Chapter 11
Chromatography in Microstructures

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1. Introduction

1.1. Background

The possibility to separate a (complex) mixture of chemicals in a sample to gain understanding of the composition of the sample and the amounts of the individual components within the sample, or both, is among the most important and often used analytical techniques. Whenever specific enough sensors are not available or when many components of the sample are of interest, or when these components are contained within a demanding matrix (e.g., proteins in blood), separation techniques are the only available choice to prepare the samples before the next step in the analytical process. Typically, an aliquot of the sample is injected into the separation system, where – if successful – the individual components are separated in time and/or space and, subsequently, monitored, detected, identified, and/or quantified. It is important, in this context, to remember that steps preceding and succeeding the actual separation always also contribute to the overall performance of the systems, which is why they always have to be included in an overall evaluation. Electrophoresis, chromatography, and related variants of these two are among the predominant separation technologies used today.

Calvin Giddings, undoubtedly one of the dominating figures in developing and describing modern separation sciences, succinctly summarized separations as “the art to maximize differential (separative) transport, while minimizing dispersive transport” [1]. It is in this little sentence that

all of the challenges of realizing high performance separations are contained. And it is also this statement where some of the advantages of performing separations in microstructures can be derived from. Because, while phenomena pertaining to the separative transport are in essence identical in microstructures compared to more conventional formats, the strengths of microsystems lie in the possibility to – literally – miniaturize the effects of dispersion, and hence, arrive at high performance separations. Let us get back to this important aspect further down.

First, when using the term miniaturization in this chapter, it is not meant in the sense of purely shrinking capillaries or tubes further and further. Instead, a more important aspect is that as many parts as possible of the separation systems are fabricated and integrated on a typically flat substrate, e.g., a piece of silicon, glass, or polymer. The final consequence of this idea is to have all required functions of a separation, or indeed of the entire analytical process, combined on that one piece of real estate, a so-called monolithic integration. This avoids external manipulation of the sample between different steps, unnecessary transfer lines, and any sort of connectors, which all can add to diminishing the performance of the analytical system. At the same time, shrinking dimensions can add other benefits, such as low sample, reagent, and energy consumption, low waste production, high surface to volume ratios, faster analysis times, higher throughputs, and more reliable analysis through parallelization and easily built-in redundancy. Of course, these and related advantages have already been discussed elsewhere in this book and the final products are labeled micrototal analysis systems or lab-on-a-chip systems.

This chapter will concern itself with microsystems invoking chromatography as the separating technique [2, 3]. We use the term chromatography to describe separations that require at least two different phases to be present. Often, one of them is stationary, while the other is mobile and pushed, moved or percolated through or past the stationary phase. Different components within the sample are then moved along with the mobile phase and will be delayed to different extents during their passage through the stationary phase. This is achieved by exploiting various physicochemical phenomena, such as (reversible) adsorption to the stationary phase or differential distribution between the two phases. A range of interactions have been used to achieve chromatographic separations, and thus make this technique very universal, i.e., available for charged and noncharged molecules, for highly polar and very nonpolar molecules, and for small molecules and all the way to complex biomolecules and even polymers. A more in-depth discussion of the many variants of chromatography and the mechanisms behind are beyond the scope of this chapter. The reader is referred to the many monographs on this topic (e.g., [1, 4, 5]). Also, purely electrophoretic separation

techniques, where charged molecules are separated in an electric field according to their charge to mass ratio, are discussed in a different chapter in this book. Hybrid techniques, such as electrokinetic chromatography (EKC), which have attributes of both electrophoretic and chromatographic techniques, are also not discussed further here – the reader is referred to a recent monograph on this topic containing a chapter detailing EKC on chips [6].

1.2. Short Overview of Some Variants of Chromatography

1.2.1. Gas Chromatography (GC)

Here, the mobile phase is normally an inert gas (such as nitrogen or helium) that is pushed through a column or capillary containing the stationary phase by overpressure from a gas storage container. In modern GC, the stationary phase is typically a thin highly viscous film on the capillary walls. Interactions exploited in this technique are manifold, but are largely restricted to interactions with the stationary phase. The mobile phase is almost exclusively a transporting medium only. In GC, temperature is frequently used as a means to tune separations.

1.2.2. Pressure-Driven Liquid Chromatography (LC)

In this variant, the mobile phase is a liquid and the stationary phase is either a solid or a material with properties of a liquid immobilized on a solid carrier. Here, interactions take place with both the stationary and the mobile phase, i.e., the differential distribution of a sample component over the phases is exploited. Historically, the first LC separations were performed such that the stationary phase was hydrophilic (e.g., silica), while the mobile phase was hydrophobic (e.g., hexane). This was referred to as *normal-phase LC*, while the opposite case (stationary phase is hydrophobic and the mobile phase is hydrophilic) was termed *reversed-phase LC*. However, nowadays, the latter is much more common than the former, mainly due to its simpler and more reliable operation and the possibility to immediately use aqueous samples, as often obtained in biochemistry and medicine. The mobile phase is driven by overpressure, which in most cases is generated by a pump. The stationary phase can be realized in many ways, as a packing of porous or nonporous beads, as a surface coating, as a porous in situ polymerized monolithic bed, or as an array of micromachined pillars, to name but the most important variants. In most cases, the above-mentioned

solutions only constitute carriers, where the actual interacting media need to be added by proper surface modification afterwards.

1.2.3. Electrochromatography (EC)

The main difference to LC is that here the mobile phase is moved through or past the stationary phase by means of electric fields and by exploiting electroosmosis as a phenomenon to induce motion of bulk electrolyte solutions under an applied voltage. This poses different requirements to equipment, mobile phase composition, and, in some cases, detection as compared to pressure-driven techniques. An often cited advantage of electrodriven techniques is the fact that the electroosmotic flow has a flat flow velocity profile over the cross section of the capillary or channel, whereas pressure-driven flows are characterized by a parabolic flow velocity profile, which has immediate consequences in terms of dispersion of chromatographic bands (see further down). On the other hand, pumping by pressure is fairly independent of the composition of the mobile phase, whereas the electroosmotic flow is directly and strongly dependent on pH, ionic strength, and percentage of added nonpolar modifiers, and thus reacts strongly to changes in the composition of the mobile phase, whether by design or by accident. As far as the selection of the stationary phase is concerned, both LC and EC can exploit a variety of interaction mechanisms, such as, e.g., hydrophobic interactions, ion exchange interactions, size exclusion mechanisms, and more specialized interactions, such as charge-transfer or affinity interactions. This technique is for historical reasons often better known as capillary electrochromatography (CEC).

1.2.4. Miscellaneous

This category includes techniques such as shear-driven chromatography and hydrodynamic chromatography. In shear-driven chromatography, neither pressure nor electric fields are used to drive the mobile phase. Instead, two adjacent walls enclosing the mobile phase are moved relative to each other, thereby inducing shear in the mobile phase, which again leads to relative movement between the mobile and the stationary phase and thus allows chromatography. This technique obviously comes with its own challenges with regard to instrumentation and the realization of proper injection and detection protocols. Hydrodynamic chromatography is, despite its name, not a true chromatographic technique as it does not employ two different phases. It makes use of a parabolic flow velocity profile and very small channel dimensions, so that differently sized molecules will sample different percentages of the parabolic profile and hence spend different

amounts of time traversing the system. Larger molecules are faster in this technique than smaller molecules, since the latter can get closer to the wall regions with their center of mass and therefore also experience the slower velocity regions near the walls. On average, they will therefore move more slowly than larger molecules. This technique works best if the inner diameter of the capillary or the depth of the channel are only a bit larger than the largest radii of the molecules to be separated.

