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Liquid Chromatography on chip

Karine Faure

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**Liquid Chromatography on chip**

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3 Review for Electrophoresis – deadline February 1st, 2010
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7 **Liquid Chromatography on chip**

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9 Karine Faure

10 Laboratoire des Sciences Analytiques,
11 UMR UCBL - CNRS 5180, Université de Lyon
12 Université Claude Bernard Lyon 1
13 43 boulevard du 11 Novembre 1918
14 69622 Villeurbanne, France
15 karine.faure@univ-lyon1.fr
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19 **Abstract**

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25 Liquid chromatography is one of the most powerful separation techniques as illustrated by its
26 leading role in analytical sciences through both academic and industrial communities. Its
27 implementation in microsystems appears to be crucial in the development of μ TAS. If
28 electrophoretic techniques have been widely used in miniaturized devices, liquid
29 chromatography has faced multiple challenges in the downsizing process.
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32 During the past five years significant breakthroughs have been achieved in this research area,
33 in both conception and use of liquid chromatography on chip. This review emphasizes on the
34 development of novel stationary phases and their implementation in microchannels. Recent
35 instrumental advances are also presented, highlighting the various driving forces (pressure,
36 electrical field) that have been selected and their respective ranges of applications.
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deadline February 1st, 2010

Liquid Chromatography on chip

Karine Faure

Laboratoire des Sciences Analytiques,
UMR UCBL - CNRS 5180, Université de Lyon
Université Claude Bernard Lyon 1
43 boulevard du 11 Novembre 1918
69622 Villeurbanne, France
karine.faure@univ-lyon1.fr

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I. Introduction

The beginning of the 21st century has witnessed explosive growth of chip-based analytical systems. This is no coincidence based on craze for miniaturized widgets, but the result of the attractive features chip-based analytical systems exhibit. Separation technique has been one of the first operations taking benefits from “Lab-on-a-Chip” conceptualization. As early as 1970, Terry and co-workers developed a gas chromatography on a silicon wafer [1]. When downsizing conventional separation systems to the micron scale, many improvements are observed in terms of separation performances, instrument versatility and effective costs. In most techniques, analysis times are reduced, efficiencies are improved. The integration of injectors minimizes the injected volumes to picoliters, which benefits to both sample consumption and band broadening. The zero-dead volume interconnections to detection systems contribute as well to this gain in efficiency. Moreover, microfabrication techniques allow the integration of multiple operations (sample preparation, microreactions) and the creation of multiple or parallel analytical devices, reducing the cost of the whole analytical chain. Mass production of these miniaturized devices and the extended use of thermoplastics participate at the reduction of costs and therefore encourage the development of disposable analytical microsystems, providing a suitable platform for samples that generate cross-contamination in conventional instruments.

Electrophoretic techniques (CZE, MEKC, IEF, ITP) are perfectly suited for miniaturization owing to the smaller separation lengths and resulting increase in electrical fields. This suitability is illustrated by the tremendous widespread of electrophoretic applications on chip, especially in the biochemistry and proteomic areas which are mostly dealing with charged solutes. However, there is a major need for additional selectivity on charged solutes and a definite necessity of solutions for the separation of neutral compounds.

Liquid chromatography is by far the most widely spread separation technique in conventional format. The separation mechanism provided by the interaction of solutes with a stationary phase is a powerful, robust, yet versatile answer to the analysis of a wide range of compounds. Chip-based liquid chromatography equally benefits from the features that have done the success of electrophoresis on chip. Surprisingly, compared to the large diffusion of electrophoretic techniques in application areas such as biochemistry, very few groups are actually working on chromatography at microscale level. This delay is owed to the technical challenges that scientists are facing to introduce efficient stationary phases in microchannels. In the first part of this review, I will present the recent advances that have been reported in the field of novel chromatographic stationary phases at the microscale level.

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In the development of chromatography on chip, another interesting point has come into sight. The question of the driving force involved in the implementation of chromatography in Lab-on-a-chip devices is a great concern. Two strategies are considered: a “top-down” method consists in the miniaturization of conventional liquid chromatography instruments and will therefore use pressure as the liquid driving force, while the “bottom-up” idea consists in the implementation of a stationary phase in pre-existing miniaturized separation techniques and therefore employs electroosmosis mechanism to bring the fluids into movement.

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A semantic issue arise from these two approaches. In the literature, a multitude of names have been given to chip-based separation techniques, leading to certain confusion. We will use throughout this text the prefix “Chip-“to confine the technique to miniaturized devices and to avoid any misunderstanding. Chip-LC will be restricted to pressure-driven liquid chromatography, while Chip-EC relates to chip-based electroosmosis-driven chromatography, i.e. electrochromatography.

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If Chip-LC has already emerged on the commercial market, Chip-EC has been the most popular technique so far in research labs. The decision between these two techniques is usually taken in regards to personal scientific culture, whether chromatography on chip is regarded as a top-down or bottom-up strategy. In the second part of this review, I will try to point out the complementary character of these two approaches and the technological issues that arise from them.

II.A brief overview of stationary phases in chips

The aim of this section is to cover the time span of the last five years while focusing on the emergence of novel stationary phases on chip. The simplest way to integrate a stationary phase (and the first performed in chips) is to coat the channel walls with interacting solid phase, to realize open-channel chromatography. Increasing the available wall surface can be done by microstructuration conducting to the fabrication of pillar-type structures. A more conventional preparation of stationary phase is by complete filling of microchannels with externally prepared material such as silica particles and I will show some recent contributions in this field. Finally, the stationary phase can be created in-situ in the microchannels and I will illustrate the different materials that have been chosen for this purpose.

1. Open-channel chromatography

The very first LC –dedicated microsystem was fabricated in 1990 by Manz et al. [2], some 20 years ago. Based on Terry's GC-chip design, it involved a silicon wafer chemically etched to provide a $2\mu\text{m} \times 6\mu\text{m} \times 15\text{cm}$ column hermetically sealed with a glass wafer. This work reflected the suitability of microsystems to open-channel chromatography. Reduction of the inner dimensions of the channels to 10 μm , ease of fabrication and low pressure drop were arguments in favour of the miniaturization of this separation technique. Although the very first open-channel chromatography actual separation was performed some 15 years ago, when Jacobson and co-workers coated a glass chip with octadecylsilane [3], for the successful separation of dyes with theoretical plate heights down to 4.5 μm , this technique has not widely spread in chip format. From 1994 to 2005, very little work has been carried out in this area, as illustrated in Pumera's review [4] who reported only 12 papers in 11 years, mainly performed by only four research groups [5-8]. Since 2005, coating of microsystems has been performed using many techniques such as sol gel [9] or nanoparticles deposition [10], but most of these surface treatments were used to reduce the adsorption character of microsystem bulk material, which contribution is especially detrimental when analysing biological solutes such as proteins. Very few strategies have been brought up to actually perform a selective adsorption or partitioning onto the wall surface for open-channel chromatography.

Functionalization of glass chips with C_8 -tetraethylorthosilicate (C_8 -TEOS) has been performed [11] following a previously reported procedure [6]. On the resulting open-channel Chip-LC, the authors demonstrate the reversed phase behavior of the coated stationary phase through separation of phenol and methylphenol. The paper focuses on the issue of continuous sampling and introduces a pressure-driven gated injection scheme.

