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Review

Liquid phase chromatography on microchips

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ABSTRACT

Over the past twenty years, the field of microfluidics has emerged providing one of the main enabling technologies to realize miniaturized chemical analysis systems, often referred to as micro-Total Analysis Systems (uTAS), or, more generally, Lab-on-a-Chip Systems (LOC) [1,2]. While microfluidics was driven forward a lot from the engineering side, especially with respect to ink jet and dispensing technology, the initial push and interest from the analytical chemistry community was through the desire to develop miniaturized sensors, detectors, and, very early on, separation systems. The initial almost explosive development of, in particular, chromatographic separation systems on microchips, has, however, slowed down in recent years. This review takes a closer, critical look at how liquid phase chromatography has been implemented in miniaturized formats over the past several years, what is important to keep in mind when developing or working with separations in a miniaturized format, and what challenges and pitfalls remain.

Contents

1. Introduction

Giddings is frequently cited for his statement that “separation is the art and science to maximize differential (separative) transport and minimize dispersive transport” [3], and while this was formulated well before the advent of microfluidics and miniaturized chromatographic systems, it still holds true. We are well advised to take Giddings’ statement as a strong guiding principle when designing and developing separation systems on microchips. Quite often, a less than satisfying separation performance of a miniaturized chromatographic system can be traced to a serious design flaw, wrong operating conditions, or generally a diffuse understanding of some of the fundamentals of separation science, as, e.g., outlined in the remainder of Giddings’ treatise [3].

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In extension of this, we must always critically ask ourselves whether a given separation problem is indeed best tackled by a miniaturized system. The initial and most stormy phase in the development of miniaturized separation systems, where it was important to show that (a) traditional separation methods could successfully be ported to the miniaturized (planar) format, and (b) entirely new separation approaches exploiting physics at the micro and nanoscale could be realized, is essentially over. We have now entered a phase of pruning, improving, fine-tuning, making practical, commercialising, and introducing miniaturized separation systems into larger workflows. We will touch upon most of these aspects in this review article. As a consequence of this development, it is important to clearly establish the benefits that come with miniaturization. Beyond the continued exploitation of micro and nanoscale physics and the potentials offered by microfabrication, it is imperative to demonstrate significant improvements in the typically used figures of merit (analysis time and throughput, separation performance (plate numbers, peak capacity), sample volume, limit of detection) as compared to more traditional solutions to justify the need for miniaturized separation systems. Improvement, in this sense, should really exceed one order of magnitude to make an impact.

This review is not intended to give a comprehensive overview of the entire field of miniaturized separation systems. Instead, it will focus on highlighting and critically evaluating important aspects and recent developments within the last several years in the area of liquid phase chromatographic systems, and in particular systems with a true stationary phase (as opposed to, e.g., pseudo stationary phases [4]). Electrophoresis-based separation methods and separation methods using non-chromatographic principles (e.g., deterministic lateral displacement devices) are not covered here. For recent reviews on similar and related topics the reader is referred to these publications [5–7].

2. Chips and capillaries

The advent of microchips developed for chromatographic separations in a planar format with all the entailing potential benefits has not stopped the evolution and progress of capillary-based chromatographic systems. If anything, microchips might probably have boosted research activities related to improving and developing chromatography in more classic formats even more. While the capillary format and the planar chip format could be considered competitive, there is also a large degree of cross-fertilization, which moves the entire liquid chromatographic development forward. Consequently, it is almost impossible to "just" look at what is going on with respect to chip-based chromatographic systems, because important work in, e.g., synthesis of novel stationary phase materials or understanding of topologies of particulate or monolithic stationary phases is just as crucial for improved chip-based separation systems.

The advantages of miniaturization and the effects of scaling, also for (chromatographic) separations, have been argued for and discussed many times in the literature and need not be repeated here [1,2,8,9]. One important effect of miniaturization is, however, that there is very little difference in what separation performance can potentially be obtained whether one is driving the chromatographic system electrokinetically or via pressure differences, as the short diffusion lengths tend to make diffusion a fast enough process to quickly equal out distortions stemming from parabolic flow profiles. This was already pointed out early on as well as experimentally proven by Hjertén and co-workers [10], even though later computational fluid dynamics studies by van Theemse et al. questioned this conclusion again [11]. Still, under the appropriate operating conditions and using appropriate channel and stationary phase geometries and topologies, the often pre-conceived notion of pressure-driven flows leading to increased band broadening needs to be re-considered. Nevertheless, until very recently, the vast majority of miniaturized chip-based chromatographic systems relied on electrokinetic means to drive the mobile phase through or by the stationary phase. The main reason for this lies in the fact that it is simply much easier to work with electrokinetic flows on microchips. Electrodes and a power supply will usually suffice to generate flow, whereas using either micromachined or external pumps or pressure sources, and the resulting necessary plumbing and connections, usually is much more challenging from an engineering point of view. Also, the planar format of most chips and the available connection technologies are often not well suited to be used with the high pressures required for appropriate operation of pressure-driven separations on chips. This is, in fact, aggravated by further miniaturization, where channel dimensions are further reduced, smaller particles are used for packings, or longer channels are designed to achieve a necessary separation performance. Jacobson et al. showed in a model calculation that as one is decreasing channel dimensions (to achieve faster separations) less and less voltage is required to obtain a certain separation performance if done electrokinetically, while ever increasing pressures need to be applied in the pressure-driven case [12]. While it could be argued that this can be overcome by proper engineering, it already points strongly towards electroseparations as being the most likely candidate for fast, reliable, portable and remotely operating miniaturized separation equipment, just because they can potentially be run on 1.5 V batteries. Clearly, though, this does not pre-empt the possibility to utilize pressure-driven separation systems for a number of other applications. After all, the big advantage of pressurized flow is that it is largely independent of the chemistry of the separation system (mobile and stationary phase, as well as sample constituents), which is not true for electrokinetic flow. For example, if you depend on a sufficiently strong electroosmotic flow (EOF) as a means for bulk transport then you typically have to operate at higher pH values. This, in turn, can seriously limit the types of chromatography that can be implemented, such as, for instance, hydrophilic interaction liquid chromatography (HILIC), which is mostly run at low pH values.

In the following, I would like to go through the main important aspects that should be kept in mind when designing or working with miniaturized liquid phase separation systems, and offer the reader some food for thought as well as starting points for further, more in-depth reading.

