silica wafers by wet-etching techniques and the monolith was polymerized with photoinitiation. A microvalve injector device was fabricated by creating a cylindrical fluoropolymer element on-chip through laser-induced polymerization. This served as a switching valve between two input channels and the separation channel.

Casting the monoliths in plastic chips instead of in glass or quartz substrates has many advantages. Polymers are inexpensive substrates and the protocols for the fabrication of microchannel systems are simple. Techniques such as hot embossing or milling can be used for series production. Cyclo-olefin copolymer (COC) has shown to be particularly suitable as substrate for the production of monolithic micro separation systems [61]. COC is transparent to UV light, which is required when photoinization and/or photografting is to be applied. Moreover, COC wafers can be irreversibly bonded under mild conditions of temperature and pressure. Stachowiak et al. studied the possibility of effectively anchoring acrylate-based monolith in microchannels machined in COC chips [61]. This was achieved by UV-photografting of ethylene dimethacrylate
(EDA) to the channel walls before the monolith-forming step. In this latter step the unreacted double bonds on the channel surface were incorporated in the monolith. The authors demonstrated the possibility to use the same photografting protocol for polypropylene (PP) chips. Poly-(ethylhexyl methacrylate-co-ethylene glycol dimethacrylate) monolithic stationary phases can also be created by UV-polymerization in COC chips [62]. The microchannel device was produced by hot embossing and sealed by a temperature and solvent-enhanced pressure-bonding method. The COC chip could withstand 1000 psi without leaking or rupture.

The possibility to make microfluidic channels with silica-based monoliths has also been demonstrated. For generating silica monolithic material, Ishida et al. [63] filled glass microchannels of approximately 40-cm length with the reactant solution. The position of the sol in the channel was adjusted with air pressure to leave the injection port free. The sol was heated and the silica dried at temperatures up to 200 °C. The silica surface was afterwards modified to yield an ODS-silica material suitable for reversed-phase liquid chromatography. Since monoliths can also be created in curved channels, the effective separation length and the number of theoretical plates can be easily increased. The entire 40-cm column length could be fitted on a 3.5-cm square by choosing a serpentine-like geometry. In one channel design the cross section of the column was tapered at the turns. The authors reported lower band dispersion in this tapered-turns microcolumn. Efficiencies up to 18 000 plates/m were reached for catechins.

A photopolymerized sol-gel (organic-inorganic) monolith in a glass chip was used for the separation of two coumarines [64]. Figure 12 shows a scanning-electron-microscopy (SEM) image of the channel. The separation occurred in 4.5-cm channels in less than 4 min, with an efficiency of (at most) 18,500 plates/m. However, the two compounds could be base-line separated in 80 seconds.
Another advantage of monoliths is their relatively easy removal from a chip. This is relevant when expensive chips, for instance made of quartz, are used. Throckmorton and coworkers [59] used thermal incineration followed by NaOH washing. An alternative method is laser destruction [57].

2.3.3 Micro-fabricated structures.

Micromachining techniques can be used to make channels that are structured with ordered arrays of pillar. The pillar bed can act as (a support for) the stationary phase in chromatographic separations. There are several potential advantages of micro-pillar structures in comparison with conventional packed beds. Frits or other retaining structures are not necessary. Pillar size, shape, and collocation can be controlled and chosen according to an optimal design. The most relevant difference between pillar beds and conventional packed beds or monoliths, however, is the degree of order of the structure. Inside a monolith or a packed column flow paths differ in length, width and tortuosity. Therefore, the flow velocity is not the same at each point in the column or microchannel. The resulting variations in flow paths are an important source of band dispersion in liquid chromatography, particularly when the flow is pressure driven.