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1 The main restriction of open-channel chromatography is the limited specific surface. The phase ratio
2 (ratio between volume of stationary phase and volume of mobile phase) can be increased by generating
3 a thick 3D coating. An illustration of this strategy is brought by Zeng [12]. PDMS chip was coated
4 with hydrophilic polyacrylamide copolymerized with allyl- β -cyclodextrins. The resulting 3D-
5 stationary phase allowed the discrimination of a mixture of enantiomeric FITC-labelled dansyl-valine
6 and dansyl-aspartic acid within 100 s.
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8 Another way to increase phase ratio can be performed by coating channel walls with particles.
9 Reversed-phase particles were embedded in a porous polymethacrylate stationary phase, as shown in
10 Figure 1 for the successful separation of coumarins by shear-driven chip chromatography [13].
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12 Gold nanoparticles have also been used in a multilayered coating procedure [14]. After
13 functionalization of the glass channel with (3-mercaptopropyl)-trimethoxysilane, the resulting thiol
14 groups linked BSA-gold nanoparticle complexes onto the surface. Immobilization of gold
15 nanoparticles increase the amount of linked BSA, therefore increasing the capacity of the resulting
16 stationary phase.
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18 In open channel electrochromatography, efficiency is nearly as elevated as in zone electrophoresis,
19 since only molecular diffusion has to be taken into account in band broadening. To counteract limited
20 mass transfer, open-channel chromatography requires small channel dimensions, typically 10-20 μm ,
21 which is provided by microfabrication technologies. This technique is therefore highly suited for
22 implementation in Lab-on-a-chip design. However, the reduction in specific surface drastically limits
23 sample capacity and therefore the range of applications.
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2. COMOSS, pillars and nanotubes

Microfabrication of pillars is an elegant strategy, taking advantages of microfabrication technology to combine small channel dimensions and large interacting surface [15]. Microfabricated columns, that are extremely difficult to create on the macroscale, present many advantages on a chromatographic point of view. The fabricated structures are inherently more regular than randomly packed particles, order that has a direct influence on eddy-diffusion. Theoretically, properly dimensioned pillars should hence result in higher efficiency [16].

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In 1998, Regnier's group [17, 18] introduced COMOSS (collocated monolith support structures), which is based on the direct fabrication of pillars during the microsystem fabrication process (Fig 2).

While the very first COMOSS were etched in quartz devices, it appears that very narrow channels induce frequent clogging, especially during post-fabrication functionalization and therefore discarding the chips was frequent. Quartz became too expensive and plastic devices were considered. PDMS COMOSS were fabricated as cheap, disposable microsystems with channels of 5 μm x 10 μm , ideal dimensions for open-channel chromatography [19]. To increase stability and selectivity, it was demonstrated that the introduction of an organic stationary phase on PDMS walls was compulsory. C₈- and C₁₈- moieties were introduced by silane modification procedure to improve peptides separation. Negative surfactants were then required in the mobile phase to generate sufficient electroosmotic mobility.

Another approach was developed the following year by Regnier's group to achieve surface modification of PDMS COMOSS with simultaneously charged and hydrophobic groups [20]. Radical polymerization was achieved on the PDMS pillars using Ce(IV) as a radical initiator. Mixed stationary phases such as acrylamidomethylpropylsulfonic acid-C₁₈, poly (styrenesulfonic acid), poly (vinylsulfonic acid) or poly (acrylic acid) were investigated to provide both reversed phase interaction and electroosmotic flow. Efficiencies reached 600 000 plates/meter with analysis time around 5 min. PDMS, however, may not appear to be the best material option, due to its tendency to swell in hydroorganic solvents and to absorb hydrophobic solutes.

Cyclic olefin copolymer (COC) has demonstrated many positive features in terms of microfabrication, optical properties and chemical inertness. Using Regnier's design [17] to nanoimprint COC microsystems, Gustafsson et al [21] developed an array of native COC pillars (here, TOPAS[®]) of 4 μm wide by 8 μm long by 5 μm deep, generating separation channels of 2 μm wide for electrochromatography purposes (Fig 3). The hydrophobic character of polymeric substrate has been cleverly exploited to use the microdevice substrate as a stationary phase. Not only native TOPAS[®]

1 material can generate an electroosmotic flow reaching a value of $4 \times 10^{-4} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$, but it exhibits
2 reversed-phase behavior towards neutral analytes. At 20 % ACN in the mobile phase, three
3 fluorescently labelled amines were separated according to their respective hydrophobicity. Observed
4 plate heights reached the exceptional level of $0.5 \mu\text{m}$ for unretained compound due to electrokinetic
5 stacking effect. Plate heights of $3.4 \mu\text{m}$ and $22 \mu\text{m}$ were achieved for Atto-532-hexylamine and Atto-
6 532-octylamine, respectively, while the retention factors remained low ($k = 0.4$ and 1.5 , respectively),
7 mainly due to low carbon loading on the accessible surface.
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9 Pressure-driven chromatography has also been carried out on such COC pillars design [22]. Again,
10 separation of coumarins was achieved with high efficiencies (plate height around $5 \mu\text{m}$ for retained
11 coumarin, see Fig 4) while retention factors remained low ($k = 0.5$ for coumarin C480 at 70/30 (v/v)
12 water/methanol).
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17 On the positive side, COMOSS fabricated structures lead to high efficiencies due to the reduction of
18 eddy-diffusion. The microfabrication technology allows close-to-perfect column-to-column
19 reproducibility and full control of pillar density. On the other hand, the use of non-porous pillars
20 generates low phase ratio (in comparison with fully-filled channels) and therefore low sample capacity,
21 limiting the analysis of samples with a wide range of concentrations. This remains the main limitation
22 of native material as stationary phase.
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27 As an answer to this issue, porous pillars have been etched in silicon chips [23]. A 550 nm porous
28 layer was generated by anodisation with mesopores ranging between 5 and 15 nm , as shown in figure
29 5. This porous layer had no major influence on efficiency, but the authors calculated a 200-fold
30 increase in the exchange surface, which could provide an answer to limited sample capacity.
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32 A different approach regarding the same issues was considered in 2008 by Fonverne et al. [24]. The
33 increase in phase ratio on ordered microfabricated pillars was obtained by growing carbon nanotubes
34 on their surface. COMOSS were etched in a silicon wafer using DRIE etching process and in-situ
35 growth of nanotubes was carried out before sealing the microsystem with Pyrex. Homogeneous layer
36 of carbon nanotubes (30 nm diameter, $0.5\text{-}1 \mu\text{m}$ long) were obtained on silicon pillars (figure 6). Not
37 only carbon nanotubes increased specific surface of COMOSS and hence capacity, but it also proved
38 to be more retentive than C_{18} -modified pillars towards polar solutes.
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44 Pushing pillar strategy to a further extent, Goswami et al. [25] and Wu et al. [26] recently grew
45 vertically-aligned carbon nanotubes directly in the microchannel as filling stationary phase (figure 7).
46 If the first group demonstrated proof of concept with the separation of tryptophan-tryptophan from
47 unretained compound on native carbon nanotubes, Wu and co-authors [26] showed that the
48 functionalization of carbon nanotubes, either with carboxylic groups or with 1-pyrenedodecanoic acid
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could provide versatility to this novel stationary phase, with modification of the selectivity when separating three dsDNA fragments by electrochromatography. Interestingly, the plug of solutes was less subject to band broadening in the carbon nanotubes stationary phase than in open channel. It was suggested by the authors that the very laminar flow field generated in the nanotubes environment was less susceptible to perturbation for dispersion. Efficiency in such a novel stationary phase reached up to 3.8×10^5 plates/meter.

3. Packed Particles

The transfer of liquid chromatography onto chip format can also be addressed as a simple miniaturisation challenge. Taking advantages of all the knowledge on silica particles that have traditionally been employed in LC columns as stationary phase is an attractive option, as the catalogue of available surface chemistry is wide and the expected chromatographic performances of such particles are well known. Two major issues can arise from the packing technology: reproducibility of the packing process and immobilization of particles. Three different strategies have been explored to pack particles in microchannel for chromatographic purposes.