3. Formats, channel sizes and geometries

The planar format is the dominating format for microfluidic separation devices. In this context, it is interesting to note that the word "microfluidic" often is understood synonymously with "microchip-based", but in the literature it is sometimes also used for separation systems based on other formats. It is recommended to think of microfluidics as an enabling technology, which is used extensively, but not exclusively, by microchip-based devices. The planar format is a result of the commonly employed machining methods used to fabricate microchip devices. Here, techniques such as photolithography, etching, deposition, ablation, and embossing, are used to produce channel networks in a number of substrates, with geometries closely dictated by the possibilities and limitations of these production techniques. The resulting channel size, shape and, in particular, the aspect ratio are important and can have quite a pronounced impact on the separation efficiency, as well as other performance parameters of the entire lab-on-a-chip system.

A reduction of the channel dimensions (its width or depth or both) is usually associated with reduced diffusion lengths to (a)

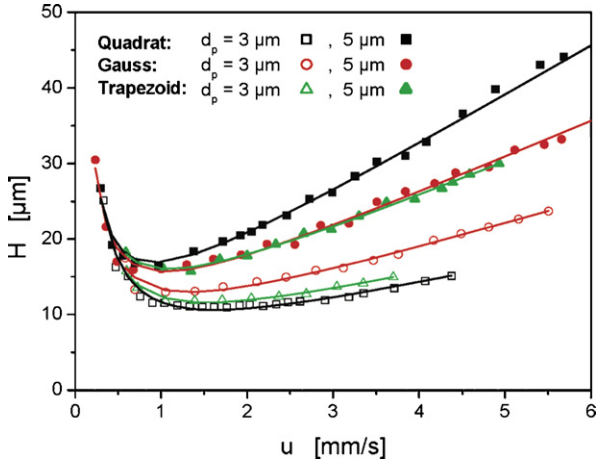

Fig. 1. Experimental plate height curves for microchannels with quadratic, Gaussian or trapezoidal cross sections and packed with 3 or 5 µm porous C18 particles; mobile phase: ACN:water 70:30 (v:v); analyte:*n*-pentylbenzene with a retention factor of 3.9.

From [15], used with permission.

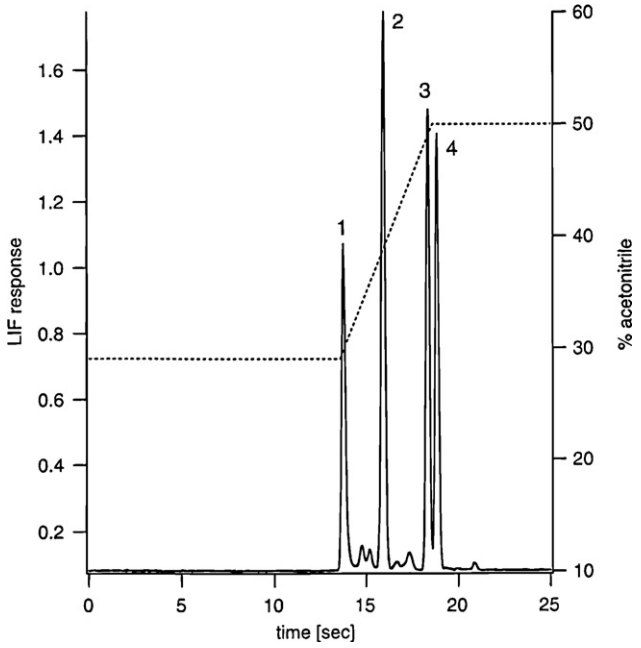

Fig. 2. Fast open channel electrochromatography of four coumarin dyes (here, with a gradient elution). The channel used was 5.2 µm deep and coated with a C18 silane; separation could be achieved within 20 s at a field strength of 700 V/cm.

From [22] with permission.

faster reach stationary phase molecules on the walls (mainly in open channel variants) and (b) to faster equal out flow path differences across the channel lumen (mostly for particulate packings, monoliths and pillar arrays (see Section 4)). For packed channels, the ratio of the channel width/depth to the particle diameter should also be considered [13] to avoid what is often referred to as the "wall effect" where, e.g., the quality/density of the packing or the flow behavior can be very different than what is found in the bulk of the channel/packing. This seems to be more pronounced for the typically used ratios in microfluidic systems (around 10, i.e., channel width or depth is about 10 times the used particle diameter or equivalent feature size), whereas for small values (particle size is of the same order as channel depth) or large values (very small particles or relatively large channels) the effects are much less pronounced, either because the wall and the bulk region become very similar (in the former case), or because the wall region only constitutes a small part of the entire channel cross section (in the latter case).

The influence of the conduit cross section on the flow behavior, and, in fact, on the band braodening is well known for open channels [14], but has almost gone neglected for packed channels. In open channels, slight variations in channel width can lead to pronounced differences in flow resistance across the depth of the channel and thus induce dispersion. This is especially important for deep, narrow channels (e.g., in pillar arrays) and great care in the fabrication is necessary to avoid large deviations. Recent investigations by Tallarek and co-workers demonstrate the influence of various cross-sectional shapes on the separation performance of packed channels [15] (see Fig. 1). Here, the shape can have an impact on both the achievable packing densities (and hence the degree and distribution of "order" in the packing), and also on how much of the lumen is fluidically accessible as corner regions tend to constitute regions of more stagnant flow.

The capillary format (cylindrical cross section) is not typically used in microchip systems, even though capillaries have been coupled to or embedded in microchips, e.g., as spraying emitter for coupling to a mass spectrometer [16]. Despite the occasional marriage between capillaries and microfabricated channels, the planar format is overall more flexible and more conducive to coupling several different functionalities together, e.g., a sample cleaning or enrichment step prior to a separation (see Section 7). In all fairness, it should be mentioned that the absence of the cylindrical cross section in the planar format is mainly because of the associated difficulties and challenges to fabricate those on microchips – other cross sectional shapes (including semi-cylindrical shapes) are much easier to obtain.

Another important aspect of the format (tying in with the material properties, see Section 5) is the impact of Joule heating on the separation performance for electro-driven methods. Planar systems can provide larger thermal mass to get rid of excessive heat more effectively, whereas traditional capillary systems are more amenable to be fitted with a liquid cooling system [17]. In any case, the format and the cross sectional shape of the channel do matter, but a clear guideline is hard to establish as the various processes associated with Joule heating cannot be de-convoluted easily.