1.3. Some Theoretical Considerations

This section cannot be a replacement for an in-depth treatment of chromatographic theory, but we need to prepare at least a foundation for some discussions concerning performance, to define figures of merit for comparison and to understand scaling effects when going from conventional formats to microchips. Further, we will restrict ourselves to discussing elution chromatography, where a defined sample aliquot is injected, as opposed to, e.g., frontal chromatography, where sample is continuously infused.

The injection plug is ideally infinitesimally narrow, but has of course a certain width in the longitudinal direction and is often approximated by a top-hat function. As the separation proceeds, different types of analytes within the sample plug will be retained within the system to different degrees resulting in discrete zones or bands containing, ideally, only one type of analyte (separative or differential transport). At the same time, however, these bands widen in the longitudinal direction due to a number of processes collectively called band broadening mechanisms (dispersive transport). At the same time, the concentration profile of the original top-hat shapes evolve into Gaussian shapes. The standard deviation, σ, i.e., half the width of the Gaussian shape at the point of inflection, can now be used as a means to describe the effects of band broadening, and hence the efficiency of a separation process. Two figures of merit – in essence borrowed from distillation theory – are important, the plate height, H, and the plate number, N. H is defined as the variance, σ^2, per unit separation length, L, while N then computes as L/H. The overall variance of a system is typically the sum of a number of variances related to various band broadening mechanisms, such as, e.g., the injection plug length, the detection width, longitudinal diffusion, Joule heating effects, influences arising from the parabolic flow profile in pressure-driven systems, and so forth. Both, N and H, are easily accessible from experimental data, such as the measured arrival times of the centers of mass of the bands at a detector and their widths in the graphical representation of the detector trace, the chromatogram. Strictly speaking, both N and H mainly include information about the amount of

dispersive transport and hence another parameter, resolution *Rs*, is introduced, combining the effects of differential and dispersive transport.

The goal of modern separation technology is to provide fast and efficient results. In practical terms, this corresponds to pushing the sample through the stationary phase as fast as possible (i.e., maximizing the linear velocity, *u*) while maintaining efficient separations (i.e., minimizing the plate height). A plot of plate heights measured vs. the linear velocity typically results in curves with a minimum corresponding to the optimum linear velocity at which the smallest plate heights are achieved. These curves can be described following the van Deemter formalism [7]:

$$H=A+B/u+Cu, \tag{1}$$

where the term *A* includes contributions to *H* due to differences in pathlength through packed/porous stationary phases, the term *B* relates mainly to longitudinal diffusion, and the term *C* includes contributions based on the mass transfer kinetics related to the establishment of an equilibrium between the analyte concentration in the mobile and in the stationary phase. These plots are most often used as practical tools to investigate separation efficiencies, identify main sources for band broadening, and find the most suited linear velocity.

From the point of view of miniaturization, at first glance it seems obvious that miniaturized separation systems would suffer from a lack of sufficient available separation length, which consequently would lead to small numbers of theoretical plates and, hence, inefficient separations. However, microsystems can make up for this "shortcoming" by offering smaller diffusion lengths than their more conventional counterparts, which reduces, for example, the term *C* in the van Deemter equation and therefore allows separations to be run at higher speeds without significant losses in efficiency. On the other hand, ways to "compact" longer separation lengths onto the small footprints of microchips have since been developed and successfully tested [8, 9]. Also, due to the possibilities in design, integration, and material, miniaturized separation systems can minimize the contributions from a number of notorious sources for band broadening, such as the injection plug length, Joule heating, and excessive connectors and transfer tubing. Finally, the short separation lengths allow the establishment of high electric fields with relatively small voltages, opening up the possibility to have electrodriven portable separation units running off batteries.

Two excellent and more in-depth discussions on the benefits of miniaturization for separation techniques can be found in the literature [10, 11].

2. Examples of Chromatography on Microchips

2.1. Gas Chromatography (GC)

The first report of a microfabricated device for chemical analysis was published by Terry and co-workers at Stanford University in 1979 [12]. They had constructed a planar gas chromatograph by using photolithography and wet-etching techniques, as previously mainly employed in the microelectronics field. The gas chromatography column was made by etching a spiral channel into a 5-cm-diameter silicon wafer, resulting in a 1.5-m-long, 200 μm-wide, and 30-μm-deep column.

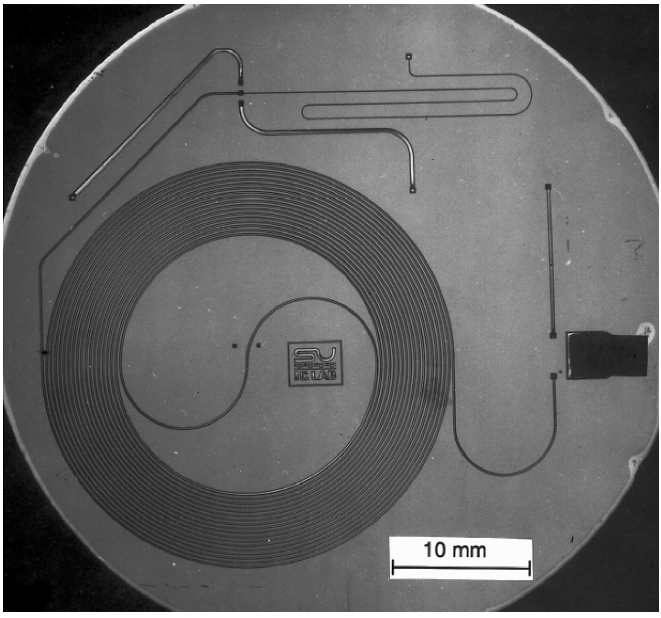

Fig. 1. Gas chromatograph with integrated sample injection and thermal conductivity detector. The chromatography column is the spiral channel. The channels on the top of the wafer make up the injection valve and on the right side of the wafer the thermal conductivity detector is mounted [13]. Reprinted with permission

The system had a sample injection valve fabricated onto the wafer, and a thermal conductivity detector that was mounted on the wafer. The whole device is pictured in Fig. 1. The system was sealed by a Pyrex glass plate that was anodically bonded to the silicon wafer. Pressurized gas was then coupled to the chip to introduce carrier gas and a sample gas stream. A viscous liquid stationary phase was introduced into the chip by filling the

chip with a mixture of the stationary phase and a volatile solvent. The organic solvent was subsequently blown out of the capillary, leaving a thin layer of the stationary phase on the channel walls.

Using this setup, a separation of eight nonpolar substances was performed in less than 10 s, whereas a comparable separation by conventional column required several minutes. The effective plate numbers achieved in the separation ranged from 385 to 2,300 plates, which is significantly lower than what can routinely be reached in a conventional capillary column with the same length (up to 15,000 plates [7]).

This microfabricated GC device was far ahead of its time and hardly recognized by the analytical chemistry community, and it was not until the early 1990s that the miniaturization of analytical methods and devices really started gathering momentum when Manz and co-workers fabricated a miniaturized liquid chromatograph on a silicon wafer [13].

More recently, Reidy et al. from the University of Michigan reported on a microchip gas chromatographic separation of 24 analytes in around 6 min (see Fig. 2). The sample was a mixture of aromatic and aliphatic hydrocarbons with boiling points ranging from 30°C to 216°C. The peak capacity of the 3-m-long column was over 100 and an efficiency of around 12,500 plates was achieved using air as carrier gas. Figure 2 shows a separation of the mixture [14]. Compounds that co-elute are compounds with very similar boiling points, for example compounds 11, 12, and 13, which are 2,3-dimethylheptane, *m*-xylene, and *p*-xylene and have boiling points that are 141°C, 139°C, and 138.3°C, respectively.