The first approach considers particles packing in microchip as the direct continuation of the miniaturization trend that has undergone chromatographic column, consisting in the definition of a trapping zone using weirs [27-29]. Between 2000 and 2005, proofs of concept were achieved but the resulting chromatographic beds were fairly short due to the difficulty of packing process. On this path, Agilent HPLC-Chip has recently aroused most interest in the separation science community [30]. Made of polyimide, this chip is packed with slurries of conventional silica particles under 400 bars and with ultrasound assistance. Thanks to the wide range of particles available in Agilent catalogue (C_8 -, C_{18} - and TiO_2 functionalized silica particles, various porosities), the number of application of the HPLC-Chip has dramatically increased in the literature. Not only this commercially-available system meets a large public avid of highly reliable micro-LC devices, but it also allows a better understanding of the influence of packing parameters on peak efficiency, such as channel geometry [31], particles size and packing procedure [32, 33]. Another commercially available chip packed with silica particles, called cHiPLC, has recently been brought up to the market by Eksigent [34]. Based on glass material, the chip is presented on a docking station that allows its connection to any compatible detector and offer C_{18} -functionalized silica particles of various sizes.

In a recent publication [33], Ehlert et al. assert that the gain of efficiency noticed in the HPLC-Chip compared to nano-LC is due to the fact that micromachined frits retaining the particulate bed in the microchip channel appeared to generate less dispersion than sintered frits. Reduction or elimination of frits has been regarded as one of the many challenges in the setup of stationary phase in microchannels. This idea of fritless chromatographic beds in microchips was already explored in the work by Ceriotti et al. [35] who defined tapered chromatographic column as to provoke a keystone

1 effect during packing. This packing procedure allowed immobilization of 3 μm particles in a
2 reproducible way in a taper 16 μm wide at the narrowest end. If efficiencies reached 290 000 plates/m
3 for unretained compound and 120 000 plates/m for retained compounds, the stabilisation of the bed
4 appeared to be somehow uncertain and required a thermal treatment. Following this path of fritless
5 particle immobilization, Gomez's group [36, 37] exploited the elasticity of PDMS material to entrap
6 particles. The authors notice that during packing process executed at an intermittent pressure of 2 bars,
7 the wall of PDMS channels would deform in an elastic way. The release of pressure induces a
8 constraint on the packed particles that results in clamping of the whole chromatographic bed. Particles
9 adjacent to PDMS walls are partially embedded in the elastomeric bulk of the microchip material. If
10 particles seemed to be properly anchored, once again, a thermal treatment was necessary to assure the
11 stability of the bed during chromatographic process. Evaluation of the chromatographic performances
12 of such beds is currently under investigation.
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18 In the search of the ideal packing procedure in microchannels, the difficulty stands in the production of
19 a homogeneous bed. Packing of conventional particles has been achieved by low pressure in PDMS
20 chips [27, 35-37], by high pressure in the HPLC-Chip [33] or even by electroosmotic flow [28, 29].
21 One can also take advantage of surface-tension capillary forces resulting from a proper design of the
22 microfluidic components to gain self-assembly of silica particles.
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24 Recently, the use of self-assembly colloidal silica beads as the packing material has emerged from two
25 groups. In Wirth's group [38], a 20 μm thick colloidal crystal of silica particles was obtained by
26 evaporation and subsequent self-assembly of 200 nm calcinated silica colloids. After C_{20}
27 functionalization, the phase was used for the electrochromatographic reserved-phase separation of
28 three dyes having similar electrophoretic mobilities. The authors demonstrated that, as expected, the
29 high uniformity of packing generated by the crystallisation process induces a decrease of the A-term
30 (eddy-diffusion) in the Van Deemter representation, reaching 2.5 μm , while the sub-micron size of the
31 nanoparticles results in a much faster mass transport and therefore a low C-term of 1.2 ms. The self-
32 assembly packing process was carried out on a quartz plate and subsequently enclosed by a PDMS
33 cover, which led to a small volume of chromatographic phase.
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40 Park et al. [39] develop the same self-assembly packing technique to fill a microstructured open
41 channel with dimensions 100 μm x 80 μm x 25 μm , with 800 nm colloidal silica nanoparticles, as
42 shown in figure 8. This considerably increased the volume of stationary phase. Unfortunately, the
43 particles were not derivatized and only electrophoretic separation was carried out in this column.
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47 4. Monoliths and continuous structures

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49 Monoliths have been extensively studied as stationary phase in miniaturized format such as capillaries
50 and microchips. Their synthesis, based on the polymerization of monomers in the presence of porogen,
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1 can easily be conducted in-situ and the resulting continuous bed exhibits a bimodal structure with large
2 through-pores for flow and small pores for retention. The chromatographic characteristics can either be
3 tuned before synthesis by adjusting the prepolymer composition, or modified in-situ by
4 functionalization of the resulting bed. Many reviews are available, focusing on the study of monoliths
5 in capillary format [40, 41]. The size of the microfluidic channel being relatively close to capillary
6 internal diameter, the transfer of monolith synthesis towards microchip is in theory quite
7 straightforward. The very first syntheses of organic monoliths for EC purposes were carried out in
8 glass chips [42-45], which material is similar to fused-silica capillaries. As in capillary, the silica
9 walls were pre-treated with an adhesion promoter and then the channel was filled with the
10 acrylate/methacrylate prepolymer before chemically-[42] or photo-initiated polymerization [43-45].
11 Since 2005, the technical issues for the synthesis in glass chips seem to have been answered and the
12 work in this kind of chips has taken a step forward and is now focusing on specific issues, such as the
13 integration of a extraction/preconcentration step before separation [46-48] or the establishment of a
14 linear gradient elution in electrochromatography technique [49].

15 The growing market of chips fabricated in synthetic polymers reflects the enthusiasm towards a cheap,
16 disposable, versatile device. However, special issues have to be faced up when implementing organic
17 monoliths in plastic devices. The most widely spread polymeric material for cheap microfluidic
18 application is polydimethylsiloxane (PDMS). The implementation of monoliths in this material was
19 particularly difficult, as absorption of monomers and porogen into PDMS was recurrent.

20 Zeng et al. [12, 50] prepared the γ - or β -CD bonded polyacrylamide monolithic column on a PDMS
21 microfluidic device after surface functionalization using (trimethoxysilyl)-propyl methacrylate. In this
22 case, crosslinker allyl- γ - or allyl- β -CD played as a chiral selector for enantioseparation of FITC-
23 labeled Dns-AAAs. Covalent coating with a first layer of acrylate polymer was found to limit adsorption
24 and subsequent photopolymerization of organic acrylate monolith was attainable [51].

25 Besides PDMS, other plastic materials were found to be less problematic in terms of absorption.
26 Polyimide, which was chosen by Agilent as the material for their commercial HPLC-Chips, is
27 chemically inert. Its non-transparency forbids UV polymerization, but thermal polymerization was
28 successfully achieved in this chip. Various monoliths have been synthesized, such as lauryl
29 methacrylate, polystyrene-divinylbenzene [52] or methylstyrene-bisvinylphenyl ethane [53].

30 Cyclic olefin copolymer (COC) is another material of interest. Indeed, the soundness of this material
31 eases its machining and enhances its durability. Furthermore, its transparency over a wide range of
32 wavelength and its inertness towards most organic solvents, such as acetone, seems to offer a
33 promising future to this material in term of analytical systems design. UV polymerization can be
34 carried out in COC microdevices, which allow photopatterning and hence localization of the stationary
35 phase in dedicated areas. One of the specifications of monolith synthesis in COC microchannels is the
36 total exclusion of water in the polymerization mixture. Indeed, no polymerization is observed in COC

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1 microchannels when water is present in the porogen solution. This phenomenon may be due to the
2 highly hydrophobic character of COC which disturbs homogeneity of the polymerization mixture. On
3 the opposite, polymerization occurs if the porogen is constituted of only organic solvents (usually
4 alcohols).

