Chapter I

Introduction
Introduction

Over the last centuries the world has undergone breathtaking changes that are unprecedented in human history. Humanity has never been so far advanced in knowledge, in so many different areas, and over such a wide spectrum. We have progressed through the age of technology to the information and digital age. Driven by insatiable curiosity, we are gaining a lot of insight into biological processes in living systems, including the functions of the human body at different levels, which holds great promise for the improvement of public health. Moreover, with limited resources on a limited planet, we have become aware of the influence we have on our planet, and the environmental problems that arise as a consequence of our actions.

Further progress in improving the quality of human life on the one hand and controlling environmental change on the other is not possible without reliable and high-resolution instrumental methods. Today in many fields, such as environmental and food analysis, and the analysis of biological material in proteomics and metabolomics, scientists have to deal with samples that may contain literally thousands of constituents. This has generated a need for improving existing, or even creating new, methods for analysis of larger numbers of compounds in more complex samples in the most comprehensive way.

Nowadays, one of the most powerful separation techniques is liquid chromatography (LC), in which a dissolved sample flows through a column packed with a solid adsorbent. Each constituent of the mixture interacts differently with the column material, which leads eventually to different elution times from the column and, therefore, the separation of components. Liquid chromatography has assumed a very important position among the modern analytical separation techniques, mainly for the analyses of samples with low complexity (active pharmaceutical ingredients, food industry, etc.). However, LC often does not provide sufficient resolving power (the ability to distinguish different compounds from each other) for the separation of complex samples. For example, biological samples of interest in proteomics and metabolomics can contain thousands of components; in a more extreme example, samples that are analyzed in experiments investigating expression of the human proteome in different tissues may contain up to 200,000 components after digestion with trypsin. However, there are few examples to be found in the literature where calculated theoretical peak capacities (the maximum number of peaks that can be separated per single run) reach even a few thousand
peaks. Under typical circumstances in an LC separation, theoretical peak capacities tend to be on the order of 200, whereas the actual separation will yield a more modest number of peaks. Approximately 50 - 75 peaks are usually recorded, which means that observed peaks are likely to contain a number of overlapping single-component peaks.

A good alternative for improving the separation power of LC is to develop a multidimensional system that combines two or more separation mechanisms in series to significantly improve resolving power and achieve peak capacities that are greater than in one-dimensional LC.

**Basic principles of two-dimensional liquid chromatography**

In 1978, Erni and Frei introduced a two-dimensional liquid chromatography system, constructed by coupling two LC columns, for the separation of complex plant extracts. Their intention was to first separate samples on a gel permeation stationary phase, followed by the complete transfer of all the eluate coming from the first column or “first dimension” (1D) to a second column (second dimension, 2D) containing a reverse-phase stationary phase. The 1D eluate was collected in fractions and re-injected to the 2D for a second separation. Though both the separation and the set-up were imperfect, this early work is regarded as “pioneering” in the area of comprehensive LC, as the idea was to subject all of the 1D eluate to a second separation.

A certain level of automation was incorporated into this first 2D-LC system in the form of an 8-port switching valve equipped with two sampling loops of identical volume for sampling and storing eluate for transfer between dimensions. In 1990, Jorgenson and co-workers utilized comprehensive 2D-LC for protein separations. However, two-dimensional gas chromatography (2D-GC) had reached a higher degree of maturity by the late 1990s than 2D-LC, due to inherent advantages such as faster separation as a result of faster diffusion and mass transfer, easier coupling between dimensions and no need for thermal re-equilibration between successive second-dimension runs.

Two-dimensional LC has found its practical applications now, two decades later, as the required instrumentation nowadays is much more advanced. State-of-the-art 2D-LC exists in three forms: off-line, on-line “heart-cutting” (LC-LC) and comprehensive (LC×LC). In the off-line techniques, the fractions that elute from the first column first are collected off-line, with subsequent re-injection into the second dimension. This approach does not require additional equipment, as the effluent portions are simply collected, but it does take a lot of
work and time. In the on-line version of 2D-LC, two dimensions are coupled via an interface (also called “modulator”). In many cases, it consists of a switching valve with a varying number of ports and two identical sampling loops, connected to the valve. By switching back and forth between loops, these loops first collect, store and later re-inject the effluent from the first column (\(1^D\)) into the second dimension (\(2^D\)), where the second separation takes place. The process of sampling, storing and re-injection of small \(1^D\) effluent fractions into the second dimension is called modulation. The separation of fractions injected onto the \(2^D\) should be fast and in most cases complete before the next transfer occurs. In the “heart-cutting” mode, only one or a few chosen \(1^D\) fractions are collected and immediately re-injected into the second column for separation, while the remaining effluent is directed to waste.