Pioneering work on micro-machined pillar-structured channels for chromatography was described in 1998 by He and Regnier [65]. The devices were named collocated monolith support structures (COMOSS) by the authors. The channels were etched in quartz wafers to a total length of 4.5 cm, a width of 150 μm, and a depth of 10 μm.
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channels were structured with square shaped posts with a length and width of 5 µm. The pilarto-pillar distance was approximately 1.5 µm. As can be seen in Figure 13, the inlet channel of the COMOSS system bifurcates several times until it reaches the structured column, allowing a uniform distribution of the liquid flow. The combined cross section was kept constant and equal to the joint cross section of all inter-pillar microchannels. The outlet region was a mirror image of the inlet. The system could be operated in the CE or CEC modes, with voltage gradients of up to 5000 V/cm without significant problems associated with Joule heating. The authors’ explanation for this was that in every part of the column the mobile phase was within 5 µm of the wall of the channel, which facilitated the heat dispersion. Moreover, all the pillars are in contact with the top and bottom walls, which contributes to the heat dissipation. For reversed-phase separations the inner surface of the structured microchannel was modified with γ-aminopropyl silane. Polystyrene sulfate could be immobilized as stationary phase on this surface by electrostatic forces. With rhodamine 123 as test compound an efficiency of 770 000 plates/m was recorded in a CEC experiment. With packed columns comparable efficiencies can only be achieved if 1-µm beads can be used successfully. The remarkable efficiency was attributed to the high degree of order of the COMOSS structure.

Figure 13  Inlet distributor of a COMOSS CEC column. The microchannel from the buffer reservoir bifurcates several times until it reaches the separation column. (Reprinted with permission from Ref. 65)

The homogeneity of the COMOSS is compromised at the side walls of the channel; in this region band spreading can increase due to the open spaces between the pillars.
and the channel wall, which may give rise to stagnant pools of liquid. The authors empirically tried to minimize these effects by machining the compensating architecture shown in Figure 14 [66]. In a later paper it was shown that the depth of the channel is the main parameter determining the diffusion time constant of the system [67].

The first COMOSS separation reported was a reversed-phase CEC separation of a fluorescein isothiocyanate (FITC) modified tryptic digest of ovalbumin [68]. The surface of the COMOSS was coated with a C_{18} stationary phase to obtain sufficient retention. However, the surface area coated with stationary phase is much lower in a COMOSS system than in columns packed with the conventional porous silica particles. Therefore, the peptide mixture could be eluted in an isocratic run. The peak capacity was claimed to be comparable to that of a conventional gradient HPLC separation. The low surface area of micromachined structures compared to porous silica columns has serious disadvantages. The amount of sample injected in the micro-fabricated channels must be sufficiently low to avoid over-loading, yet high enough to allow detection of the analytes. The resulting dynamic range is extremely narrow. To broaden it, the width of the structured channel may be increased. COMOSS channels as wide as 1 mm have been tested [69].

COMOSS devices machined in PDMS have been considered as an alternative to quartz, since PDMS is more suitable for series production [70]. A mold was created using a positive photo-resist. Then the mixture of polymer and curing agent was poured on the mold and the polymer was cross-linked according to well-known protocols. The structure formed was closed with another slab of PDMS; after plasma oxidation the two substrates could be bonded irreversibly. Pillar dimensions had to be larger than with quartz, due to the softness of the PDMS. The PDMS surface inside the COMOSS channel was functionalized with two different reversed phases: polystyrene sulfonate and dimethyl-octadecyl-silyl groups. The FITC-labeled ovalbumin digest was separated using both stationary phases. Under identical conditions the C_{18} COMOSS yielded to higher number of identified peaks in a shorter time. The highest efficiency reported in this work was 560 000 plates/m.

Upon plasma oxidation the surface of PDMS becomes rich in silanol groups, which are available for further modification. Several surface modifications of PDMS through cerium-initiated polymerization have been described [71]. Different pillar sizes and
geometries in PDMS devices have been compared [72]. The main conclusion of this study was that the efficiency increased with the number of cross-over (coupling) points between the different flow paths through the structure.

A PDMS micro-analysis system for comprehensive proteomics incorporating a COMOSS column was presented by Slentz et al. [73]. Three operations could be performed on-line, i.e. tryptic digestion, affinity chromatography, and CEC. Trypsin was immobilized on (5-µm) beads confined in a channel in the first part of the microfluidic device. The particles were retained by a micro-fabricated frit, with a structure similar to the inlet and outlet distributors of the COMOSS. The second part of the device was packed with 5-µm particles modified for affinity chromatography, to retain the peptides containing histidine. The final part of the microfluidic device was a COMOSS separation column coated with a polymeric stationary phase. The device was integrated with several buffer reservoirs and switching possibilities. The fluid manipulation was by electroosmosis during all steps of the analysis. The tryptic digestion took place in a continuous-flow mode.