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5 Knapp's group has developed a UV-initiated synthesis of organic monolith in COC microchip to
6 achieve the pressure-driven reversed-phase chromatography separation of proteins [54, 55]. This
7 monolith is prepared from a mixture of ethylhexyl methacrylate and EDMA as monomers and 1-
8 decanol as porogen. Functionalization of the COC channels with benzophenone and
9 trimethylolpropane triacrylate (TMPTA) was required to firmly anchor the monolith to the channel
10 wall, as advised by Stachowiak et al. [56], to avoid the formation of voids at the monolith-wall
11 interface and to prevent movement of the monolith under high pressure. The resulting chip was then
12 applied on the interface with a MALDI-TOF and an ESI mass spectrometry system for analysis of
13 peptides mixtures. Peptide separation was achieved in Chip-LC-MS on this device but no
14 chromatographic performances such as efficiency or retention factors are given.

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15 Recently, Faure et al. synthesized a lauryl methacrylate-based monolith by UV initiation in COC
16 microchip [57]. The porogenic system was a mixture of 1-propanol and 1,4-butanediol and no surface
17 modification was performed before the introduction of the monolith prepolymer. The voids that may
18 occur between monolith and channel walls were not detrimental to the separation of neutral
19 compounds in electrochromatography mode. Efficiency reached 85 000 plates/meter.

20 DeVoe's group reported a COC microdevice filled with polymethacrylate monolith for Chip-LC with a
21 conventional LC gradient pumping system [48]. A 15 cm long monolith was formed in a serpentine
22 channel to increase peak capacity. The plastic device and needle interfacing connections could
23 withstand the outstanding pressure of 238 bar and 400 bar, respectively. The ability to locally
24 synthesize monoliths by UV initiation enabled the definition of two zones for sample cleanup/
25 enrichment and chromatographic separation, respectively. This setup allows the separation of FITC-
26 labelled peptide by gradient elution.

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27 Inorganic monoliths, such as silica monoliths, are prepared via a sol gel reaction of an alkoxy silane
28 solution. In glass chip, no surface pretreatment is required since the silanols of the microfluidic
29 channel walls are directly incorporated in the sol gel reaction. The chromatographic properties of silica
30 monoliths are simply tuned since the skeleton size dictating permeability and the microporous
31 structure dictating efficiency can be independently controlled. Another positive point in the use of
32 silica monoliths as stationary phase in chromatography is their non-swelling behavior towards organic
33 solvents, which may happen with some organic monoliths.

1 One major drawback that has refrained the use of silica monolith in Lab-on-Chip strategies is the
2 difficulty associated with their localization in precise zone. On the contrary of photopolymerized
3 organic monoliths, silica sols must be confined in areas before gelation occurs.

4 To circumvent this problem, Morishima introduced a hybrid silica-acrylate monolith that could be
5 photopolymerized [58]. Sol-gel reaction of a hybrid monomer, namely 3-
6 trimethoxysilylpropylmethacrylate resulted in the localized in-situ synthesis of acrylate-silica
7 monolith. The reversed-phase behavior of such a monolith was confirmed by the separation of two
8 neutral coumarin dyes with efficiency reaching 18 000 plates /meter.
9

10 Another approach consisted in the localization of a sol plug, thanks to a push/pull technique using air
11 pressure control. Ishida et al. [59] introduced Chip-LC with a porous monolithic octadecyl-modified
12 silica column prepared within a 15-cm serpentine separation channel of a glass microchip. Pressure-
13 driven LC separation of catechins was carried out and the influence of tapered turns in the serpentine
14 design was evaluated. This LC chip provided plate height of 55 μm .
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16 Another technique consists in the complete filling of the microchip with silica monolith and the
17 subsequent destruction of the monolith in appropriate zones, using NaOH. Silica monoliths from C1-
18 TMOS and C2-TMOS have been synthesized in-situ in glass chips using a 60 °C thermal treatment
19 [60]. The monolith in injection and elution channel was removed by flushing with 3M NaOH. C1-
20 TMOS stationary phase was tuned with 10 % C2-TMOS monomer to increase hydrophobicity and
21 therefore resolution in the separation of nitroaromatic and nitramine explosives. In regards to the low
22 hydrophobicity of this stationary phase, it appears that the incorporation of longer alkyl chains is
23 compulsory in the development of this chromatographic support.
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31 Monoliths have demonstrated many interesting features for the miniaturisation of chromatographic
32 stationary phase. A better understanding of the various synthesis parameters and their relationship with
33 chromatographic behavior is still required for the widespread of these supports in microfluidic
34 environment. But given the ease of fabrication in microchannels, no doubt monoliths will play an
35 important role in the future development of chromatography on chip.
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39 5. New materials for chromatography

40 To fabricate size-controlled nanostructure, some approaches have taken advantages of the recent
41 advances in nanotechnologies. The pre-cited COMOSS stationary phases are one example. Another
42 example, as continuous bed, is the use of membranes integrated in microfluidic devices.
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45 Porous anodic alumina was investigated as a stationary phase [61]. Demonstration of chromatographic
46 behavior was achieved on porous alumina membrane incorporated in a chip. The honeycomb array of
47 300 μm thick with channels of 100 nm diameter was tested and revealed a normal-phase character.
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Another interesting strategy based on a widely used technique in catalyst fabrication is the idea of “sacrificial” material. In order to design nanopores, Chen’s group [62, 63] introduced sacrificial silica colloids in microchannels. Silica colloids are grown inside the microfluidic device using evaporation induced self-assembly process. Then the voids between the ordered nanoparticles are filled with SU8-photoresist. After curing, the embedded nanoparticles are dissolved using a buffer oxide etch solution. The resulting continuous bed consists in a bimodal porous structure, with large nanopores corresponding to the removed nanoparticles (300 nm) and smaller interconnecting pores (30 nm) due to the contact between nanoparticles, as shown in figure 9. This nanoporous bed was only used as a sieving matrix for electrophoretic separation of DNA molecules till now. However, due to the efficient and simple control of pore size, this “sacrificial nanoparticle” approach is worth mentioning and would be of interest for chromatographic purposes.

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When investigating the various stationary phases that have been implemented in chips, it appears that commercially available designs are aiming towards existing solutions, namely porous silica beads that have given full satisfaction to HPLC users, while the recent years have seen the emergence of a new class of stationary phase, particularly pointing towards smaller dimensions (nanotubes, nanoparticles). If the realization of particle packed-bed is now fulfilled by few companies, scaling down benchtop instruments, the new materials coming up may invite analysts to apprehend bottom-up way of thinking, especially in terms of fluid manipulation.

30 31 32 33 **III. Instrumental advances and technological issues**

34 35 36 37 38 39 40 41 42 43 44 45 46 47 **1. Benchtop Chip LC: User-friendly NanoLC**

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NanoLC, which consists in pressure-driven liquid chromatography in capillary columns (10-100 μm i.d.), has well established itself in laboratories as not only the miniaturization of conventional HPLC systems, but more as a complementary technique. The use of capillary format allows the injection of smaller sample quantities. Moreover, the flow rates of few hundreds of nanolitre/min facilitate easy coupling to highly sensitive MS detection. All these features are highly suitable for proteomic area and in other specific domains of application such as enantiomeric analysis, as illustrated by a recent review [64]. Moreover, downscaling of HPLC is also addressing the issue of solvent consumption and waste management which find echoes respectively in economics (as illustrated by the recent acetonitrile shortage) and in the regulation of more environmental-friendly analysis.

There are now on the market as many nanoLC systems as HPLC suppliers. However, it appears that the packed capillary columns with numerous connecting interfaces with injectors and detection systems may impair the robustness of the technique. Moreover, practical considerations need to be

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addressed to improve a user-friendly nanoLC system, such as micro leaks and band broadening due to external effects. An argument towards integration on chip is the possibility to achieve zero dead-volume connections. With the first commercially available integrated LC-on-chip solution, Agilent Technologies has taken a top-down approach by miniaturizing both injection loop and separation column, limiting the risk of micro leaks and providing a robust instrument for nanoLC [65]. They later demonstrated that dispersions both before and after the chromatographic bed could be reduced when using chip format, illustrating the fact that not only there is a gain in instrumentation facility, but also an improvement in separation performances [30].