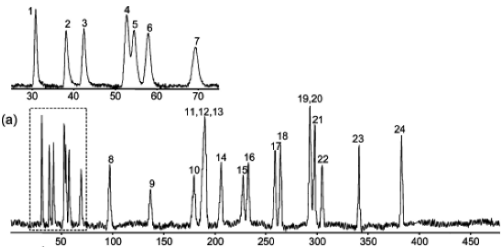

Fig. 2. Microchip gas chromatographic separation of 24 aromatic and aliphatic hydrocarbons. The upper image shows an expanded view of the first seven peaks [14]. Reprinted with permission

The same group from the University of Michigan reported in 2005 about using two 3-m-long microfabricated gas chromatography columns in tandem [15]. The two columns contained different stationary phases, one of them having a nonpolar dimethyl polysiloxane stationary phase and the other a moderately polar trifluoropropylmethyl polysiloxane stationary

phase. Compounds have different retention factors in the two columns. One drawback of such a system is that some peaks that are separated in the first column can be recombined in the second column if the first compounds to elute are more retained in the second column. To overcome this problem, Lambertus and co-workers designed the system so that the first column could be bypassed for a certain time so that the flow was essentially put "on hold" for a few seconds in the first column. This allowed for greater control of the separations in the column ensemble. Figure 3 shows chromatograms of a 14-component mixture when operating with and without stopping the flow in the first column. Several components that otherwise coelute from the column ensemble can thus be separated very well by stopping the flow through the first column a few times. Because of the short separation columns, all of the 14 compounds are separated in less than 5 min using the stop-flow operation.

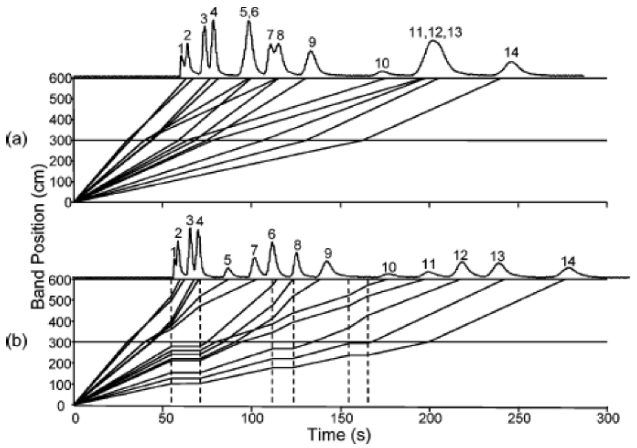

Fig. 3. Band positions in the two columns vs. time and the corresponding chromatograms. The band trajectories through the columns are indicated with lines. *Horizontal lines* mean that an analyte has stopped in the column. (**a**) No stop flow pulses. (**b**) Three stop flow pulses in the first column are indicated by *dashed vertical lines*. Analytes: (1) Methanol, (2) ethanol, (3) pentane, (4) isoprene, (5) propanal, (6) cyclopentane, (7) acetone, (8) 1-hexene, (9) isopropyl ether, (10) butanal, (11) benzene, (12) ethyl acetate, (13) cyclohexene, (14) heptane [15]. Reprinted with permission

Work is also being done on integrating several microfabricated elements to make complete gas chromatography systems. One such system was demonstrated in 2005 where a gas chromatograph containing a 3.0-m separation

column, a chemiresistor array for detection, a multistage preconcentrator/focuser, and a calibration vapor source were assembled and tested [16]. The system was able to analyze complex mixtures of common indoor air contaminants in the part-per-billion range in less than 90 s. Figure 4 shows the assembly of the μGC from the microfabricated parts.

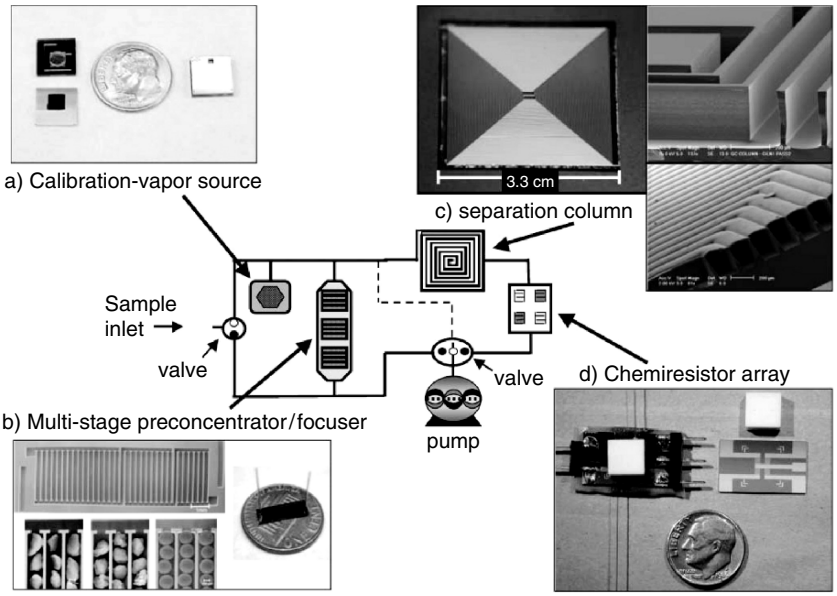

Fig. 4. A schematic of a miniaturized gas chromatograph. (**a**) Calibration vapor source, (**b**) Preconcentrator, (**c**) Separation column, (**d**) Chemiresistor detection array [16]. Reprinted with permission

Some groups have opted for using packed microfabricated columns to avoid the problem of uniformly coating the microchannels with a stationary phase, but this greatly increases the analysis time and reduces the efficiency of the separation. A separation presented by Zampolli et al. took 25 min to perform on a 75-cm column [17]. Figure 5 shows a photo of the microfabricated GC column packed with commercial (Carbograph + Carbowax™) stationary phase. The particles are between 150 and 250 μm in diameter.

Fig. 5. Encapsulated silicon spiral column packed with a commercial gas chromatography stationary phase [17]. Reprinted with permission

In 2006, Stadermann and co-workers from Lawrence Livermore National Laboratory and the University of Washington published an article about ultrafast gas chromatography on microchips using single-walled carbon nanotubes as a stationary phase. The carbon nanotubes were grown on the bottom of a 50-cm-long separation channel by chemical vapor deposition. The carbon nanotubes have a very high surface to volume ratio and therefore have great potential as a stationary phase. Furthermore, the microchip had integrated heaters that allowed for fast temperature programming. Separation of two 4-compound mixtures was achieved in less than 1 s when using an appropriate temperature gradient [18].

2.2. Pressure-Driven Liquid Chromatography (LC)

The first microfabricated liquid chromatography column was described and fabricated by Manz and co-workers in 1990 [19]. Their design was based on a 15-cm open tubular column that was 6 μm wide and 2 μm deep, similar to the "Stanford gas chromatograph" presented earlier, but no separations that were performed on the device were presented. The authors claimed that using microchannels for open tubular LC instead of glass capillaries is attractive because the possibilities offered by microfabrication allow for the reduction of the inner diameter of the chromatography column, reduction of the column length, and the integration of a detection sensor onto the chip. Open tubular LC columns offer relatively short analysis

times for a given performance. On the other hand, the main disadvantage of using open tubular system is the low capacity of the column as a result of the low surface-area-to-volume ratio and hence a low phase ratio (volume of the stationary phase to volume of the mobile phase).