4. Stationary phases: physical and chemical realization

There are four main variants to introduce a stationary phase into a micromachined channel: (a) coating only the wall with the chromatographically active moieties, leading to what is the equivalent of open tubular chromatography, or, here, open channel chromatography; (b) using particles modified with the retentive material to prepare packed beds of these particles (a variant of this uses self-assembly of particles inside the channel); (c) creating monoliths, i.e., a porous scaffold, which either already includes the retentive species or can be grafted with it later; and (d) micromachined pillar arrays (in the literature also often called collocated monolithic stationary phase supports, COMOSS), whose surfaces can then be modified to offer retention. In the following, to avoid confusion, only stationary phases of type (c) will be called "monoliths". There are significant differences between these four main flavors, and some of them are almost religiously advocated over others. After discussing the important features of each variant, I will present a short comparison at the end of this section.

4.1. Open channel chromatography [18–22]

This is the simplest way to perform chromatography on chips (Fig. 2). Here, the material of the channel walls (e.g., glass or a

polymer) is directly chemically modified or derivatized to yield the desired retentive ability. Often, classical silane chemistry is used, or sol–gel approaches, or grafting techniques for polymer materials. The big advantage of the open channel format is the ease of preparation and the relatively small pressures that are sufficient for driving the mobile phase through. Even when going to channel depths of 5 μm or less (which is important to get short diffusion lengths and thus a fast mass transfer to the stationary phase), the necessary pressures are acceptable. Kato et al. recently demonstrated open channel chromatography in submicron channels and could perform separations with pressures in the range of 1–3 bars (14–43 psi) [18]. An alternative to using pressure is to use shear forces between a stationary and a moving plate to push the mobile phase [21,23].

The main disadvantage is the limited loading capacity of the stationary phase. Since only small amounts of analyte can be injected to avoid artifacts from concentration or volume overloading, this has immediate consequences for the detection, where often only the most sensitive methods (e.g., fluorescence) can be used together with open channel chromatography. A so far little used way around some of these challenges lies in the possibility to work with thick film stationary phases or the equivalent of porous layer open tubular (PLOT) approaches. To my knowledge, there are to date basically no mentions of these possibilities for chip-based separation systems in the literature; however, Faure mentions a few related alternatives in her recent review [6].

4.2. Packed tubes or channels [24–27]

This variant is simply an extension from the "classical" stainless steel packed HPLC column (Fig. 3). The immediate big advantage is that basically all the many particulate materials that have been developed over the years for LC can be adopted to the planar format. This wealth of available packing materials means that stationary phase chemistries for basically all possible scenarios are at one's disposal. The next aspects to consider are then the particle size, the size dispersity of the particles, as well as their porosity. It is extremely important to have a good understanding of all experimental parameters before choosing, e.g., the most appropriate particle size. It is a common misunderstanding that smaller particles are necessarily better and give better separation performances than larger particles. In fact, most generalized conclusions similar to this one are unlikely to hold true as they are inevitably based on too many simplifications. As such, the particle size should never be taken alone as the determining factor, but must always be seen in a context, together with such parameters as monodispersity, porosity, pressure capabilities of the system, achieveable packing quality, and more. One approach to optimization is via kinetic plots, as nicely explained in this recent review [28]. In an early example of LC on a chip, 5 μm particles were packed into a 20 cm long, 312 μm × 102 μm channel [29], but because only an insufficiently high pressure could be applied to run the chips (because of the mechanical properties of the chip itself), the chip was essentially operated far away from the optimum conditions resulting in fairly broad, dispersed peaks. Recently, Gaspar et al. have used ground C16-modified silica aerogel particles (below 1 μm in size) to pack short channels in polymer chips [30]. These columns were operated at 3 bar (maximum of the used pump) but it is not clear whether this constituted the optimum run conditions. In-depth studies over the past few years have begun to unravel the intricacies of packed beds and how their performance is influenced [15,31–40]. One of the main conclusions appears to be that the degree of order in the packing is the biggest challenge on the way to perfectly packed beds. While modern particulate material is ever more monodisperse, the limitation of achieving high order packings lies now in the used packing technique. Here, it seems that commercial vendors on account of many years of experience and their possibility

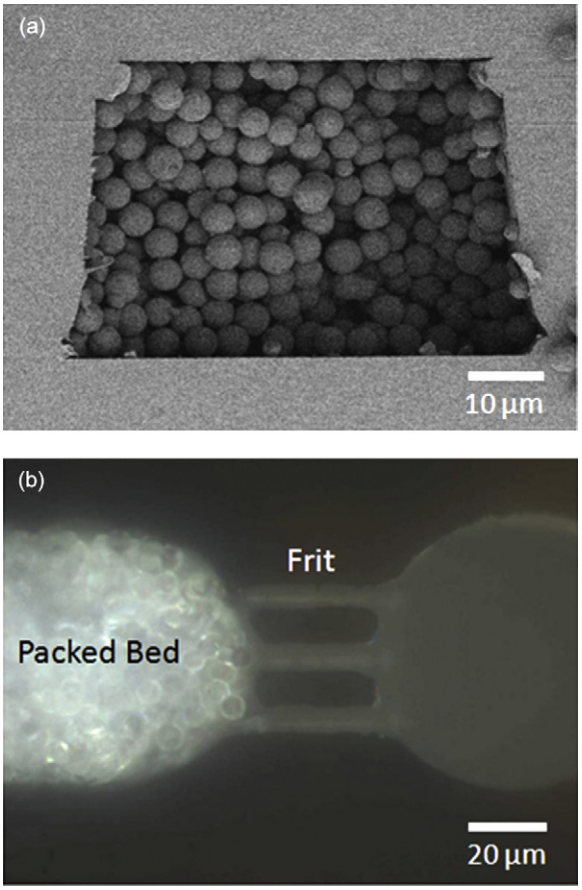

Fig. 3. SEM of a polyimide microchannel with trapezoidal cross section packed with 5 μm C18 particles (upper panel); several smaller channels constitute a frit-like structure to contain the packed particles (lower panel).
From [25] with permission.

to set up dedicated packing equipment will always have an edge over academic groups in terms of achievable packing quality and reproducibility.