The pumping system is an essential instrumental part of Chip-LC. Usually, it consists in conventional pumps that are connected to the chromatographic chip through a split connection [30, 48] which use annihilates the argument of on-chip low solvent consumption, as most of solvent volume is deviated to waste to achieve an adequate flow rate. To follow the logical pathway to miniaturization, micropumps able to deliver nanolitre/min rates should be promoted. The fabrication of such micropumps has been a technological challenge. Two interesting reviews on the various options available is cited as references [66] and [67]. A first commercial approach in the coupling of micropump and Chip-LC is provided by Eksigent company [34], whose cHiPLC Nanoflex system presents a leak-less connecting station between a splitless micropump and a glass chip for Chip-LC purposes. The Microfluidic Flow control consists in two pneumatic pumps used to amplify pressure in solvent reservoirs. Two flow sensors measure the flow coming out of each individual pump heads and use the feedback to control the driving pressure. Performance of such a pump relies on fast response of the flow sensor and proper feedback from the control loop. The risk underlies when very low flow rate is requested from one pump at the boundaries of the gradient profile.

If gradient elution is nearly systematic in conventional benchtop LC analysis for complex sample, its realization on microchip is not straightforward. As mentioned before, the beginning or the end of the gradient profile requires pumps delivering few nanolitre/min. The gradient delay is another important matter when aiming at high throughput analyses. Even if mixing chamber and tubing are downsized to few nL, the low flow rate increases delay duration. Brennen et al. [68] have addressed this problem by integrating the gradient mixing chamber on chip. The approach realized on Agilent polyimide chip consists in the microfabrication of so-called "timing channels", which are parallel channels of various lengths, interfaced through a single point to the LC column, as shown in Figure 10. At initial time, the channels are filled with starting mobile phase A. As the analysis is carried out, more eluent mobile phase B enters every channel. Every time a channel is filled with B, the overall mobile phase composition sees its B-content increases. The resulting step profile can be assimilated to a linear profile if the increments in length between channels are small enough. The actual Agilent gradient

1 generator chip is made of 20 channels to get a smooth profile, imposing a 2.4 μl gradient volume. Flow
2 rate is induced by a single pump controlling B solvent entry in the parallel channels and governed by
3 microfabricated restrictors. Unfortunately, three other pumps are required to condition “timing
4 channels”, flush LC chip and inject sample, which adds complexity to the instrumental setup. Through
5 this approach, the delay duration is minimized (delay volume of few nanolitre) by directly connecting
6 gradient chip with LC chip, while the gradient volume (volume used to change from 100 % A to 100
7 % B) is permanently defined by the microchip design.
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11 Chip-LC is a mature technique which has recently seen the emergence of commercially available
12 instruments dedicated to its miniaturized environment. Miniaturization on chip has brought solutions
13 in the downsizing of dead volumes, either before (on-chip injection, on-chip gradient) or after the
14 column (direct coupling to ESI-MS). The resulting performances are expected to be remarkable,
15 proving Chip-LC to be the technique/instrument of choice in the analysis of complex sample such as
16 proteomics.
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22 **2. Portable Chip-LC: a higher level of integration**

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25 The development of liquid chromatography on chip can also be seen as an adequate answer to on-site
26 analysis. Portable analytical systems can be required for analysis directly conducted at the point of
27 sampling to provide a fast response to a very specific problem. Various situations can be encountered
28 such as point of care or point of consumption. As point of care is concerned, the market for portable,
29 user friendly analysers has already exhibited interest towards microfluidics, as illustrated by the many
30 commercially available miniaturized assays (pregnancy, diabetes, iron content). Diagnosis or follow-
31 up of medical treatment will in the future require the realization of a full analytical process (sample
32 treatment, separation, detection) in a non-chemist environment (costumer or trained person). Point of
33 consumption is another area avid of miniaturized on-site analysis, to control food freshness or water
34 quality with a fast response time.
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39 Chip-LC systems are betting on high performances in complex sample analysis. If the user-friendly
40 setup is indisputable through commercial instruments (docking station, automatic chip alignment), the
41 overall analytical chain remains a cumbersome, benchtop-sized system, usually involving two or three
42 external pumps and MS detector. The instrumentation remains impressive and hardly portable.
43 Miniaturization of pumping systems is still in its infancy and the cost of micro valves prohibits their
44 discarding. Miniaturization of the detection technique is another aspect that we will not address in this
45 discussion.
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1 An intermediate solution to on-site chromatography consists in the use of electrically activated pumps.
2 The generation of fluid flow using electrochemistry or electroosmosis is perfectly suitable to
3 miniaturization, as it delivers flow rates in the range of few hundreds nanolitre/min. On the
4 technological point of view, it requires only sets of electrodes and is running off batteries. Involving
5 cheap technology (no moving parts) it is directly integrated on chip and can be discarded after use.
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8 Electrochemical pumps are based on the generation of gas in enclosed reservoirs through electrolysis.
9 This mechanism requires low voltages, builds up pressures around 15-20 bars and can induced
10 hydrodynamic flow rates in the range of 80 nL/min through an open-tubular column [69] or a packed
11 bed [70]. In the most accomplished reduction of dead volume and delay volume, Xie and colleagues
12 [70] presented an electrochemical pump with two 20 μ l –reservoirs linked to a 1.5 nL static mixer to
13 perform a mobile phase gradient through a 1.2 cm long packed bed. The corresponding delay volume
14 was only 15s. In comparison, the gradient time was set to 30min.
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20 Electroosmotic pumps are another option to generate a flow rate under electrical actuation. Through
21 the creation of an electrical double layer on charged surface, the fluid is propelled with a velocity
22 directly related to electrical field. The resulting flow rate is then directed downstream in a field-free
23 region where the chromatographic bed sits. Integrated EOF pumps have been implemented to Chip-LC
24 systems by Lazar et al. [71] and by Collins et al. [72]. In the first case, the EOF pump consists in a
25 series of 200 unpacked nanochannels etched in glass to provide the source of electroosmosis. The
26 chosen configuration allows the implementation of gradient elution, EOF valving and injection in the 2
27 cm-long column packed with 5 μ m C18 silica particles. The main concern focused on the backpressure
28 that is issued from the chromatographic bed and requires a consistent hydraulic resistance of the
29 nanochannels.
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34 This concern was partially answered by Collins' group [72] who used a design including two
35 stationary phases, as illustrated in Figure 11. The first bed is packed with silica particles to generate
36 EOF, while the downstream separation bed is filled with silica monolith, well known for its high
37 permeability. This setup minimizes backpressure from the field free region and therefore optimizes the
38 delivered flow rate, 5.30 nL.min⁻¹.kV⁻¹ in 20 mM Tris, 60 % acetonitrile solution, in this case.
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43 A limitation to the spread of electrokinetic pumps is the low backpressure that they can withstand.
44 Indeed, if the material generating the electroosmotic flow is too permeable in comparison with the
45 following chromatographic bed, backpressure from the head of the column can result in hydrodynamic
46 backflow into the EOF/ electrochemical pump, reducing the actual flow rate. Investigations will
47 proceed to match pumping systems and downstream columns.
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3. Chip-EC: both portable and disposable

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3 If portability and user-friendly character are essential in the investment of this analysis-on-site area,
4 the fact that health is concerned makes the disposability another reason to miniaturize chromatography.
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6 If electrokinetic pumping- LC illustrate a solution towards total integration, it involves the synthesis of
7 two different beds separated by a specific membrane, with sufficient hydrodynamic restrictions to
8 avoid siphoning between solvent reservoirs or back flush upstream from LC column. Moreover, the
9 use of hydrodynamic flow in injection and separation units makes tightness a crucial point. The overall
10 technology is complex and may be costly.