![Figure 1](image)

In a comprehensive mode, the entire \(1^D\) effluent is transferred in fractions for analysis on the second column. Due to the different separation mechanisms applied in each dimension, poorly resolved peaks from one column may be completely separated on another column.
The entire 1D effluent is collected on-line in small-volume fractions, which are transferred one by one to the 2D column in multiple repeated cycles. Every second-dimension run is recorded by a detector as a separate chromatogram (for each individual 1D fraction). A fraction may contain just one component, or several components, as the case may be (Figure 1Ba). Thus, one sample component may be observed in several successive chromatograms, spanning over the width of one first-dimension peak (Figure 1B). All two-dimensional data, recorded by the detector, can be presented in the form of a colour plot (similar to Figure 2).

**Theoretical aspects**

In a two-dimensional separation process three steps can be distinguished: the first-dimension separation; sampling, storing and re-injection of small 1D effluent fractions into the second column (“modulation”); and the second-dimension separation. Each of these steps makes a contribution to the overall resolution of a two-dimensional liquid chromatography experiment.

The separation in the individual dimensions of the 2D-LC system is dictated by the same parameters as those in separations performed in one-dimensional systems. However, specific for the 2D systems is the need for selection of suitable “orthogonal”-phase-system combinations in both dimensions, and for selecting conditions for the fraction transfer between dimensions that result in as little loss of resolution as possible.

**Sampling rate (modulation time)**

The modulation interface determines whether the resolution obtained in the first separation can be maintained in the 2nd dimension and is hence the heart of any LC×LC system. The frequency at which the interface performs operations with each 1D fraction indicates how often the fractions are transferred into the second dimension (the so-called sampling rate or modulation time, $t_R$, Figure 1B). In practice, re-mixing or re-dispersion of the collected fraction before its transfer to the second dimension is the main reason for the resolution loss in overall LC×LC separation. The volume of the 1D fraction contributes to the total band broadening in the second dimension, which decreases at shorter modulation times (higher sampling rate). Hence, the sampling rate strongly affects the quality of the 2D separation, and a fast and reliable transfer of the 1D effluent is needed. Moreover, the internal volumes of all the components making up the interface determine the extra-column band broadening; as such, the volume of these components should also be minimized.
Choosing the appropriate sampling rate (number of samples per 1D peak, \(n_s\)) is a major decision that must be made when designing a comprehensive 2D-LC separation method.\(^6\) A fundamental rule (Murphy-Schure-Foley rule)\(^{17}\) for comprehensive 2D separation is to sample each 1D peak 3 or 4 times. The effect of different sampling rates on the resolution of peaks in close proximity to each other in 2D-LC is shown in Figure 2. Where the sampling rate (\(n_s\)) decreases from 4 (in this case the sampling time \(t_R\) is 30 s) to 1 (\(t_R=120\) s), three peaks that were observed at \(n_s = 4\) combine into a single peak. Furthermore, Murphy, Schure and Foley\(^{17}\) showed that even if the initial 1D peak is narrow, the effective width of the peak as it enters the second column will depend on the sampling rate.\(^6\) Recent research indicates that it is possible to simplify the technique and still obtain adequate resolution with only 2.5 - 3 cuts per 1D peak.\(^{18,19}\)

In practice, it is difficult to achieve both good resolution and high throughput. For that reason, the first-dimensional column is very often operated at a very low flow rate in order to sample a sufficient number of fractions across the 1D peak, which often leads to some broadening.\(^8\) Moreover, a high sampling rate requires that the second-dimension separations should not be longer than the duration of a modulation period to ensure an adequate number of samplings. Thus, the 2D column should be very short, which leads to a relatively low number of theoretical plates.\(^{15}\)
Figure 2. Simulated demonstration of the effect of the first dimension sampling time ($t_R$) and sampling rate ($n_s$) on the peak resolution when: (A) $n_s=4$, (B) $n_s=2$, (C) $n_s=1.5$ and (D) $n_s=1$ (modified from 6).