The most common chromatography columns in LC to date are packed columns, where the packing consists of small porous particles throughout the entire lumen of the column resulting in a high phase ratio. Realizing packed columns in microfluidic channels is nontrivial, however, both from a purely practical point of view but also because retaining structures (e.g., frits) and nonuniform packings can reduce the separation efficiency [13]. Still, some groups have been successful in making packed microfabricated LC columns. Agilent Technologies currently sell a microfluidic chip for peptide analysis that integrates a packed LC separation column, a preseparation sample enrichment column, and a nanoelectrospray tip for introduction of the eluate into a mass spectrometer [20]. The microchip is fabricated by laser-ablating polyimide films, resulting in a 45-mm-long column with a 75 μm × 50 μm cross-sectional area. The separation and the sample enrichment columns are filled with a 3.5–5 μm stationary phase packing material. External pumps are used to drive the mobile phase, and a rotary switching valve is used to control flow between sample loading and elution/separation on the chip.

A system such as this is very useful for proteomic analysis where the proteins are obtained from 2D gel spots and diluted to low concentrations in microliter amounts of solvent. The samples are then concentrated in the on-chip sample enrichment column before they are separated in the chromatography column. Figure 6 shows base peak chromatograms for a tryptic digest of bovine serum albumin (BSA) separated on the LC microchip using an octadecylsilane stationary phase for five different flow rates. The separation times in these runs are quite long for a microchip separation, up to 40 min. The fluid velocity in the column is quite low because of the increased backpressure when using small particles as a stationary phase support in pressure-driven chromatography.

One way around having to rely on packed columns that is becoming increasingly popular is the use of porous polymer monoliths for a stationary phase. These types of stationary phases are made by mixing monomers with the desired properties (e.g., monomers with octadecyl side chains, cross-linking monomers, porogens, etc.), introducing this low-viscous cocktail into the channels and polymerizing them in situ. By controlling the monomer composition and the solvents, it is possible to control the pore size in the polymer so that the resulting polymer monolith still allows adequate transport of liquid through it. The monoliths have a phase ratio that is similar to packed columns. For added control over the monolith

formation, i.e., to make sure that it only forms in the separation channel and not in the rest of the microfluidic channels, a photoinitiator is used to start the polymerization. By masking all the channels except the separation channel, and then exposing the chip with UV light, polymerization will only occur in the separation channel. After polymerization, the unreacted monomers are removed from the channel network, only leaving a porous polymer monolith in the separation channel. Figure 7 shows a SEM image of a porous polymer monolith in a microchannel.

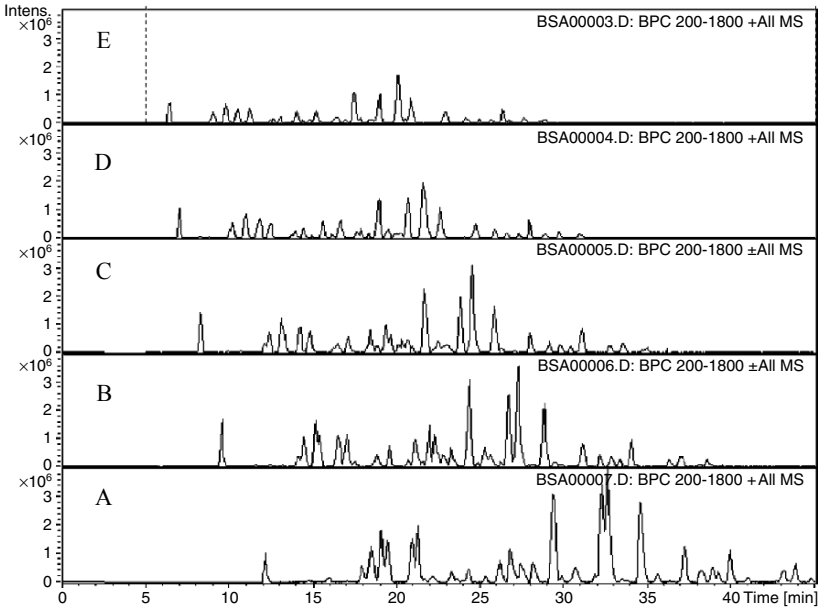

Fig. 6. Microchip LC separation of a tryptic BSA digest. Base peak chromatograms from the mass spectrometric data at different flow rates. (**A**) 100, (**B**) 150, (**C**) 200, (**D**) 300, and (**E**) 400 nL min^{-1} [20]. Reprinted with permission

An ion chromatography separation of four proteins on a microchip is shown in Fig. 8, where the stationary phase was a porous polymer monolith. The separation was performed on a 4.5-cm effective length column that was 40 μm wide. All of the analytes eluted within 1 min. The system used an external rotary valve for injection and also controlled the mobile phase gradient externally. Note that the peaks are very sharp due to the zone-sharpening effect of the mobile phase gradient [21].

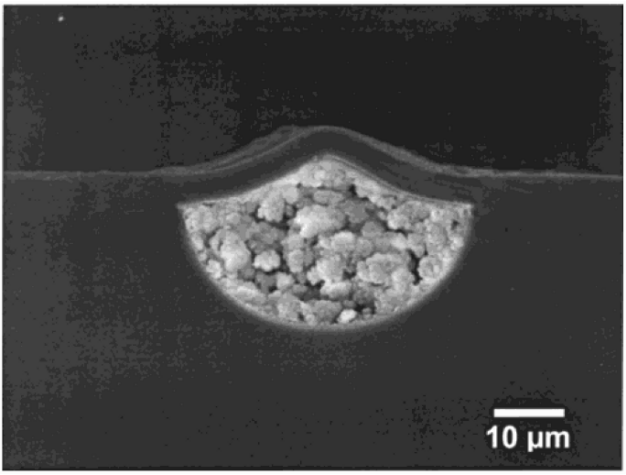

Fig. 7. Porous polymer monolith for anion exchange chromatography in a microfluidic channel (cross-sectional view) [21]. Reprinted with permission

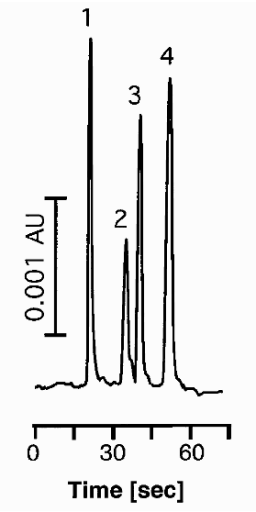

Fig. 8. Microchip anion-exchange chromatography of four proteins. The stationary phase is a continuous bed derivatized with alkylammonium moieties. UV-absorption detection at 230 nm. Sample: (**1)** myoglobin, (**2)** conalbumin, (**3)** ovalbumin, (**4)** trypsin inhibitor [21]. Reprinted with permission

In the case described above, the sample injection into the microfabricated LC column was done by using an external rotary valve, like the ones used in regular LC. This reduces the efficiency of the separation as the defined plug will start to disperse even before it reaches the chromatography

column, mainly due to extracolumn broadening in the transfer lines. To reduce this dead volume work has been performed on on-chip injection systems. In 2004, Reichmuth and co-workers from Sandia National Laboratories published an article on an on-chip high pressure picoliter injector for microchip applications [22]. Their design consisted of a mobile polymer monolith that blocked the sample inlet, and was then moved to inject sample into the separation column (see Fig. 9). The injection volume was controlled by the time the sample injection channel was open resulting in reproducible injection volumes down to a few hundreds of picoliters. The authors also demonstrated that this system was capable of rapid sample switching without any carryover [23].