One of the main challenges with using particles in microchannels is the fact that some sort of "device" or structure is needed to keep the particulate phase inside the channel, first during the packing and then during operation (see also Fig. 3, lower panel). The necessity to employ, e.g., frits makes the use of particles for many a less elegant solution, and a solution that may never fulfill its true potential as frits (conventional or micromachined) and other ways of retaining the particles (weirs, tapers, constrictions) can introduce sources of band broadening and thus reduce separation performance. The desire to avoid the need for frits entirely – together with the inherent challenges of achieving near perfect packings – was one of the driving forces to investigate in situ monolithic phases.

4.3. In situ generated monolithic phases [10,41–48]

Rather than having to pressure pack particles into microchannels and accepting less than satisfying results, the concept of synthesizing a porous monolithic skeletal support structure as well as the stationary phase in situ, and possibly in one step, seems much more appealing. The advantages are obvious: solution cocktails including monomers, chemical moieties to offer interaction sites for the analytes, and porogenic chemicals can easily be introduced into the channels and then polymerized right there, either thermally or photo-induced, and no frits or similar tricks to avoid

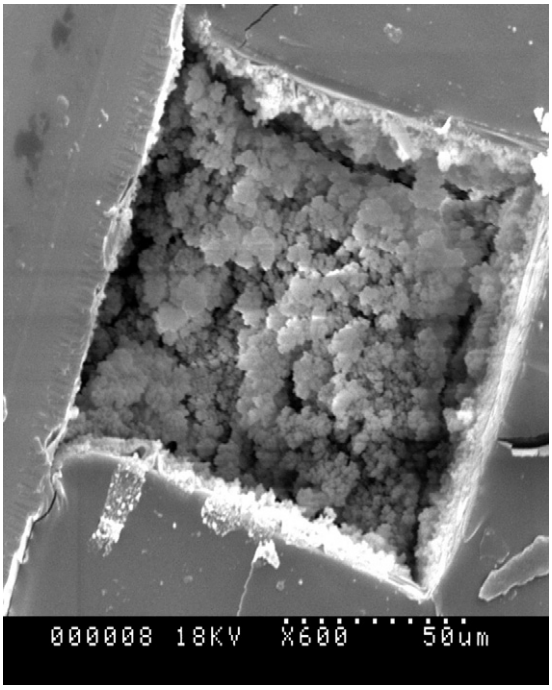

Fig. 4. SEM picture of a microchannel with quadratic cross section (100 μm × 100 μm) filled with a photoinitiated hexyl acrylate monolith. From [46] with permission.

flushing out particles are necessary. Indeed, using photo-induced polymerization gives the added benefit of being able to spatially control where polymerization is meant to happen and where not, using masks that shield parts of the channel manifold from the initiating UV radiation [49]. By carefully mixing the cocktail, one can tailor both the selectivity of the resulting stationary phase as well as the porosity, and even include chemical moieties that support the electroosmotic flow, if desired.

Monoliths are typically either silica-based or polymer-based (e.g., acrylates, Fig. 4). They often have a higher permeability than comparable particulate materials, and can therefore be run at lower pressures, without sacrificing loadability. In fact, much like particles, monoliths can come in a variety of porosities on the mesoscale, thus providing counterparts to solid particles, porous shell particles and fully porous particles. Monoliths have in recent years also been the focus of in-depth studies to improve our understanding of their properties and thus support the development of novel materials for improved separation performance [50–58].

One of the disadvantages often associated with monoliths is that it is more difficult to provide acceptable batch-to-batch reproducibility. This may in part be due to the fact that the involved chemistry often is more complex and still comparatively new in contrast to the very well studied and understood chemistry of making silica particles. I have, in the past, likened this to black magic in an offhand remark trying to convey my opinion that small variations in experimental conditions during in situ generation might lead to larger than anticipated variations in the performance of the resulting monolith. Of course, researchers working in this field are not seldom very gifted organic chemists, and significant developments have been achieved in the area of monoliths since my tongue-in-cheek remark [59].

Another issue that has plagued monoliths early on was shrinkage of the monolith during polymerization and the resulting detachment from the channel wall. As such, the chemistry chosen to prepare a monolith is thus also never entirely independent of the material chosen for making the microfluidic manifold.

4.4. Microfabricated pillar arrays

Instead of "just" implementing already existing formats on the microchip, Regnier and co-workers suggested to really exploit the possibilities offered by microfabrication to realize micromachined pillar arrays (or collocated monolithic support structures, as the authors called them), a regular array of posts with identical size and shape and equally distributed over the area of a larger channel, thus mimicking a perfectly ordered packed bed [60,61]. The main limitations with this approach lay in the resolution limits of UV lithography. Until recently, the lower limit was around 1 μm in feature size, which was thus also the minimum distance between pillars. In order to decrease this distance and thus enhance mass transfer kinetics, it was suggested to thermally oxidize the pillars, which allows decreasing the distance after the lithography step because the oxide takes up more space than the original silicon material [62]. Recently, advances in deep UV lithography have allowed smaller feature sizes without having to invoke more costly and time-consuming methods such as e-beam lithography.

Great care needs to be given to the design of the pillar arrays in order to minimize contributions from eddy dispersion (based on differences in flowpaths through the array) and dispersion due to hindered mass transfer, as well as other effects caused by the chosen geometry [63,64]. The group of Desmet has been very active in studying the optimum design parameters of pillar arrays using computational fluid dynamics approaches and comparing them to experimental findings [11,58,65–72]. Some of the main conclusion are that fully porous pillar arrays should perform better than the best packed particulate beds, and that elongated hexagonal pillars appear to be the best shape to optimize performance, a finding very much in line with early suggestions by Knox [73]. In this context, it proved again very important to focus one's attention not only on the separation column itself. The best designed column will be rendered almost useless if up- and downstream components do not match the performance of the column, but instead introduce extra band broadening (see also Section 7). The importance of distributing sample and flow in an optimized manner from the, typically narrow, injection channel to the much wider beginning of the pillar array was demonstrated and designs were suggested and experimentally tested (Fig. 5) [74]. Similar considerations are necessary when designing the region where the pillar array ends towards the sides and meets the channel walls. Microfabrication allows to create channel walls that follow the contours of the pillars, thus alleviating the impact of larger voids as one tends to get when packing spherical particles into a cylindrical lumen [63]. However, designs still needed to be tuned finely to minimize the impact of this "wall effect".