Over the last decades, many different interface configurations have been reported. In most cases, they consist of a two-position/10-port\textsuperscript{20-22}, several 6-port\textsuperscript{23,24} or a two-position/12-port valve\textsuperscript{25,26} having two sampling loops (with the same volume).\textsuperscript{25,27,28} One example of a 2D-LC set-up with an 8-port switching valve is presented in the Figure 3. While Loop 1 collects the 1D effluent, Loop 2 is simultaneously being emptied, transferring its content to the second dimension.\textsuperscript{15} The sampling loop can be replaced with a trap-column.\textsuperscript{26,27,29-31} In this case, focusing (pre-concentration) of the solutes occurs on a trap column prior to their analysis in the second dimension.\textsuperscript{8} The same principle is used for other reported configurations, but using two or more columns operating in parallel in the second dimension.\textsuperscript{28,31-33} Of course, such an approach would increase the amount of sample to be analysed. However, the interfaces with trap- and parallel-columns are difficult to implement in practice, due to the need for additional instrumentation, which increases the complexity of the system.\textsuperscript{8} For these reasons, the interfaces that contain a valve with loops are the most common in 2D-LC.\textsuperscript{34}
Orthogonality

In 2D chromatography, the orthogonality of the two dimensions (i.e. of the two separation mechanisms) is of prime importance. Orthogonality refers to the differences between the properties of the coupled dimensions.\textsuperscript{15} If the two separation mechanisms exhibit minimum correlation in separation selectivity, a 2D separation is considered to be orthogonal.\textsuperscript{1,8} Orthogonality not only depends on the chemical properties of the stationary and mobile phases (i.e. the structure, polarity, charge, etc. of the stationary surface and the eluent), but also on the properties of the solutes being separated (hydrophobicity, charge, etc.). Since solute properties will of course vary from one solute to the next, the universal orthogonal 2D-LC combination does not exist. Nowadays, a large variety of stationary phases is available. They differ in pore size, surface chemistry, support material used, etc. At the same time, properties of the mobile phase can be changed by modifying pH, adding ion-pair agents or adjusting the temperature.\textsuperscript{8,16}

(In)compatibility of the mobile phases

It is important to make an appropriate choice of both stationary and mobile phases, especially from the perspective of mobile-phase compatibility between dimensions, as even slight incompatibility may adversely affect the overall separation. Incompatibility can mean that either two liquid phases are simply immiscible, or a so-called solvent-strength mismatch is observed. The latter occurs when a ‘strong’ eluent having high elution strength in one mode is a ‘weak’ eluent having a low elution strength in the other mode. In both cases, not only
Retention selectivity may be significantly affected, but also band broadening and/or peak shape in the second dimension.

In order to maximize the reduction of the fraction volume to improve resolution as discussed above, the sample should be retained more strongly on the 2D column than on the 1D column. For this reason, the 1D mobile phase should preferably act as a weak solvent (having low elution strength) in the second column. In this situation, a transferred fraction is retained (focused) at the beginning of the second column in a more or less compressed narrow zone before its elution with 2D mobile phase. This effect, called “on-column fraction focusing”, improves resolution, peak capacity and detection sensitivity, due to suppression of the band broadening that occurs when the fraction is transferred to the 2D. However, in practice, it is rather difficult to achieve. For instance, the aqueous-organic mobile phases that are used in 2D reversed-phase (RP) - hydrophilic interaction liquid chromatography (HILIC) systems are miscible. However, the RP×HILIC combination still presents significant solvent-strength mismatch problems. In this case, an organic-solvent-rich 1D mobile phase would act as a strong solvent in a RP 2D, whereas a water-rich mobile phase used as a weak eluent in a RP 1D would act as a strong eluent in a HILIC 2D. This would cause reduced retention, increased band broadening and asymmetrical or even split peaks.

Moreover, if the solvents used as mobile phases are not completely miscible, serious difficulties could arise, resulting in some separation modes being almost impossible to combine. For instance, coupling normal-phase (NP) and reversed-phase (RP) liquid chromatography – a combination that in terms of orthogonality is very useful – is generally not easy to achieve due to mobile-phase immiscibility. In this case, if RP were to constitute the 1D, the high concentrations of water in the RP mobile phase would almost certainly deactivate the polar adsorbent of the 2D NP column, since NP utilizes non-aqueous mobile phase (e.g. n-hexane).