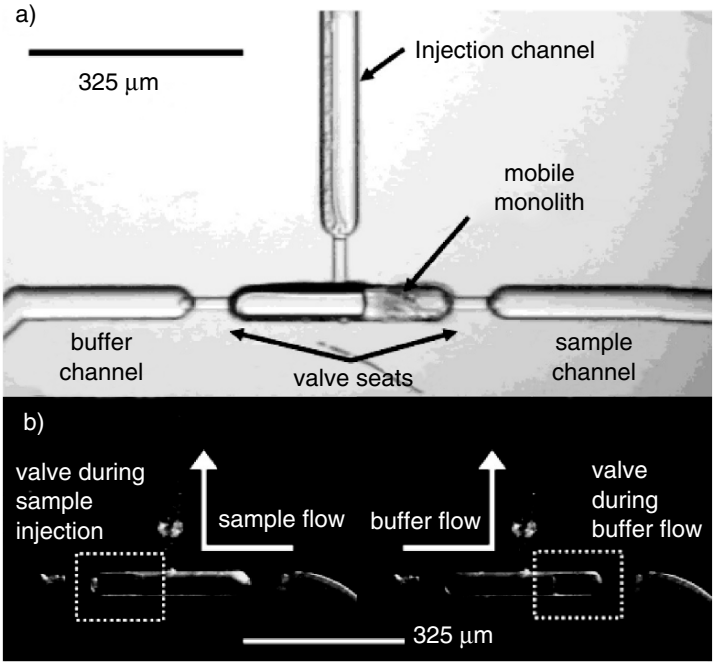

Fig. 9. (**a**) Photograph of a microfabricated injection valve. The mobile monolith plug blocks the sample channel, isolating it from the rest of the system. (**b**) Movement of the mobile monolith allows control of the flow into the separation column [22]. Reprinted with permission

While LC is a very well established and robust method, it is somewhat less suited for use in microfluidic systems. As the dimensions of the channels are reduced, the fluidic resistance increases dramatically making it harder and harder to pump liquid through the conduits by using a pressure

difference. Even so, in most cases, an external pump needs to be connected to the microchannels, but in order to make small, compact, and even mobile systems, a large pump to achieve high pressures is not feasible. Therefore, external miniaturized pumps, such as an electroosmotically driven pump [24], can be used to accommodate the size requirements. Another pumping method that has been suggested for liquid chromatography is evaporation-driven pumping, where the constant evaporation from the column outlet and the refilling of the channel by capillary forces will "pump" the mobile phase through the channel [25].

However, with these challenges in mind, most of the work on chromatographic separations in liquid phase in microchips has been directed toward capillary electrochomatography (CEC), where, instead of a pressure-driven flow, the electroosmotic flow generated inside the microchannels is used to transport liquids.

2.3. Capillary Electrochromatography (CEC)

Capillary electrochomatography has quickly established itself as the most common form of liquid phase separation investigated in microchips. It is a hybrid technique between capillary electrophoresis (CE) and liquid chromatography. The mobile phase is moved by an electroosmotic flow (EOF), as in capillary electrophoresis (see also the dedicated chapter in this book). The main separation principle, on the other hand, is again based on a specific interaction with a stationary phase in the separation column, just as in liquid chromatography. In the case of charged analytes, an electrophoretic separation based on their mass/charge ratio may be superimposed on the chromatographic separation.

One of the great advantages of CEC compared with LC is that complex fluid-handling protocols can be realized fairly easily in CEC for several channels by applying and controlling different voltages at all the inlets and outlets of the chip. Another advantage when using EOF instead of pressurized flow is that there is no increase in backpressure as the channels (or stationary phase packing) get smaller. This is because the EOF originates from the surface of the channels and on the surface of the stationary phase (beads/porous polymer monolith). This offers the possibility of using stationary phases with smaller particles (packed) or smaller pores (porous polymer) and thereby achieving higher separation efficiency.

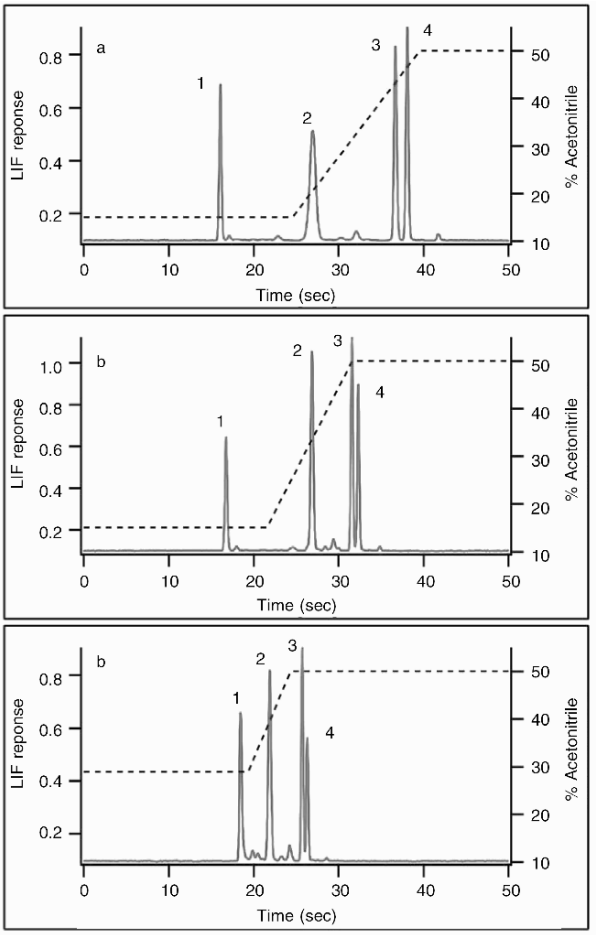

Fig. 10. Gradient elution in microchip open channel electrochromatography. Chromatograms (*solid lines*) for the separation of (1) Coumarin 440, (2) Coumarin 450, (3) Coumarin 460, and (4) Coumarin 480 using laser-induced fluorescence (LIF) detection. The LIF response is on the left axis, while the *dotted line* showing the acetonitrile concentration in the mobile phase relates to the right axis (**a**) Gradient from 15 to 50% in 15 s, starting 8 s after injection, (**b**) gradient from 15 to 50% in 10 s, staring 5 s after injection, and (**c**) gradient from 29 to 50% in 5 s, starting 3 s after injection. Adapted from Kutter et al. [27]. Reprinted with permission

Microchip CEC was first reported by Jacobson and co-workers at Oak Ridge National Laboratory in 1994. They used an open channel electrochromatographic approach (OCEC) where an octadecylsilane stationary phase was attached to the surface of a glass channel. They were able to achieve plate

heights as low as 5 μm for a retained coumarin dye [26]. The same group has since published many different variations and applications of OCEC. In 1998, Kutter et al. reported on using solvent programming on microchips in combination with OCEC [27]. Solvent programming or gradient elution means that the mobile phase composition is changed during the run to gradually affect the interactions between the analytes, the stationary phase and the mobile phase. This can, for e.g., be used to speed up later eluting analytes by increasing the concentration of an organic modifier (such as methanol or acetonitrile) in the mobile phase.

Figure 10 shows the effect of changing the acetonitrile concentration during a run using different gradients. By increasing the acetonitrile concentration and increasing the slope of the gradient, the peaks become much more closely spaced, while still retaining baseline resolution. This results in a faster separation where all components have eluted in around 25 s in Fig. 4c while in Fig. 4a the last analyte elutes at close to 40 s.

Gottschlich et al. presented a 2D separation on a microchip, where the effluent from a 25-cm spiral OCEC column was directly injected into a 1.2-cm CE column without the need for any interconnects or transfer line, and thus being dead-volume free. Figure 11 shows an image of the microchip used. Note that the spiral is one way of compacting a long separation length onto small footprint, while avoiding detrimental effects stemming from too tight and improperly designed turns [28].

The injections on the chip were controlled so that a TRITC-labeled tryptic digest of β-casein was first injected for 0.5 s into the relatively slow OCEC column (first dimension). Every 3 s a 0.2-s plug of the effluent from the OCEC column was injected into the second dimension (CE separation). This corresponds to about 9% of the total elution volume from the first dimension being injected into the second dimension, and as such does not constitute a comprehensive 2D separation, and loss of some information has to be accepted. An example of a 2D separation performed in the device is shown in Fig. 12.