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12 Electrochromatography technique appears to be the simplest answer to carry out chromatography on
13 the field in disposable devices. Not only technological requirements (electrodes, batteries) are suitable
14 to portability, but the absence of injection valves (as opposed to Chip-LC) by the integration of
15 electrokinetic injection on-chip allows the throwing-away of the overall analytical chain. This ability
16 to discard both injectors and columns i.e. the overall parts that have come into contact with sensitive
17 sample is especially interesting for cross contamination issue (biological samples) or specifically
18 damaging domains, like nuclear environment.

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20 The answer proposed by electrochromatography is to combine both pump and column, by initiating
21 electroosmosis directly from the chromatographic stationary phase. On the instrumental point of view,
22 it is the simplest unit as only one stationary phase is required and proper sealing of reservoirs is less
23 essential than in hydrodynamic flow. Electrochromatography exhibits specific features when compared
24 to pressure-driven chromatography [73]. In electroosmosis, the fluid movement arise from the surface
25 of the material, which theoretically results in identical velocity inside and outside the porous structure.
26 This results in a minimal eddy diffusion (A-term) and reduces mass transfer contribution to band
27 broadening (C-term) since the volume of stagnant mobile phase is reduced. It may counterbalance any
28 discrepancy in packing homogeneity as well. Moreover, as the flow rate is independent of the particle
29 size, it can be interesting to use smaller and smaller particles to achieve better chromatographic
30 performances, which has dramatic consequences in pressure-driven technique. Sub-1 μm particles
31 were actually investigated in CEC [74] and many new stationary phases within the sub-micron size
32 range that are now developed in microchannels will find optimal working conditions in
33 electrochromatography mode [4, 75, 76].

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35 Another point in favour of electrochromatography is the mixed-mode separation mechanism that
36 occurs during analysis. By combining electrophoresis with a stationary phase interaction, this
37 technique is able to separate both neutral and charged compounds. Besides this, He et al. [77] showed
38 that CEC can improve LC and/or CE separation by a proper selectivity on proteins, such as ovalbumin.
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Of course, when Chip-EC and CEC have not break through as competing separation techniques on the LC market, it is because drawbacks are still multiple. While hydrodynamic flow rate is externally monitored, electroosmotic flow rate is directly dependent on surface chemistry, mobile phase and electrical field in the separation channel. Electrical field finds limitations in Joule heating and instrumental resistance (usually few kV/cm), while surface chemistry can be altered over time by pH change, solute adsorption, making this technique less robust than pressure-driven chromatography. This argument becomes sterile if one considers that the two techniques are complementary and that the disposable character of Chip-EC means that the device will not be subject to the same high throughput as laboratory-based Chip-LC.

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Another challenge in electrochromatography is the dependency of flow velocity to mobile phase composition. This results in a real challenge to program gradient elution profile if complex samples are considered. Ramsey's group focus on this problem since the late 90's [5, 78]. Using an open-tubular format as the separation column, they designed a solvent programming where the separation electrical field remains constant over analysis duration, while the amounts of solvent A (less eluent) and B (more eluent) varies as a function of time. Three elution schemes are evaluated: isocratic elution, step gradient and linear gradient. If the first results were promising, the linear velocity developed through the analysis duration evolves as the mobile phase composition changes.

Recently, Watson et al. [49] address this problem by using velocity-matched pairs of buffers. In the design illustrated as Figure 12, an acrylate monolith is cast in the separation region to provide the stationary phase, while two inlets are connected to a serpentine mixer to achieve proper solvent composition. The authors have chosen to tune ionic strength in buffers in order to maintain constant flow mobility when varying acetonitrile content. This was done by calibration of the individual velocities to properly select pairs of velocity-matched buffers. When the two inlet voltages were ramped according to elution program (fig 12b), the overall flow rate remained constant, as both electrical field and electroosmotic mobilities were fixed in the separation channel.

If Chip-EC has not yet achieved robustness of benchtop Chip-LC, it addresses issues of portability and disposability and proved to be a simple solution to simple problems. Currently limited to isocratic mode, this technique has recently seen elegant advances to establish gradient elution at constant mobile phase velocity. Despite exhibiting a high potential for the future of separation science, especially towards novel stationary phases and sub-microns particle size, its widespread has been so far hold back, probably due to the lack of commercially available Chip-EC system.

4. Alternative choices

1 It has to be mentioned some non-conventional driving forces that have been investigated to achieve
2 liquid chromatography on chip. If these methods may appear as epiphenomena, they are fully taking
3 advantages of the miniaturized environment of chips to achieve chromatography and therefore should
4 be considered as innovative.

5 Shear-driven chromatography is based on the displacement of a channel wall in respect to a non-
6 moving coated wall. If high efficiencies and fast analysis have been achieved using this technique,
7 research in this area is mostly restricted to Baron and Desmet group due to technical difficulties
8 working in nanochannels [79].

9 Evaporation driven flow was also explored as a new simpler way to move mobile phase through the
10 chromatographic bed [80]. However, this mode was subjected to a number of external parameters and
11 full control of the flow rate was tedious, involving a permanent air stream over the evaporation zone.

12 Aiming at ultra miniaturization is the very specific work by Kitamori et al. [81] who described a
13 chromatographic separation realized in the extended nanospace. The size of the quartz nanochannels
14 ($270 * 280 \text{ nm}^2$) attains the range of Debye length, which results in a repulsion of charged solutes
15 towards the centre of the nanochannel. The pressure-driven flow leading to a parabolic profile,
16 repulsed solutes localized at the centre acquire a higher velocity than less charged solutes localized
17 near the channel walls. This velocity was proved to depend on Debye Length, i.e. ionic strength. In
18 the light of the above considerations, femto liquid chromatography can be considered as an extreme
19 example of Chip-LC, involving no stationary phase but the interaction (repulsion) between solutes and
20 walls. This example may not reach everyday user, but it highlights the aptitude of miniaturization to
21 initiate novel separation mechanisms.

32 IV. Conclusion

33 Outstanding achievements have been accomplished in the past five years, both in the development of
34 novel stationary phases and in the design of innovative analytical microsystems. This modern field of
35 research that was liquid chromatography on chip has taken a step further towards maturity, leaving the
36 experimental stage to gain the market place. If pressure-driven Chip-LC has found its place in
37 laboratories as a well-established miniaturized version of HPLC, Chip-EC is still facing challenges and
38 has not yet fully expressed its potential towards simplicity and portability.

39 Further Chip-LC investigations will apprehend complex analytical problems and will carry on with the
40 implementation of existing HPLC features (2D, high temperature, high pressure) to miniaturized
41 environment. On the other hand, electrochromatography has never breakthrough in capillary format
42 [73], but is easily implemented in chip-format, which augurs a very promising future. Chip-EC has not
43 yet found its “killer application” and besides its perfect suitability towards nanoworld (nanoparticles,
44 nanochannels), is still undervalued.