The design of the interface is of primary importance for systems in which the separation modes are difficult to combine. Over the last decade, a number of interfaces have been suggested, including interfaces incorporating trapping columns, or collection of low-volume fractions from a 1D capillary monolithic column. More sophisticated approaches utilize a vacuum-evaporation interface for on-line evaporation of the 1D solvents from the loop, or thermally-assisted modulation exploiting the influence of temperature on analyte retention. While each of these approaches has unique advantages for solving the problem
of mobile-phase incompatibility, these technologies are still not developed to the extent where they could be used universally in routine LC×LC analyses.

An approach that is more generally applied is the use of an additional solvent flow (so-called make-up flow) to allow modification of the solvent composition between dimensions by diluting the collected 1D fraction with a weaker 2D mobile phase. However, this requires a good mixing device at the interface between the two columns. Referring to the above discussion, in order to be able to cope with solvent incompatibility and efficiently perform all required manipulations with the 1D effluent at the interface between two dimensions, the mixing device must satisfy three strict conditions. First, it should provide fast mixing in-line at different ratios over a wide range of flow rates compatible with typical flow rates used in 2D-LC; thus, the efficiency of the mixing mechanism should not depend on the flow rate, under which the mixing is happening. Second, a mixer for modulation should have a small volume so as not to contribute to the extra column-band broadening. Finally, the mixer must be able to withstand brief pressure pulses of up to a few hundred bar, due to its connection to switching valves used to shuttle sample from the 1D to the 2D. This is why we chose to use small-volume microfluidic devices as mixers that are placed in the interface between two dimensions.
Microfluidics

Microfluidic technology is an area of scientific research which involves the manipulation of small (µL and nL) amounts of fluids in micrometer-size channels. It has received growing interest over the last twenty years, due to its promising implementation in both industrial and academic fields, especially in applications related to analytical chemistry, and cell or medical biology. Microfluidic systems are often called “miniaturized total analysis systems” (µTAS) or “lab-on-a-chip” devices. The main advantages of these systems include a significant reduction in the amount of samples, reagents and waste products, faster analysis, limited costs, good sensitivity, small size (portability), and minimal dead volume.\textsuperscript{43,44}

Nowadays, microchip technology has entered the arena of scientific research as an attractive tool to improve or even replace conventional ‘macro’ analytical techniques. The development of chip-based microfluidic devices for integration in LC or LC-MS systems has increased especially in the last decade\textsuperscript{45–50} as a result of the miniaturization trend in separation science. In addition to the many benefits of device miniaturization mentioned above, increased separation efficiency is an expected consequence of the incorporation of microfluidic components into multi-dimensional LC separation systems. Performance in many other applications can be enhanced by the increased surface-to-volume ratios of microchannels, which facilitate high-speed reactions or interactions due to the increased surface available. Processes can be run at length scales that are more relevant for normal biological conditions (e.g. microfluidic channels can mimic blood capillaries). Throughput (defined as samples per unit time) can also be improved if large numbers of samples can be processed in parallel.\textsuperscript{51}

Nowadays, there are a great variety of materials, fabrication methods, and techniques available for the development of microfluidic devices. The most important of these will be described in more detail below.

Materials for microfluidic-device fabrication

Because microfabrication techniques for the first microfluidic devices were adapted from the microelectronics industry, they were fabricated in glass and silicon using a combination of planar fabrication techniques (photolithography, thin-film metallization, and chemical etching).\textsuperscript{52,53} Both Si and glass possess important characteristics, such as chemical inertness and excellent thermal stability, and can be used if the application of high temperatures or organic solvents are required.\textsuperscript{54,55} Glass is an important material for the fabrication of
microfluidic devices due to its optical transparency, and the fact that it is available in various compositions (e.g. fused silica, Pyrex, soda lime glass). Moreover, it is widely used as a substrate for microchannels, as well as for device covers, often in combination with other materials, as it allows microchannels and their contents to be directly observed under a microscope.