While ordered pillar arrays approach the ideal packed bed, they are only support structures and not the separative stationary phase itself. Thus, surface chemical reactions have to be performed to put the stationary phase material onto the pillars [66,75,76]. In the case of oxidized silicon pillars, this can, for example, be done by exploiting silane reactions similar to what is used to derivatize the channel walls in open channel chromatography. Getting C18 functionality onto the walls of the pillars has, however, proven to be difficult. The reasons for this are unclear, but it can be hypothesized that reactions in confined spaces (interpillar distances of around 1 μm or less) may follow different reaction pathways than in less crowded environments. Additionally, the presence of physisorbed water on all surfaces can induce unwanted polymerization reactions, which eventually lead to a clogging of the interstices between the pillars. In the literature, pillar arrays have often been used with shorter hydrocarbon chains than C18 for this reason.

A way to avoid having to perform post-production surface modifications is to directly utilize the bulk material of the chip as stationary phase. Gustafsson et al. used a COC polymer to fabricate

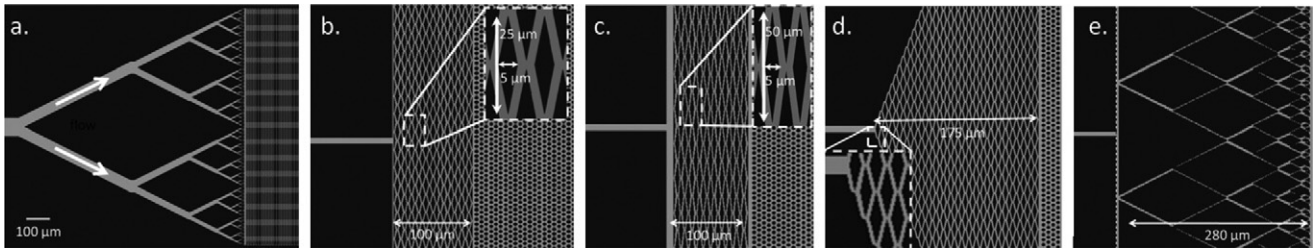

Fig. 5. Five suggested and tested flow distributor designs to aid in the optimized distribution of sample onto the pillar array; flow is from left to right in all cases. From [74] with permission.

their chips, and this material displayed reversed phase type interactions with alkylamines [77]. Illa et al. used the same material for more in-depth investigations [78–80].

Pillar arrays offer a larger surface area for interaction than open channel systems, but the area is still very limited when compared to (porous) particles and monoliths. Attempts have therefore been made to introduce mesoporosity into microfabricated pillar arrays. This proves to be challenging from a manufacturing point of view. Different approaches, such as electrochemically enhanced etching or sol–gel processes can make the surface of the pillars more porous, thus mimicking pore shell particles [81–84]. These have undeniable advantages, but the loadability of fully porous particles cannot be achieved this way. Recently, Mogensen et al. showed a pillar array where the pillars consist of beds of carbon nanotubes, thus providing mesoporosity and retention (Fig. 6) [85]. While the process used to make these pillar arrays was relatively manageable, the quest for finding easy pathways to fully porous pillars is still on.

4.5. Chemical realizations

As mentioned above, from a chemical point of view, a whole range of stationary phase materials (especially in particulate and monolithic format) is available. Still, new phase materials with new characteristics offering new selectivities or better suitability for certain ranges of analytes are continuously developed. Beyond more classical normal phase and reversed phase materials, graphitized carbon stationary phases have also been used in the microchip format; here, demonstrating retention of acrylamide and hydrocortisone [86]. Other materials, also based on modifications of carbon, which are so far mostly available through the use of microfabrication technology and only beginning to be available in bulk, have recently appeared, mainly carbon nanotubes (CNT). CNT have been added as powder during the packing process of conventional columns [87], grown on pillars [88] or directly inside channels [85,89–91] and used for separation of, e.g., small molecules and double-stranded DNA fragments. One of the drawbacks when using CNTs in connection with electro-driven methods is their inherent conductivity. To avoid this issue, it has recently been shown that CNTs can be grown directly inside channels in (photolithographically) predefined areas or patches [85] (see also Fig. 6). If the size of the patches is held to about 8 µm and the distance of the patches is such that there is no direct contact between CNTs from different patches then much larger voltages than in previous designs could be applied, thus rendering the use of CNT type phases an interesting alternative for electro-driven chip-based liquid separation systems. Finally, the rapidly increasing research activity with graphene makes it likely that this carbon conformation will be exploited sooner or later as well. However, the limitation of graphene (apart from its conductivity) stems from its planar topology, which means that it offers much less surface than, e.g., CNTs unless it can be combined with larger area stationary phase supports, such as, e.g., pillar arrays.

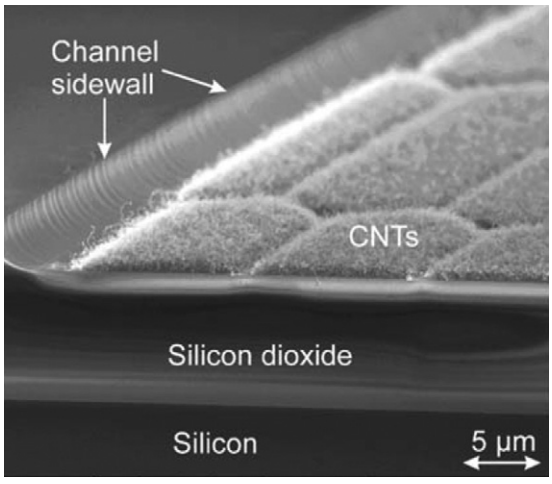

Fig. 6. SEM of the floor of a microchannel showing regular patches of grown carbon nanotubes (CNT); the patches are about 23 µm long in this case and the CNTs are grown to about 2 µm in length.
From [85] with permission.

4.6. Comparison

As can be gathered from the above said, none of these realizations of stationary phases inside microchannels is without flaws, but they certainly all have their merits. The following table (Table 1) provides a quick glance over the main pros and cons of the mentioned realizations.

5. Chip and capillary materials

The main materials used for chip fabrication were, from the beginning, silicon and glass. Silicon was favored by engineers, since it was a natural evolution to use the same fabrication methods, which had been used for quite some time in making electronic chips. Glass, on the other hand, was and is the preferred material for chemists. Its compatibility with many solvents, the large know-how of derivatizing its surface, and not least its transparency for visible light make it ideal for many applications in the life sciences. More refined, and expensive, variants of glass, such as quartz and fused silica, have also been used for chip fabrication.