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For Peer Review

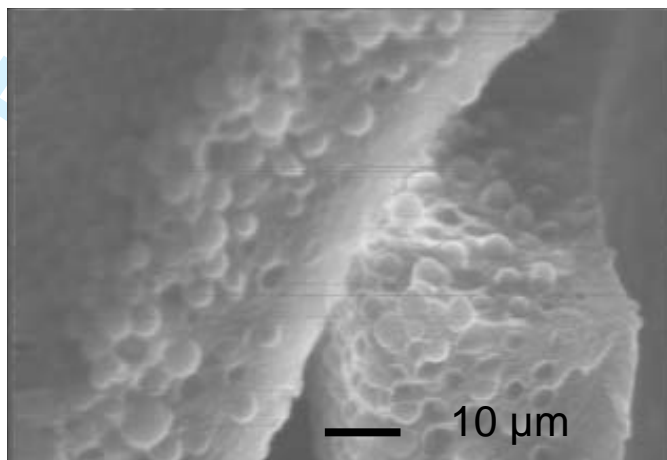


Figure 1

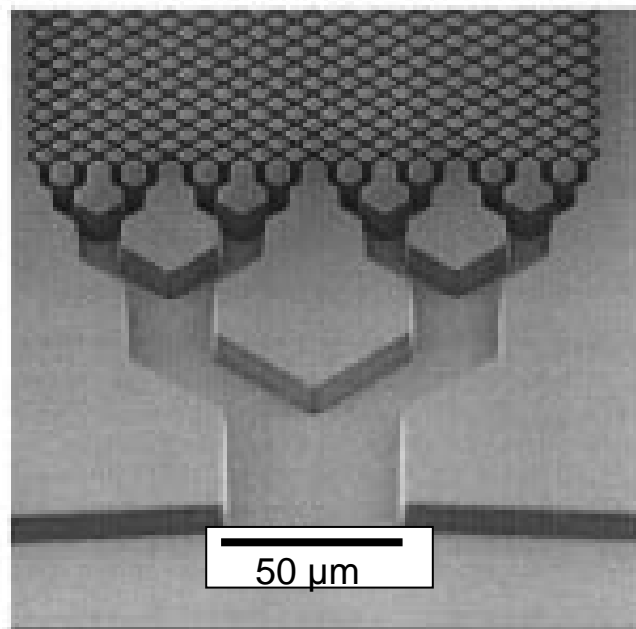


Figure 2

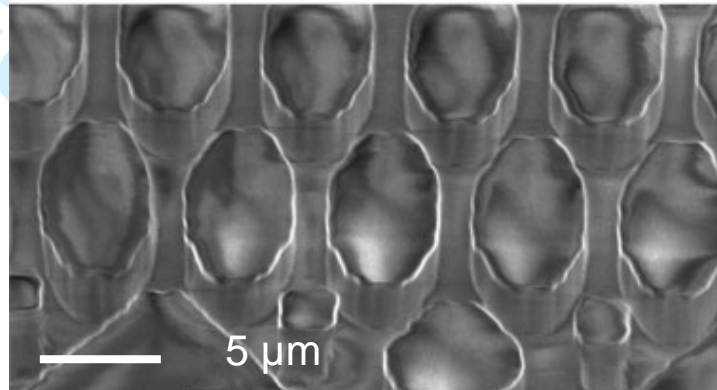


Figure 3

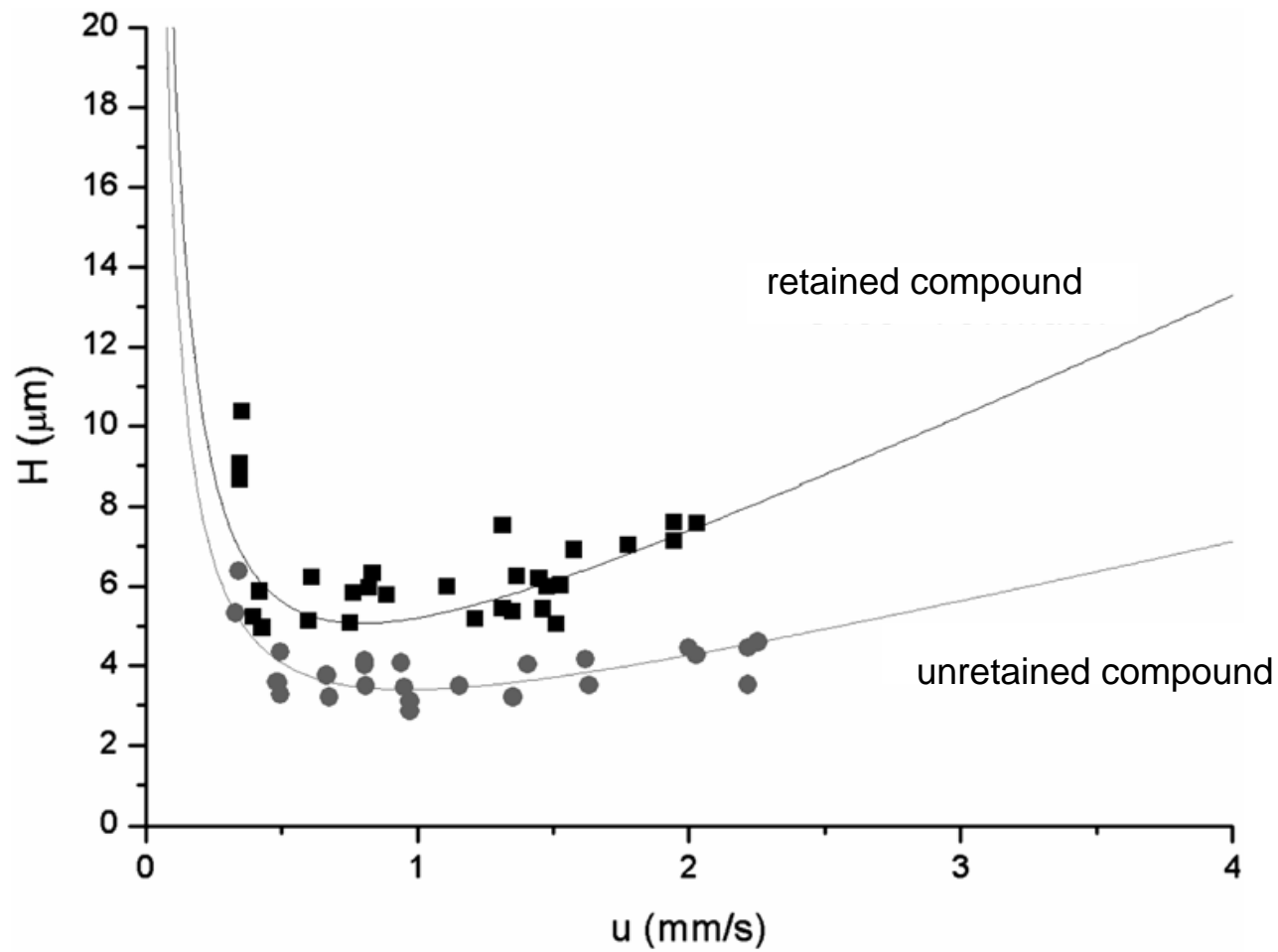


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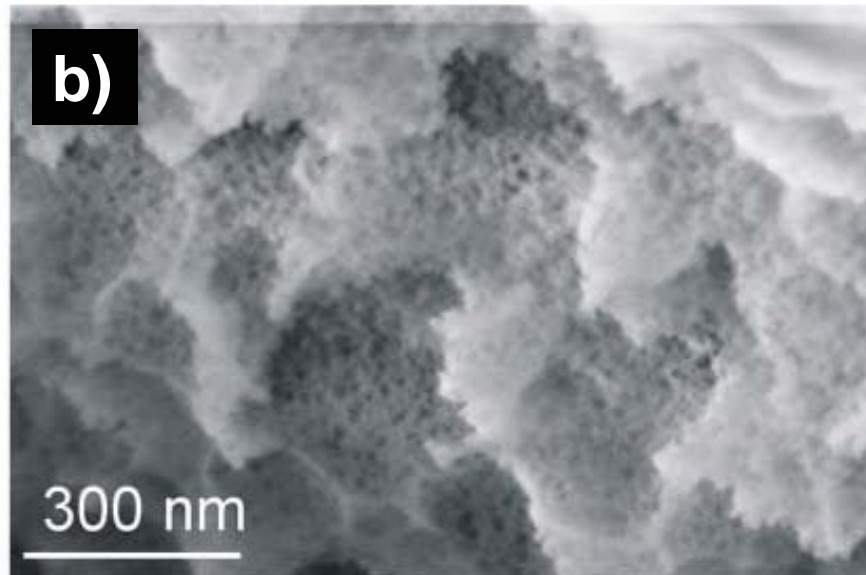
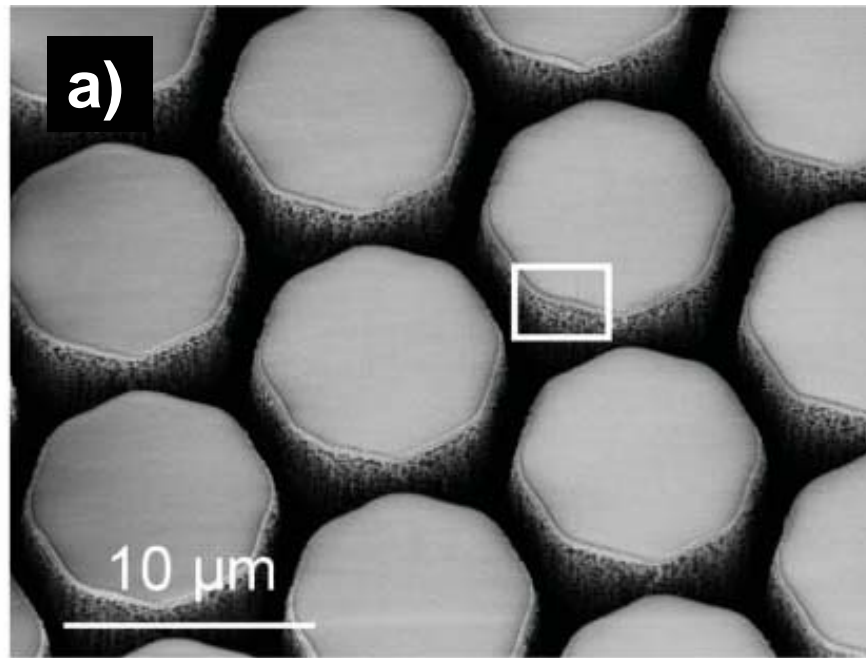