The surface characteristics of oxidized silicon and glass can be beneficial for many applications, due not only to the chemical inertness of these materials, but also because of the possibility of chemically modifying surfaces using a host of different silane chemistries. The high electrical resistance of glass also allows the application of high electric fields for induction of electro-osmotic flow, an easily implemented mechanism for fluid propulsion in microchannels, However, devices fabricated in these materials are not always easily implemented for applications with living mammalian cells.\textsuperscript{55} The fact that glass and silicon are not gas-permeable, for instance, means that perfusion media must be pre-equilibrated with oxygen and other gases before introduction into a micro cell-culture device. Moreover, conventional optical detection methods cannot be used for devices fabricated in silicon, because silicon is opaque to visible and ultraviolet light.\textsuperscript{56} Besides, the fabrication of devices from these materials is a time-consuming process that requires a cleanroom environment.\textsuperscript{53}

Nowadays silicon and glass have largely been displaced by polymers (elastomers, such as PDMS\textsuperscript{1}) and thermoplastics (e.g. PMMA, COC, PC\textsuperscript{2}) that have advantages such as optical transparency, non-toxicity and lower costs. Besides, they are chemically quite inert (though they are susceptible to surface softening and swelling in certain organic solvents), and have good mechanical properties (they are not fragile).\textsuperscript{54,57} In general, the components required for lab-on-a-chip devices are easier to fabricate in elastomers than in rigid, thermoplastic materials. This is because the former materials can be easily cast in solution form onto molds to replicate microchannels.\textsuperscript{56}

Polydimethylsiloxane (PDMS), an elastomeric silicone rubber, has widened the possibilities for utilization of microfluidic devices and has sped up their development in the academic microfluidics community. This is due to its low cost, robustness and the straightforward fabrication by replication of devices that it enables.\textsuperscript{52,58,59} PDMS cures at low temperatures, is flexible (it is a soft elastomer), and is optically transparent down to 280 nm

\textsuperscript{1} PDMS - poly(dimethylsiloxane)
\textsuperscript{2} PMMA - polymethylmethacrylate; PC – polycarbonate; COC – cyclic olefin copolymer.
Microfluidic Tools for Multidimensional Liquid Chromatography

(which makes UV/Vis absorbance and fluorescence detection feasible). In addition, it is non-toxic (allowing cultivation of mammalian cells in unmodified devices), commercially available, reasonably inert from a chemical perspective, and durable. All of these qualities have made PDMS a material of choice for many microfluidic applications, especially in the exploratory stages of research projects involving device development.

Of course, some properties of PDMS may be disadvantageous for certain applications. For example, the elastomeric nature of PDMS may cause microchannels to expand or even tear at high flow rates or under high pressures. The utilization of PDMS is also limited by its incompatibility with many organic solvents. In addition, non-specific adsorption to the relatively hydrophobic PDMS surface may occur when working with biological samples, leading to fouled surfaces having undefined compositions.

Table 1. Comparison between the most used materials for fabrication of microfluidic devices.

<table>
<thead>
<tr>
<th>Material</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>chemically inert; excellent thermal stability(^{53})</td>
<td>Opaque to visible and ultraviolet light;(^{55}) fabrication is a time-consuming process that requires a cleanroom environment(^ {52})</td>
</tr>
<tr>
<td>Glass (pyrex and fused silica)</td>
<td>chemically inert; optically transparent; wide availability in various sizes and chemical compositions(^ {53})</td>
<td>relatively expensive material; device fabrication is a time-consuming process(^ {52}) requiring a cleanroom environment</td>
</tr>
<tr>
<td>PDMS</td>
<td>optically transparent; soft elastomer; ease in fabrication; gas permeability; inexpensive, biocompatible and non-toxic(^ {51,57,58})</td>
<td>not pressure-resistant due to elastomeric nature;(^ {58}) incompatibility with some organic solvents;(^ {61,62}) absorption of small molecules into the matrix;(^ {58}) modified surfaces are generally unstable over time as the modification wears off(^ {58})</td>
</tr>
<tr>
<td>Thermoplastics (PMMA, PC, COC etc.)</td>
<td>low material cost; can easily be adapted for mass production;(^ {63,64}) optically clear; non-toxic; excellent chemical inertness; superior mechanical qualities(^ {53,56})</td>
<td>surface chemistry control required; often incompatible with organic solvents and low-molecular-weight organic solutes;(^ {51}) generally incompatible with temperatures greater than 170°C(^ {55})</td>
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</table>