Fairly early on in the development of microfluidic devices, researchers begun to look into using polymers, mainly with the idea of being able to produce cheaper, disposable devices for one-time use, or to provide fast prototyping possibilities before going to more time-intensive and expensive cleanroom-based production approaches [92]. Among commonly used polymers are flexible, elastic materials (mostly poly dimethyl siloxane (PDMS) [93]), as well as more rigid thermoset or thermoplastic type materials (poly methyl methacrylate (PMMA) [94], poly carbonate (PC), parylene, poly imide), and even photoresist-turned-bulk material

Table 1
Comparison of the main advantages and disadvantages of the four main variants of getting a stationary phase into a microfluidic channel.

Variant	+	–
Open channel	Can be easily realized and run Low flow resistance Thickness of stationary phase can be tuned Good for fast separations	Very limited loadability, which has repercussions on what kind of detection should be used with this variant
Packed beds	Many different materials available Good batch-to-batch Reproducibility of particles Easy to use right selectivity Different porosities available High loadability	Packing quality strongly dependent on packing skills A frit or other retaining device or design required Often high backpressure for porous particles
Monoliths	Much less backpressure than comparable particulate phases Different base chemistries available No packing and mostly no frits necessary	Batch-to-batch reproducibility still an issue Synthesis procedure (including possible wall coupling) may be dependent on chip material Inherent variations due to organic chemistry approach
Pillar arrays	Potentially better performance than for packed particles (reduced eddy diffusion) Can be made by nanoimprint lithography (mass producibility)	Limited loadability Hard to make porous When porous not necessarily much better than porous particles Can require sophisticated fabrication methods

(SU-8, Ordyl SY330 [95]). Recent additions are the cyclic olefin (co)polymers (COC, COP), which are thermoplastic materials with favorable chemical, electrical, mechanical and optical properties [96], as well as polymers based on thiol-ene chemistry [97], which are poised to limit or even replace the use of PDMS. Also, more exotic materials (such as diamond [98] or ceramics) have been used for making microfluidic chips.

Important questions in relation to miniaturized chromatographic systems, which anyone who wants to make such devices has to ask themselves, are: is the material compatible with packing procedures? Is the material compatible with chemistries for preparing monoliths? Is the material compatible with solvents as, e.g., used in reversed phase chromatography? Is the material interfering with the separation process (e.g., adsorption/absorption)? Is the material supporting electroosmotic flow? What are the electrical properties of the material? What are the optical properties of the material? Is the material likely to provide pathways to chemically or physically modifying its surface? Can the material be machined with the necessary precision for, e.g., micropillar arrays? Can devices from this material be mass-produced?

6. Flow generation

Flow generation is important as it distinguishes between electro-driven separation methods and those that exploit a pressure differential to move the mobile phase. As was already mentioned above, the fact that using a pressure differential creates a parabolic flow profile whereas undisturbed electroosmotic flow corresponds to a flat flow profile, does not weigh in from an overall dispersion point of view nearly as much as intuitively expected. While the flow profile and its possible effect on dispersion should be kept in mind, there is little difference between the two flow generation methods when channels and separation media are designed carefully. On the other hand, the magnitude of the flow velocity is much more important to avoid running the system outside of the van-Deemter optimum (see Section 8). It is detrimental to ideal performance if the separation cannot be executed at the optimum flow velocity, for example because the chip is designed or built such that it cannot withstand the required pressure, or the materials and chemistry used does not allow the application of sufficient voltages because short circuits, the onset of electrolysis, or overly pronounced Joule heating issues must be expected.

Another issue, which is by no means limited to miniaturized separation devices, is the question whether pumps and valves can be incorporated or integrated on chip or whether external pressure and/or voltage sources need to be employed. This has immediate repercussion as to how "portable" a system will be and how effectively it might be used outside of a laboratory setting. Finally, the never-ending compromise in finding materials that are compatible with each other, with the fabrication processes used and with the chemistry involved, is also affecting the choice of flow generation in miniaturized separation devices.

Most examples of microfluidic separation systems in the literature use electro-driven flow, but there are also examples where set-ups have been developed utilizing pressure-driven flows. This is especially the case for commercial solutions, e.g., the Agilent set-up with a true separation chip [25,27,99] or the equipment developed by Eksigent, which features parallel microfluidic pumps which can be used with conventional uHPLC columns [100,101]. Alternative pumping approaches are electrically actuated pumps, which use an electric field to create a pressure differential on chip [102–104], or the use of centrifugal forces (i.e., chromatography systems on spinning disks, often for sample preparation) [105,106], and even the use of shear-driven flows have been investigated for separations [19,21,23,107,108]. By carefully exploiting the fluidic control possibilities on- or off-chip, gradient elution for chromatographic separations was also demonstrated [22,25,44,94,109,110].

7. Upstream and downstream processes

The planar format of microfluidic chips is inherently more conducive to integrating different functionalities than the cylindrical capillary format. And, since a separation is hardly standing by itself, but instead needs at least to be coupled to an injection and a detection, this advantage of chips is very crucial when it comes to keeping outer column contributions to band broadening to a minimum [111]. Many of the functionalities surrounding chromatography can be integrated monolithically, thus completely avoiding any kind of connector and at the same time keeping transfer lines as short as possible, thereby all but eliminating two sources of band broadening that often plague more classical set-ups.

Integrated injection schemes have been part of microchips for a long time and have proven to be able to reproducibly dispense picoliter amounts of sample [112]. More elaborate injection schemes that allow distribution of sample to parallel separation channels have also been shown [113]. Other important functional elements on the front-end are sample preparation tools, such as filters, extractors, reactors, and concentrators [93,114–118]. An upconcentration step such as, e.g., solid phase extraction (SPE) [44,119,120], is often necessary to compensate for the detection challenges when working with small volumes, and this is often achieved using similar stationary phase material as used for separation purposes.