One of the advantages of using microfabrication is that it is relatively easy to combine and integrate different functional elements on a microchip. This was demonstrated by Broyles et al. in 2003, when they presented a microchip that integrated sample filtration for particles above 1 μm, solid phase extraction that increased the detected signal over 400-fold, and an OCEC gradient separation on a single microchip [29].

Some groups have opted for packed columns for microchip electrochromatography. As mentioned before, the main problem associated with using packed chromatography columns on microchips is the ineffective packing one often gets on account of the polydispersity of the particles, insufficient

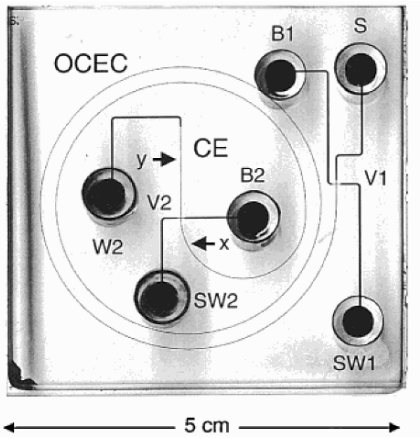

Fig. 11. Microchip for 2D separation. Black circles are chip reservoirs for the following: sample (S), buffer 1 and 2 (B1, B2), sample waste 1 and 2 (SW1, SW2), and waste (W). injection valves, V1 and V2, for dimension 1 and 2, respectively. The first dimension (OCEC) extends from V1 to V2 through the spiral channel, while the second dimension (CE) goes from V2 to the detection point y. Detection point x is used for the first dimension only [28]. Reprinted with permission

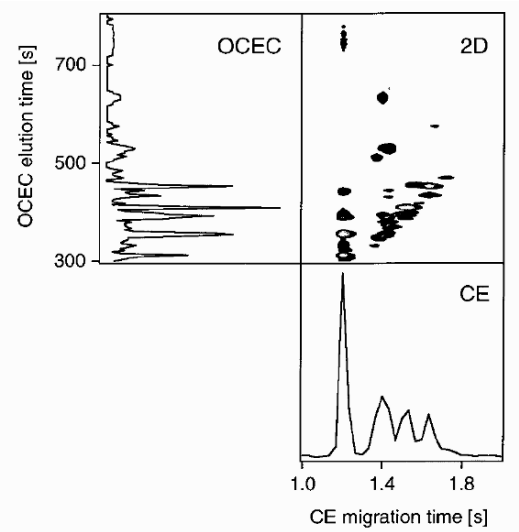

Fig. 12. 2D microchip separation of a TRITC-labeled tryptic digest of β-casein. The projections of the first dimension (OCEC) and the second dimension (CE) are shown to the left and below the 2D contour plot, respectively. LIF detection was employed [28]. Reprinted with permission

applicable packing pressures, or difficulties in filling the often noncircular cross sections of microchannels. Trying to improve the packing efficiency, Oleschuk and co-workers at the University of Alberta, Canada, published an article in 2000 where they described a method for trapping beads inside a microsystem using electrokinetic forces. They made a microfluidic channel with weirs at the start and finish of the chromatography column in order to retain the stationary phase beads while allowing mobile phase to pass (see Fig. 13).

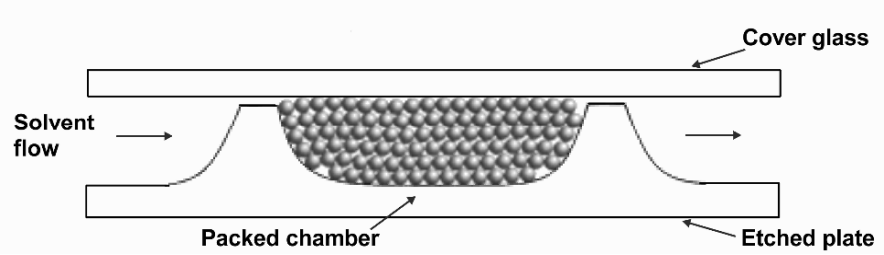

Fig. 13. Microchannel with retaining weirs to define a chromatography column [30]. Reprinted with permission

To fill the chamber with the stationary phase packing, an extra channel was fabricated on the microchip that connected to the chromatography column. Electrokinetic pumping was then utilized to direct the stationary phase packing material into the chamber. Figure 14 shows images of the packing process, where beads are "ejected" into the chamber until the entire chamber is filled with beads.

While this method is successful for making short chromatography columns, the authors note that 5-mm-long columns are difficult to prepare routinely [31]. Using these packed columns, a separation of three fluorescent dyes was performed yielding plate heights ranging from 2.8 to 4.1 μm. Other groups have tried different variations of this approach such as using a microchip fabricated in PDMS instead of glass and using pressure to fill the chromatography column with stationary phase beads [32]. This makes the fabrication of the microchip both faster and cheaper, but absorption of the analytes into the PDMS matrix limits the possible use of this polymer for CEC applications. A couple of groups have been studying methods to prevent analyte absorption into the PDMS matrix and, at the same time, increase the magnitude of the EOF by coating the microchannels with polyelectrolyte multilayers [32], using polymer grafting [33], or by mixing transition metal sol–gels into the matrix [34].

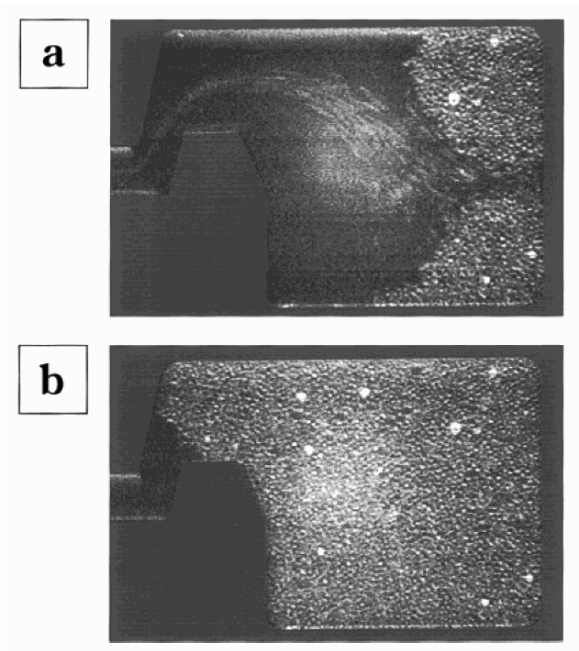

Fig. 14. Images of packing the microchip chamber. (**a**) Initial stages of electrokinetic packing. (**b**) Chamber filled with beads. The chamber is about 600 × 600 μm in size [31]. Reprinted with permission

A very different approach to emulate a "packed" stationary phase that can only be realized in microchips is to fabricate microchannels that contain support structures for a stationary phase. This was first introduced by He and co-workers at Purdue University in 1998 when they published an article on the in situ fabrication of stationary phase support pillars that were 5 × 5 × 10 μm separated by 1.5-μm rectangular channels.

Figure 15 shows the design of the inlet structure to a microfabricated chromatography column with microfabricated solid support structures in the separation channel [35].

These structures have the advantage that all dimensions and all geometrical aspects are precisely controlled by the design and the fabrication, thereby potentially reducing band-broadening effects as caused by nonideal packings. In particular, the widths of the channels between the solid support structures can be precisely controlled down to below a micrometer [36], which is expected to reduce the contribution to band broadening by mass transfer allowing faster separations without reduced efficiency. A stationary phase can be attached to the solid support structures through standard silane chemistry for glass and silicon microchips or via polymer

grafting for PDMS chips [33]. Separations using these pillar arrays have yielded plate heights around 1.7 μm, which are comparable to those of packed columns with particles less than 1 μm in diameter [37]. Figure 16 shows a CEC separation of a tryptic digest of bovine serum albumine (BSA) on a PDMS microchip with solid support structures in the chromatography column.