Thermoplastics are gaining more interest, due to the broad range of material parameters and surface chemical properties offered, allowing for optimal material selection and, thus, tailoring of a device to the required application. Compared to silicon and glass, they are

\(^3\) A polymer material that becomes pliable or moldable above a specific temperature and solidifies upon cooling.
less expensive and can be rapidly implemented in manufacturing processes for mass production. The need to control the chemistry of the polymer surface makes these materials a somewhat disadvantageous choice sometimes for microfluidic devices (compared to silicon or glass). Many thermoplastics also exhibit incompatibility with organic solvents, which tend to adsorb onto or absorb into the polymer substrate, creating some issues in real applications. Thermoplastics also generally cannot be used at high temperatures (greater than 170 °C), as they will tend to soften and deform.52

Table 1 summarizes the most-used materials for microfluidic devices together with their advantages and disadvantages.

Fabrication techniques
The selection of the microfabrication method is a crucial step in the development of any microfluidic system. This choice depends on the compatibility of the material with the (reagent) solutions used, as well as the requirements set for the final product, e.g. the feature resolution, thermal or pressure resistance, and the time available for device fabrication. The costs for device fabrication is also considered as an important factor, because for some fabrication methods the manufacturing costs for one device can be higher (e.g. due to the initial cost of making the molds) than for the mass production of this device for commercial purposes.

Today the most common fabrication techniques for microfluidic devices are replica molding (soft lithography),43,52 injection molding,67–69 hot embossing,70,71 and stereolithography.72 The first three approaches can be regarded as indirect fabrication techniques, in that the actual fabrication involves making a mold for replicating microchannels. Once the mold is made, polymer solutions are cast onto it and allowed to cure (as is done with elastomeric compounds) to form microchannels, or the mold is pressed into a hard polymer layer which has been softened at elevated temperature to form microfluidic features. In contrast, stereolithography involves the direct formation of microchannels through patterning in the center of a mass of light-sensitive material with a focused laser. Other direct fabrication methods also exist, such as laser micromachining (laser ablation),73,74 wet/dry etching after photolithographic patterning75 and micromilling.76 In all of these latter examples, microchannel formation is achieved by removing material from a substrate. An overview of these methods is given in Table 2 and they are schematically presented in Figure 4.
Figure 4. Overview of main fabrication methods for microfluidic devices with their advantages (+) and disadvantages (-). Modified from 59.

Certain methods are more suited than others to the rapid prototyping of devices, an important step in many projects involving the use of microfluidics for new applications. Injection molding and hot embossing can be considered as fast, but they are expensive methods for polymer-device prototyping, due to the high initial cost of making the molds. Having molds made can also result in significant waiting times of at least several weeks before a design can be tested. On the other hand, glass/silicon micromachining processes based on wet/dry etching create high-precision structures, but are technically demanding and time consuming. 59 Although it is possible to achieve the widest range of features for making complex 3D structures with micromilling, stereolithography (3D-printing) and laser ablation, these techniques offer relatively low resolution (around 20-60 µm), generate surface roughness, and yield limited numbers of produced devices, due to the inherent slowness of the sequential fabrication process. 59 Besides, 3D-printers with the highest resolution usually need supporting materials to fill the void spaces (i.e., a channel) during the printing process, and the removal of this materials from a very small channel (less than 60 µm in depth) is very difficult and sometimes even impossible process for designs with complex channel structures. 77

To date, however, the most common technique used for prototyping remains soft lithography, a rapid-prototyping process which is based on replica molding. As was mentioned above, it is this method that primarily makes use of PDMS. This is thanks to the extremely precise replication of all the features on a mold surface with resolution in
the nm range, but without the need for expensive equipment or advanced skills in microfabrication.\textsuperscript{78}