Equally important is integration of or coupling to a sensor/detector unit [114,121–123]. While electrochemical and optical

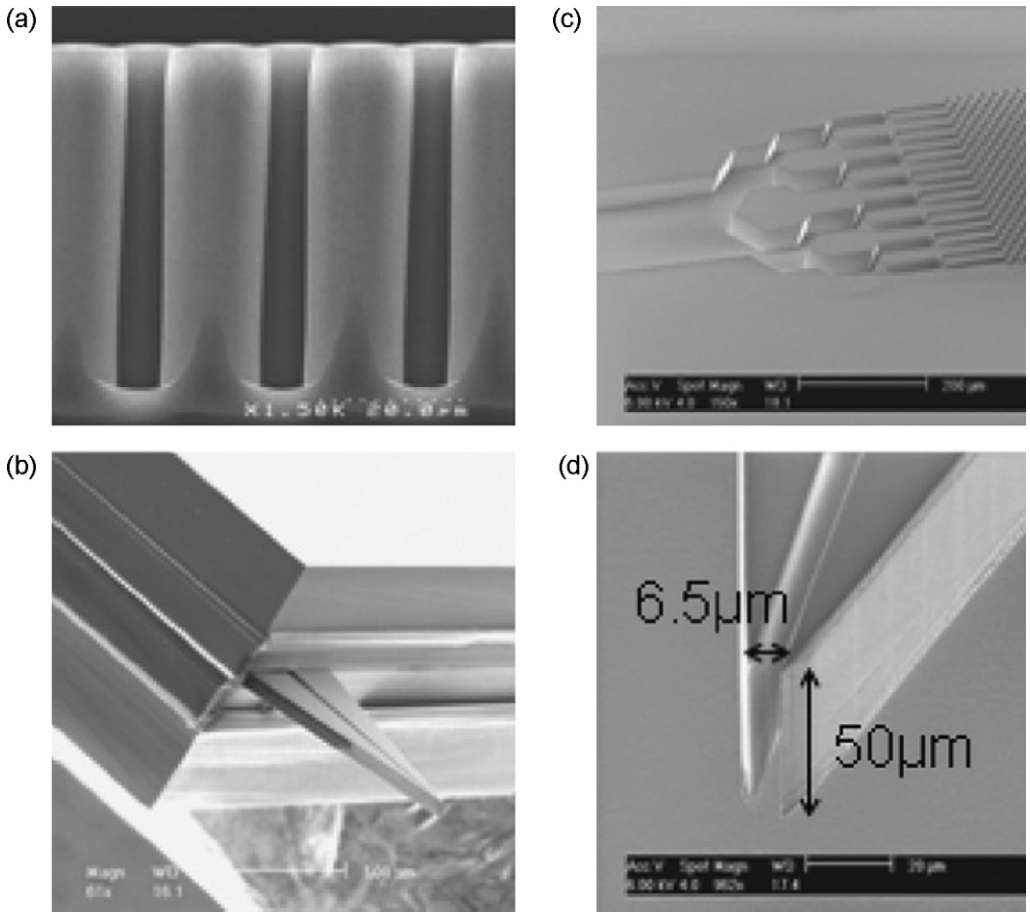

Fig. 7. SEM images of a chip with a high-aspect ratio pillar array (panels a and c) and an integrated spray tip for introduction of eluate into an MS (panels b and d). From [75] with permission.

detectors are more amenable to miniaturization and integration, this is less likely for mass spectrometers [124]. However, since MS is a powerful detection and identification tool, and microfluidic (separation) devices can act as versatile sample preparation means before MS, a coupling of these two has been the focus of a lot of research [75,125–129]. Provided that the interface is designed properly, band broadening effects after a separation can be minimized. Work has thus focused on integrating carefully designed emitter features to allow direct spray generation on the chip and introduction to the mass spectrometer (Fig. 7). More details on detection approaches for microchip devices can be found in these reviews [124,125,130–132].

The compactness of microchips and the possibilities to avoid long transfer lines and cumbersome connectors makes these devices very attractive for implementing two-dimensional separations [113,133–136] (see also Fig. 8). Fast and reproducible injections from the first into the second dimension and very fast separations in the second dimension are additional advantages of microchips, which improve the possibility for comprehensive 2D separations. However, this has mostly been shown for the combination of on-chip LC and CE, and not yet with two LC separations coupled together. The main challenge here is no different than for classical LC columns: the two separation columns must be as orthogonal in their selectivity as possible, and this can in turn put restrictions on the allowed composition of the mobile phase, and even require a change in mobile phase between dimensions.

While the possibility to monolithically combine several functionalities is certainly one of the great advantages of micromachined chemical devices, and the success of many separation

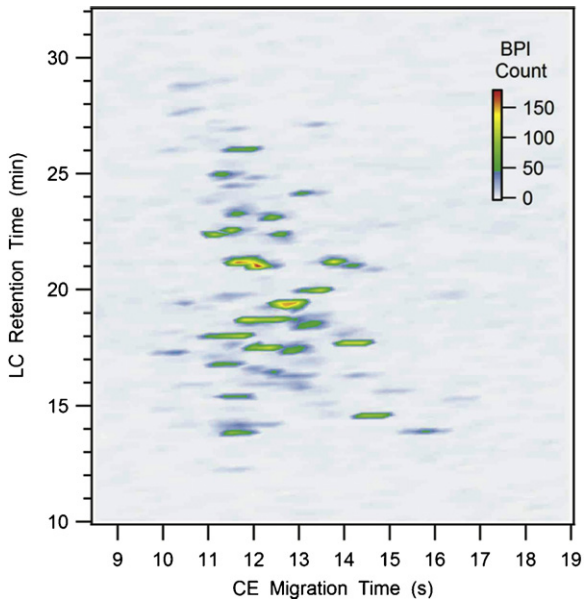

Fig. 8. Two-dimensional plot for the separation of 200 fmol of a tryptic digest of bovine serum albumin using LC–CE–MS; LC and CE performed on-chip and then sprayed from the chip into the MS.
From [133] with permission.

solutions will ultimately depend on what is connected to it upstream and downstream, there are two things always to keep in mind: (1) up and downstream processes along with the actual connection to the separation system need to be designed such as to avoid adding extensive band broadening; and (2) with all the possible complexity, experience has shown again and again that one should seek for the simplest possible solution and shy away from extravagantly complex designs and workflows.