**Figure 14** Possible side-walls architectures of COMOSS systems. The shaded areas represent stagnant zones. (Reprinted with permission from Ref. 66)

In all early applications of structured microchannels for chromatographic separations the mobile-phase flow was electro-driven. However, pressure-driven chromatography could also benefit strongly from the high degree of order, as pointed out by Knox [40]. The dispersion due to flow differences in the mobile phase is the most-important contribution to the overall band dispersion, at least in the flow-velocity range normally
used. Desmet and coworkers performed a series of computational-fluid-dynamic (CFD) simulations of pressure-driven flow through pillar arrays [74]. The cylindrical pillars were positioned in an equilateral-triangular geometry. The pillar sizes and the bed porosities considered were comparable to those of conventional packed columns. The predicted gain in efficiency compared to packed columns was estimated from the minimum plate-height ($H_{\text{min}}$) on the van Deemter curve. For non-porous pillars $H_{\text{min}}$ was about six times lower than for columns packed with non-porous particles of the same diameter. For porous pillars relative to porous particles the gain was smaller, but still considerable (approximately 2.5 times lower $H_{\text{min}}$). The back-pressure estimated from the simulations was also reduced due to the increased order of the packing. Different pillar shapes can lead to different channel performances, as reported in another simulation study [75]. Cylindrical, hexagonal, and diamond-shaped pillars were considered. The evaluation of chromatographic performance can be expressed as the channel separation impedance $E$ [76]. The impedance is defined by the ratio between the square of plate height number and the chromatographic permeability. The measurements showed diamond-shaped posts to be the best choice. Ellipsoid shaped pillars, which were included in another study [77] did not improve the impedance compared to the previously considered shapes. Within the same shape group, the more elongated is the geometry, the lower is the impedance, when at the same time the number of coupling points between the flow-through pores is kept constant. For instance channels structured with diamond or ellipsoid shaped posts lead to lower $E$ when the ratio between the longitudinal axis (i.e. along the net flow direction) and the lateral axis is increased. The effect of the (external) porosity of the pillar arrays was also investigated by CFD simulation [78].

The periodicity of a two-dimensional array of pillars is interrupted at the side-walls that confine the microchannels. The role of the side walls in the band dispersion was the object of another simulation study [79]. The distance of the last row of pillars from the wall was found to be extremely important for the performance of the channel, as it determines the velocity of the flow stream adjacent to the walls. The flow velocity at the wall should be the same as in the bulk channel and this can be achieved by optimizing the distance between the pillars and the wall.
The dynamics of electroosmotic and pressure-driven liquid flow in a two-dimensional-array of cylindrical pillars have been investigated [80]. Van Deemter plots for the two cases show the efficiency for a non-retained compound to be much higher in the electro-driven mode. This difference mostly stems from the different flow profiles, \textit{i.e.} parabolic in pressure-driven and plug shaped in electro-driven flow.

Recently, simple separations of three coumarines were reported for a microchannel structured with pillars, the surface of which was coated with a monolayer of octadecylsilane [81]. Carbon nanotubes have also been grafted on pillar surfaces to increase the loadibility [82].

2.4 Conclusions

The construction and use of structured microfluidic separation channels is an active field of research. The topographic structures that can be machined in various substrates offer completely new possibilities for DNA separations. Most research in this area is still fundamental in character, since the behavior of the DNA molecules in the devices is not yet completely known or understood. For the fabrication of chromatographic devices different strategies are being followed. Filling channels with a monolithic stationary phase is probably the easiest way experimentally. Creating ordered structures as chromatographic supports using micro-fabrication techniques may offer the greatest potential for efficient, fast separation systems. Still, the strategy of packing conventional stationary phase materials into microfluidic channels has been most successful in terms of valorization. Separation chips produced in this way and the associated instrumentation are already commercially available. This strategy may have been commercialized first because it stays closest to the generally accepted separation methods. Still, these fairly conservative devices may pave the way for the practical implementation of revolutionary concepts in the near future.
References

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