All the methods that are listed in Table 2, except stereolithography, suffer from one inherent drawback. They enable the creation of an open 2D channel network in the substrate surface that has to be hermetically sealed (closed or bonded to a second chip acting as a cover) in order to obtain a microfluidic channel. Bonded interfaces between chips tend to form a weak point for any high-pressure application, as it is typically the bond itself that fails first when higher pressures are applied. Developed more than a decade ago, Femtosecond Laser Irradiation followed by Chemical Etching (FLICE),\textsuperscript{79} also called Selective Laser-Induced Etching (SLE),\textsuperscript{80,81} has appeared as a novel powerful alternative approach for direct fabrication of complex 3D structures inside a solid transparent material, such as fused silica. Being a direct fabrication technique inside the solid piece of material, SLE provides an appealing solution for avoiding the chip-sealing step. The SLE technique also allows the fabrication of glass devices with higher resolution (10-20 µm) than is possible with wet etching, allowing the unique properties of glass (transparency, rigidity, inertness and so on) to be exploited in devices with finer structuring.
Table 2. An overview of the most used methods for microfluidic devices fabrication.\textsuperscript{59,76}

<table>
<thead>
<tr>
<th></th>
<th>Hot-embossing</th>
<th>Injection molding</th>
<th>Micromilling</th>
<th>3D-printing (stereolithography)</th>
<th>Wet/dry etching</th>
<th>Laser micromachining (laser ablation)</th>
<th>Rapid prototyping (soft-lithography)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type</strong></td>
<td>Indirect</td>
<td>Indirect</td>
<td>Direct</td>
<td>Direct</td>
<td>Direct</td>
<td>Direct</td>
<td>Indirect</td>
</tr>
<tr>
<td><strong>Materials</strong></td>
<td>polymeric materials</td>
<td>thermoplastics</td>
<td>metals and plastics</td>
<td>Photo curable polymeric resins, ceramics</td>
<td>glass/silicon</td>
<td>ceramics, metals, and polymers</td>
<td>elastomers; epoxy resins</td>
</tr>
<tr>
<td><strong>General</strong></td>
<td>polymer substrate is softened at elevated temperature and then pressed against a mold under pressure to transfer the desired features from the mold to the polymer</td>
<td>melted polymer is injected into a microstructured mold and thendemolded after cooling</td>
<td>microsize features are structured by removing bulk material using special cutting tools (end mills)</td>
<td>3D parts are created in bulk photosensitive material using a light source (\textit{e.g.} a laser)</td>
<td>remove the substrate material from a wafer by means of etching with abrasive chemical or plasma gases</td>
<td>a high-powered pulsed laser is used to remove material from the polymer substrate (through a mask or by using a laser direct-write process)</td>
<td>a prepolymer solution is cast onto a mold and is cross-linked with heat or ultraviolet (UV) light; the resulting polymer is peeled off the mold</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>fast; high-throughput; precise; rapid replication of small (low micrometer) to medium-size features with high aspect ratio; mass production</td>
<td>Fast, high-throughput when the final design has been established; enables complex 3D geometry; highly automated</td>
<td>fast transfer of designs into prototypes; cost-accessible; enabling complex 3D geometry</td>
<td>enables complex 3D features (accuracy of 20-60 (\mu)m) that may be impossible with other methods</td>
<td>enables small features with good resolution</td>
<td>cost-accessible; enables complex 3D-multilayer structures; mass production</td>
<td>easy; economical; fast; high resolution (few nm); possibility to create 3D features</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>expensive due to the initial cost of making the molds; restricted to thermoplastics, difficult to fabricate complex 3D structures; relatively long cycle times</td>
<td>expensive due to the initial cost and time of making the molds; limited to thermoplastics</td>
<td>limited throughput due to the inherent serial nature\textsuperscript{59}; low resolution (around 100 (\mu)m); surface roughness</td>
<td>expensive equipment and material; limited material availability; materials are brittle</td>
<td>technically demanding; time-consuming</td>
<td>limited number of produced devices due to the inherent feature of the sequential process; low resolution (~50 (\mu)m); surface roughness; the surface chemistry of the final products is very different due to laser treatment</td>
<td>devices are vulnerable under higher applied pressures due to microchannel deformation</td>
</tr>
</tbody>
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Mixing at the microscale

As described above, miniaturization provides many attractive features that separation science can use for its own benefit. However, some phenomena that are not very relevant in the macro world, play an important role in micrometer-sized devices. For example, on a large scale, fluids mix convectively resulting in turbulent flow patterns (think here of milk when it is swirled into coffee). In this example inertia is more important than viscosity, which is true for most fluids in the macro world. However, at the microscale this situation is reversed, and viscosity dominates in microchannels, leading to laminar flow due to the small masses of fluids involved. This means that turbulence tends not to be exhibited in microsystems, except at flow rates which are so extremely high that many devices would not survive the high pressures generated as a result. When two fluids meet in the microchannel under laminar flow conditions, they flow side-by-side, without agitation or disruptions. This makes mixing at the microscale a challenging task. In such systems, the mixing can be achieved only by diffusion, which is a passive and slow transport process where molecules of two fluid streams move across their interface.