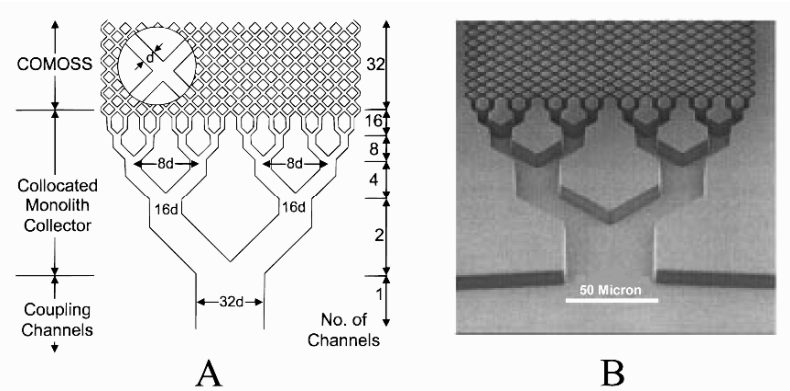

Fig. 15. (**a**) Design of the inlet channels to a chromatography column with solid support structures: The microchannels branch out into the chromatography column itself, retaining the cross-sectional area of the initial channel. (**b**) SEM image of the microfabricated structure [35]. Reprinted with permission

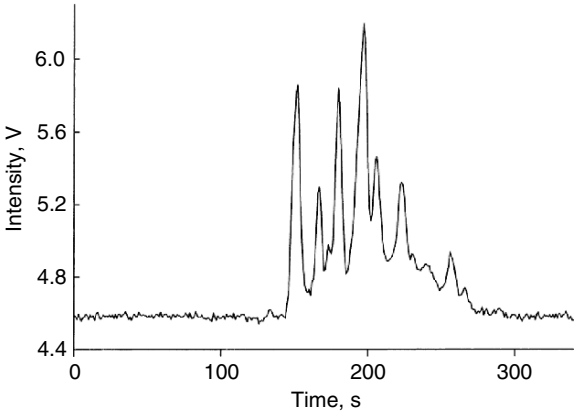

Fig. 16. CEC separation of a FITC labeled bovine serum albumine digest using a PDMS microchip with solid support structures in the separation channel. The channels are coated with a charged polymer for EOF support and C_{18} for chromatographic interaction [33]. Reprinted with permission

One of the drawbacks of micromachined solid support structures is the fact that they are, indeed, solid. Even though they help increase the surface-to-volume ratio of such a column compared to open channel chromatography, the phase ratio is still well below that of (classical) packed columns because the beads used for packing are typically highly porous. One recently suggested approach to increase the phase ratio for micromachined support structures is to make porous pillars using a microfabrication process sequence yielding the so-called "black silicon" [38], which resembles a grassy surface with silicon needles instead of grass blades.

Microchip CEC has also been reported with porous monoliths, as described for microchip LC earlier. Several groups have employed porous polymer monoliths in microchips [21, 39, 40]. One of the great advantages of using porous polymer monoliths is that there is a vast selection of monomers commercially available, and these can be mixed and matched to make a polymer monolith with desired and tailor-made properties. A typical monomer mixture for CEC includes cross-linking monomers to link together the polymer chains, a monomer with a strong acidic moiety for EOF generation, and a monomer with a side group that can be used as a stationary phase, such as lauryl acrylate [39]. Again, the monoliths can be prepared restricted to the separation channel by using a photoinitiator to start the polymerization and masking off all other channels during polymerization. This is usually an advantage as it keeps both the injection and the detection regions free of polymeric monolith. CEC separations of 13 unlabelled polycyclic aromatic hydrocarbons were reported by Fintschenko et al. in 2001 on a butyl acrylate-based polymeric monolith using UV laser-induced fluorescence detection. The separation was performed within 15 min, with 10 of the 13 analytes resolved and plate heights of around 5 μm (see Fig. 17).

Other groups have reported even faster microchip CEC separations using porous polymer monoliths. For example, a reversed phase separation of five peptides in 45 s [39] and a separation of uracil, phenol, and benzyl alcohol in less than 20 s [21] have been published.

A slight variant of the porous polymer monoliths are the porous sol–gel monoliths for CEC. These use an in situ polymerized sol–gel instead of organic polymers. The sol–gels are silica-based and have been reported to yield plate heights at around 5 μm [41, 42].

A further variant of using a porous polymeric monolith in a microchip was reported by Zeng and co-workers at the Chinese Academy of Science in 2005 [43]. They immobilized a molecule with chiral recognition abilities (γ-cyclodextrin) in the porous polymer monolith. This allowed the authors to separate enantiomers of two amino acids in less than 2 min (see Fig. 18).

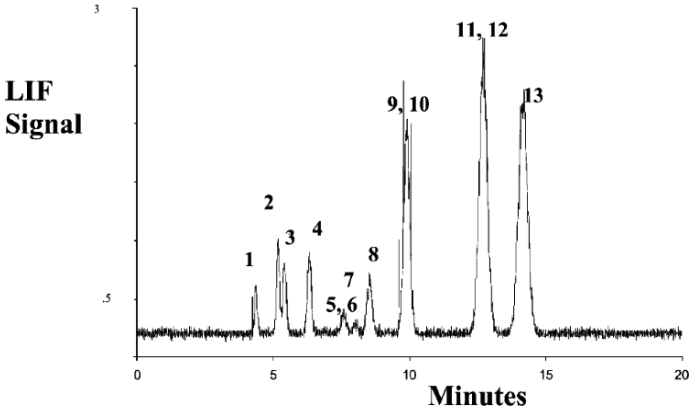

Fig. 17. Microchip CEC separation of 13 polycyclic aromatic hydrocarbons. LIF detection was employed with excitation at 257 nm [40]. Reprinted with permission

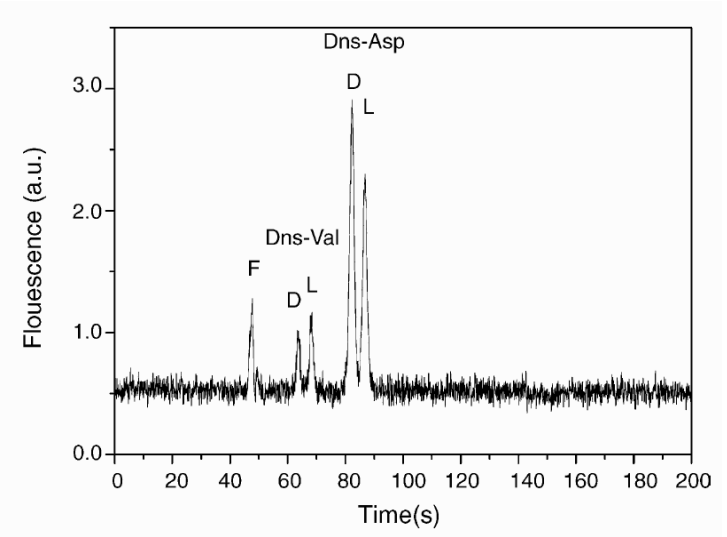

Fig. 18. Microchip chiral separations of FITC labeled Dns-valine and Dns-aspartic acid in a γ-cyclodextrin-modified porous polymer monolith. LIF detection was employed [43]. Reprinted with permission

2.4. Other Chromatographic Methods on Microchips

Shear-driven chromatography (SDC) is a variation of chromatography where flow is generated by a moving channel wall and the viscous drag exerted by this moving part on the mobile phase (see Fig. 19).