8. Performance evaluation

What, then, is the "best" liquid phase miniaturized separation device? Is there a proven way to determine when a separation is "good", "brilliant" or just "mediocre"? While Giddings' statement cited at the beginning of this review succinctly defines separation, the goal is ultimately always to deliver efficient separations (i.e., separations with narrow peaks in order to baseline resolve as many components as possible) and to do that in the shortest amount of time. Typically used figures of merit are the number of theoretical plates (N) or the height equivalent to a theoretical plate (H) to determine efficiency, and the linear velocity of the mobile phase (v) to determine how fast the system can be driven and hence how fast a separation can be delivered. Plots that relate H and v are the well-known van Deemter plots, a closer analysis of which can yield insight into the contribution of different mechanisms to H, namely the contribution from flow path differences (eddy diffusion, A term), the contribution from longitudinal (or axial) diffusion (B term, equivalent to the Péclet number formalism), and the contribution from mass transfer kinetics (C term). Van Deemter plots often show a minimum, which corresponds to the best performance and the linear flow rate at which it can be achieved. Driving a system faster or slower typically results in a decreased performance. Clearly, though, if a separation system (e.g., via the selectivity of the chosen stationary and mobile phase combination) has plenty of separation power, it can be operated outside the optimum parameters if this means a significant decrease in analysis time. Also, because of the smaller dimensions involved in miniaturized separation systems, and the corresponding reduced diffusion distances, the C term often has a very shallow slope, thus allowing operation at higher velocities without sacrificing a lot of separation efficiency. On the other hand, if mechanical or other material issues limit the applicable pressure or voltage one can risk to run the system at too slow linear velocities, which, apart from long analysis times, also results in much poorer separation performance. This is particularly dangerous as the low linear velocity regime is dominated by the influence of the B term, which often has a very steep slope, i.e., the performance becomes very sensitive to small changes in the linear velocity.

Another tool, apart from van Deemter plots, that is available to determine whether a system is run under optimal conditions is kinetic plots. Here, other instrumental and experimental aspects not explicitly covered by the van Deemter formalism are considered, in particular the relation between pressure drop, particle size (or equivalent feature size in other variants), separation column length and flow velocity (related to analysis time). Kinetic plots typically show the required analysis time to achieve a certain number of plates (quality of separation) where certain values for the above mentioned experimental conditions are chosen. A recent review describes the use of kinetic plots for optimization in liquid chromatography, also with miniaturized formats in mind [28].

Microchip-based separation systems offer strong possibilities to minimize the effects of all three terms in the van Deemter plot, thus achieving very small plate heights and hence efficient separations. The A-term contribution can be reduced most efficiently when using carefully designed microfabricated pillar arrays or "exquisitely" packed particulate beds. The B-term contributions are minimized provided the system is run under sufficiently large linear velocities of the mobile phase. And, as already mentioned, due to short diffusion lengths (small channels, small particles) the C-term influence is reduced drastically, even at larger linear velocities.

Next to paying attention to the column, it is important to minimize extra-column sources to band broadening, as already mentioned several times. Again, micromachined separation systems have the power to get these potential sources minimized and under control much more easily than in more conventional set-ups.

One aspect that is often "overlooked" when considering microchip separation devices is the fact that certain difficult separation problems require a minimum available column length to achieve baseline separation. It can pose a challenge to combine a length requirement of more than 2–5 cm with the small footprints of microfluidic chips. Solutions to this challenge have been discussed and presented in the literature, and demonstrated again very impressively how important the correct design is to avoid strongly increased band broadening [137–142]. An early example of a long meandering microchannel (16 cm length), where the correct design rules for such meanders had not been established yet, showed a plate count that did not even account for 10% of what had been theoretically predicted [143]. While it proved eventually possible to put up to 25 cm of channel length onto the typical footprint of a microchip, long channels can introduce other difficulties during operation, which can take away some of the advantages of working in the miniaturized format and might thus limit or even prevent the usefulness of extended channel systems in the long run. One challenge is to provide high enough voltages to guarantee reasonable field strengths for the separation. Long channels also require higher pressures in the pressure-driven separation modes, and the increased hydraulic resistance can actually make priming of the channel (or removing of bubbles) a serious problem. Despite these challenges, high-resolution separations performed on microdevices have been demonstrated [138].

9. Alternatives to "pure" chromatography

For the sake of fairness, I feel some alternatives to pure chromatographic systems deserve to be briefly mentioned here, i.e., systems that exploit other physico-chemical phenomena in order to achieve separation of (chemical) species in space and time. Some of theses solutions have the limitation that they only work for separation of particles or macromolecules, such as e.g., DNA fragments. Important members of this group are the deterministic lateral displacement (DLD) devices using non-retaining post arrays [144], the so-called Brownian ratchets [145] and related techniques often also collectively referred to as "vector chromatography" [146], hydrodynamic chromatography [147], as well as devices that exploit, e.g., pI differences and diffusion [148], or any number of other types of forces [149]. The big advantage of these and similar methods is the fact that they provide a continuous separation mode, where samples is continuously injected and the separated components, or at least several different fractions, can be collected continuously as well.

10. Applications examples

To wrap up this review, I would like to give a few examples of separations of real samples (as opposed to test mixtures) separated on or aided by microchip-based separation devices. Peptides and proteins [111,126,150–155], glycoproteins and glycans [156–159], enantiomers [160,161], and iso-prostaglandin [162] have all been analyzed using microchip-based chromatography. A miniaturized

system for bioaffinity chromatography has also been described [163].

11. Conclusions

This article attempted to give an overview over current developments and important aspects of microchip-based liquid chromatography systems, albeit without being able to afford the luxury of delving deeper into the many different facets that need to be considered in this context. It is my hope that the readers have gained some understanding of the complexity of the topic and found useful (if not exhaustive) pointers to literature dealing in much more detail with the mentioned issues.

In a review article published in 2000 [164] I was speculating about whether miniaturized separation devices would, in the future, be used predominantly for fast, but not very demanding separation problems in routine settings (e.g., process control), or whether there also would be a demand and an application field for microchips to tackle complex separation problems with many compounds, maybe even in challenging matrices. The picture here, almost 11 years later, is still rather foggy. On the one hand, research that strives to push the limits of separation performance of microfluidic chromatography chips is crucial, as its findings will eventually benefit solutions for both simple and complex separation problems. On the other hand, I am more inclined to believe that, in the long run, miniaturized separation devices will find their niche in providing answers by performing fast separations of not too complex mixtures, working remotely, with little sample and chemical consumption, providing ruggedness on account of accepting relaxed (i.e., not too stringent) experimental conditions, and reliability via built-in redundancy (i.e., parallel operations).

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