To overcome the problem of mixing at the microscale, a large number of micromixers have already been developed. All of these work on the fundamental principle of increasing contact areas between the solutions to be mixed, so as to reduce distances that molecules need to diffuse to achieve mixing. Micromixers can be divided into two big groups, comprising either passive or active mixers. This classification is based on the mechanism by which the solution interfaces are disrupted to achieve larger contact areas. In active mixers, the need for integration of elements for transferring energy from an external source into the mixing chamber complicates the fabrication process, limiting the implementation of such devices. In addition, the external forces involved in these mixers can negatively influence the samples studied (e.g. acoustic waves can generate heat, which could lead to unwanted reactions or damage of biological samples). Passive micromixers, on the other hand, generally function either by splitting solution flows into multiple thinner streams in a branched channel network, or by placing fixed obstacles in the flow to perturb laminar flow patterns. This makes passive micromixing, which requires no moving parts and, thus, no external energy (other than that required to displace solutions through microchannels), a more preferable choice for many applications. The reader is referred to Chapter 2 of this thesis for a more in-depth discussion about mixing on the microscale in micromachined devices.
Scope of the thesis

In this thesis, we have aimed to improve the performance of two-dimensional liquid chromatography by using microfluidic devices fabricated by different microfabrication techniques. We developed a small-volume microfluidic mixer that was implemented in the interface between two columns in a 2D-LC instrument. The problem of the mobile phase incompatibilities between dimensions was addressed by fast in-line mixing of the 1D effluent with a weaker eluent inside the micromixer before it reached the second column. Because of the small inner volume of our device (< 5 µL), we did not introduce extra dispersion to the 1D effluent fraction.

In Chapter 2 we give a broad overview of already existing micromixers based on chaotic advection, as well as chaotic advection combined with other principles, that have been proposed over the last decade. We have emphasized the link between channel geometry, operating flow conditions and the mixing mechanism adopted. We also describe the most common application areas of passive chaotic micromixers using real examples. We discuss the connection between channel geometry and possible areas of application under different flow conditions, as this influences mixing efficiency.

In Chapter 3 we describe the development of microfluidic chaotic mixers with a small volume for future applications in two-dimensional liquid chromatography. The PDMS micromixer contains staggered herringbone grooves with an optimized geometry for fast modification of mobile phases at different flow-rate ratios (1:2, 1:5 and 1:10). The microchannel is 5 cm long and complete mixing is achieved within the first 3 cm of the channel. The mixing is efficient over the whole range of flow rates tested (4-1000 μL/min).

The research described in Chapter 4 was aimed at the fabrication of a pressure-resistant microfluidic mixer inside a solid piece of fused silica using Selective Laser-Induced Etching (SLE). We report a chip containing herringbone grooves for chaotic advective mixing in a channel with lengths up to 33 mm fabricated using SLE. The pressure tests showed that fused silica chips can withstand pressures up to 85 bar.

In Chapter 5, we successfully implemented a microfluidic micromixer in the interface of a two-dimensional liquid chromatograph for analysis of real samples. For this research we used a microfluidic mixer with herringbone grooves fabricated in COC using micromilling. Using a custom-designed, robust, low-dead-volume interface, a chip was directly coupled to
the chromatographic equipment. This design could withstand pressure pulses up to 150 bar. A microfluidic mixer was implemented in a 2D HILIC×RP-LC system and an improved separation of nylon polymers was obtained compared to the system without a mixer.

In Chapter 6 we summarize and discuss the findings of the research presented in this thesis. We present future perspectives on the use of microfluidic technology for improving conventional multidimensional chromatographic techniques.
Microfluidic Tools for Multidimensional Liquid Chromatography

References

Introduction

Introduction