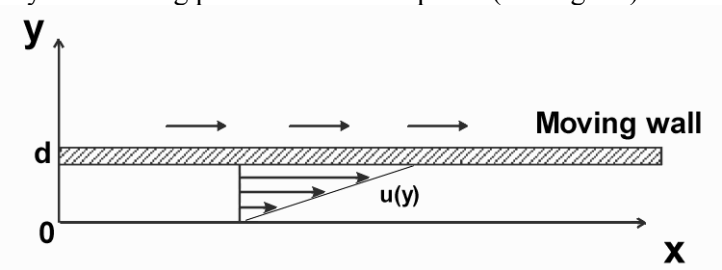

Fig. 19. Principle of shear-driven flow: The moving wall drags the mobile phase. The average flow velocity is half the velocity of the moving wall [44]. Reprinted with permission

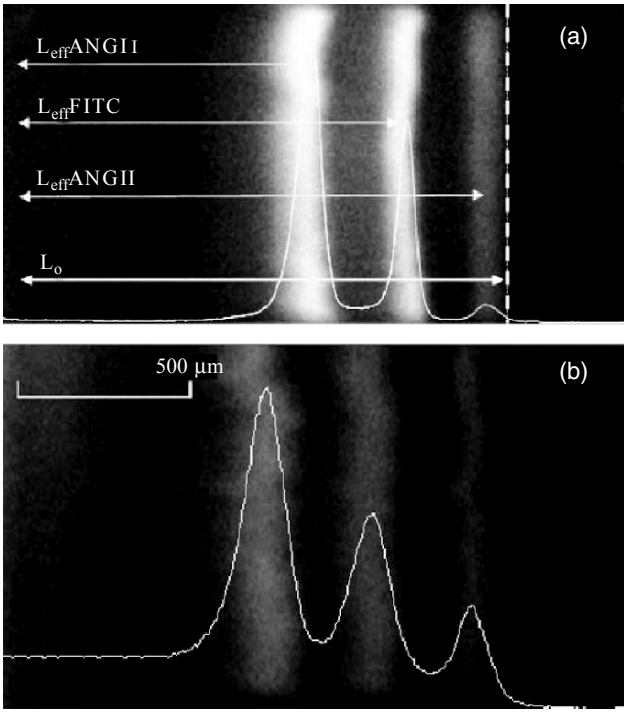

Fig. 20. Two SDC separations of FITC-labeled angiotensins. The moving wall velocity is (**a**) 2 mm s^{-1} and (**b**) 35 mm s^{-1}. The middle peak is unreacted FITC labeling reagent, while the first and the last peaks are the labeled angiotensins [45]. Reprinted with permission

The average flow velocity is equal to half the velocity of the moving part. Microchip shear-driven chromatography has been reported in channels that were 300–400 nm deep, where only the nonmoving part was coated with a stationary phase [45, 46]. Because of the short diffusion distances in such shallow channels, it is possible to achieve very low plate heights and very fast separations. Vankrukelsven et al. from Vrije Universiteit Brussel published an article in 2006 where they used SDC to separate two FITC-labeled angiotensin peptides with plate heights as low as 0.4 μm [45]. The separation was performed in less than 0.2 s. Figure 20 shows photographs of two SDC separations at different moving wall velocities.

SDC has also been reported in a microchip with a circular channel layout and a rotating moving counterpart. In essence, this yields variable length columns in the same system (and theoretically infinitely long columns), since the progress of the separation can be monitored for each revolution. However, a Fourier transform is necessary to deconvolute the signal for each of the analyte bands as the collected signal trace becomes increasingly complex for each revolution. When the analytes have completed the circular column length several times, lightly retained analytes are likely to have overtaken highly retained analytes [44].

Another chromatographic separation technique realized on microchips is size exclusion chromatography (SEC), which was demonstrated in 2003 by Baba et al. [47]. They used a nanostructured region that acted as a stationary phase, representing an artificial gel. The nanostructured region contains narrow 400-nm "gaps," which molecules can diffuse into and be held back before diffusing out into the flow again (see Fig. 21a, b). Smaller molecules (with larger diffusion coefficients) are more likely to diffuse into the narrow gaps and are therefore retained longer in the separation system. A separation of three DNA fragments with different sizes (10, 5, and 2 kbp) was demonstrated using such a device (see Fig. 21c).

Finally, we will briefly discuss the realization of Hydrodynamic Chromatography (HDC) on microchips. HDC is, despite its name, not strictly a chromatographic technique since it does not have a stationary phase. Nevertheless, it is employed to separate macromolecules or particles based on their size. The principle of HDC is to exploit the parabolic flow profile in pressure-driven flows and the fact that different sized molecules or particles only can "sample" a certain percentage of the flow velocity distribution, depending on how close their centers of mass can get to the channel walls. Additionally, for this to work, the height of the channel must ideally be not too much larger than the particles' dimensions. Then, larger particles, which cannot get as close to the wall with their centers of mass as smaller particles, will on average experience a larger velocity and thus be

separated from smaller particles [48–50]. For more details on this particular technique, the reader is referred to Chap. 12 in this book.

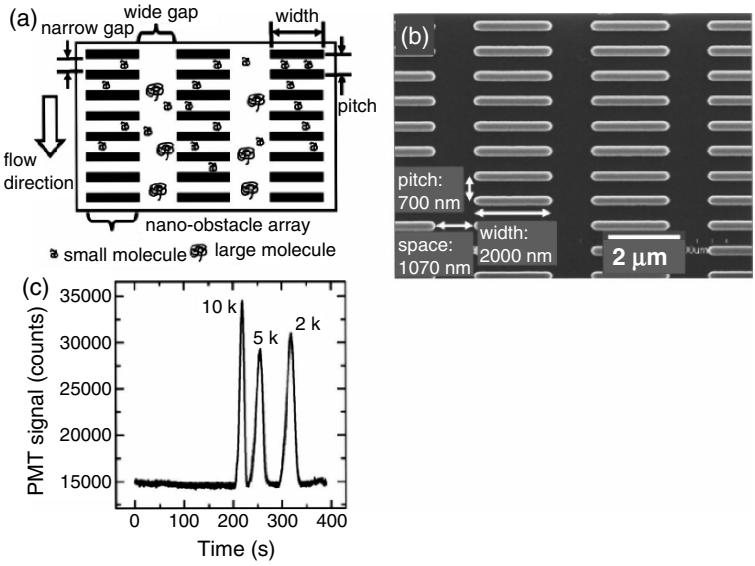

Fig. 21. (**a**) Design and principle of a nanostructured SEC column: Small molecules can diffuse into the small gaps and are retained, where as larger molecules flow through the wider gaps only and are hence much less retained. (**b**) SEM image of nano obstacles with a 700-nm pitch, the space between rows is 1,070 nm. (**c**) Microchip SEC separation of three DNA fragments with sizes of 2, 5, and 10 kbp, respectively, using a nanostructured stationary phase [47]. Reprinted with permission

3. Conclusions

Miniaturization offers a range of advantages and unique possibilities for realizing chromatographic systems on small scales. Fast and efficient microchip-based chromatography systems can become very powerful competitors or complementary partners to simpler sensor systems, with applications in all aspects of modern life, from food quality monitoring to medical diagnosis. Likewise, in more industrial settings, miniaturized chromatography systems will be employed for in-line process monitoring and high-throughput screening tasks. While the first commercial products using systems as described in this chapter are already appearing, the technical and scientific investigations and developments will continue. Even though there are ultimate

limits imposed by the sample (typically the amount of available molecules in a given volume), trends are emerging to further look into the possibilities of nanotechnology for the benefits of improved separation devices.

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