Figure 5

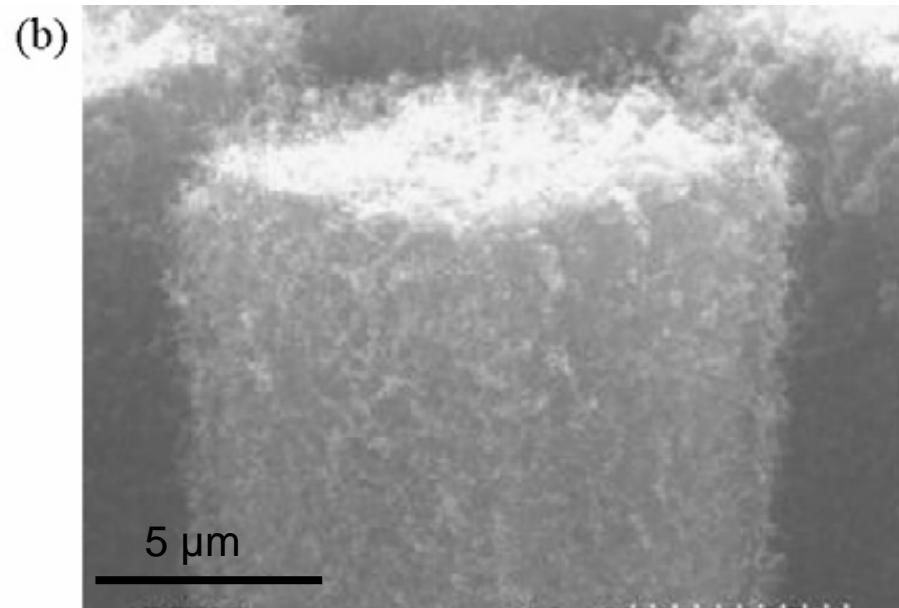
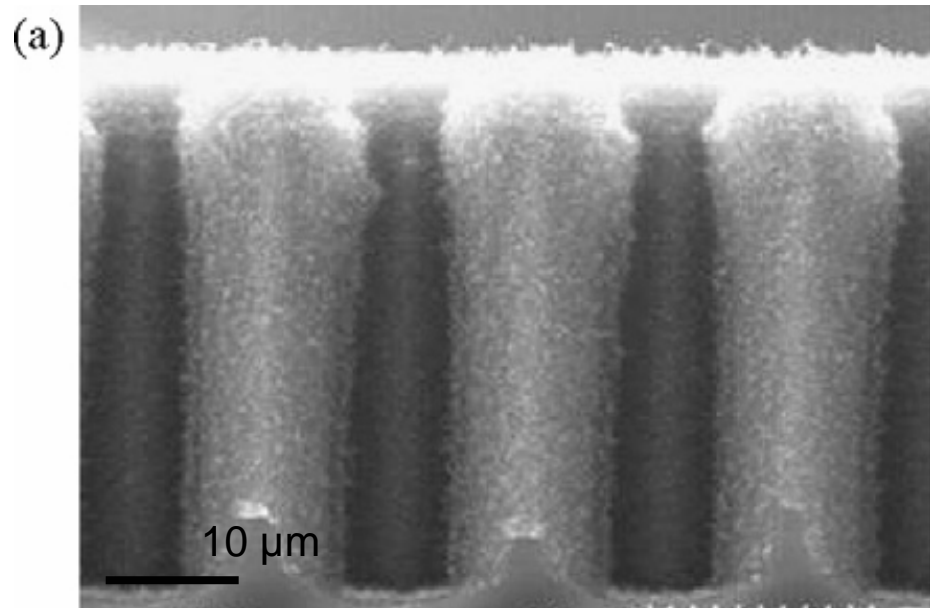


Figure 6

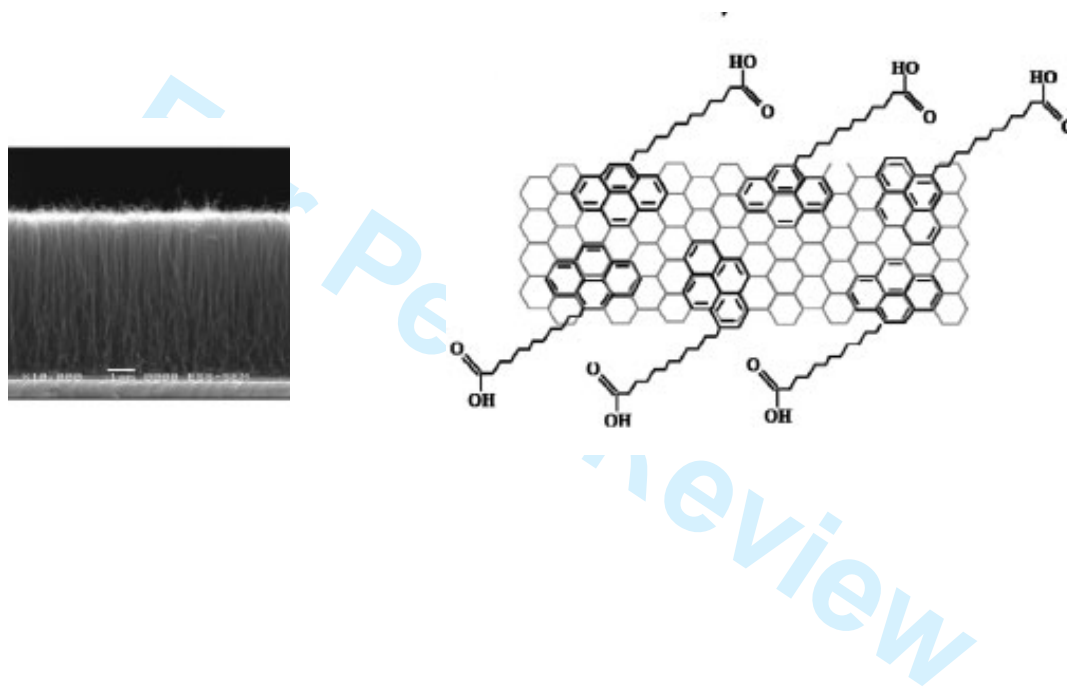


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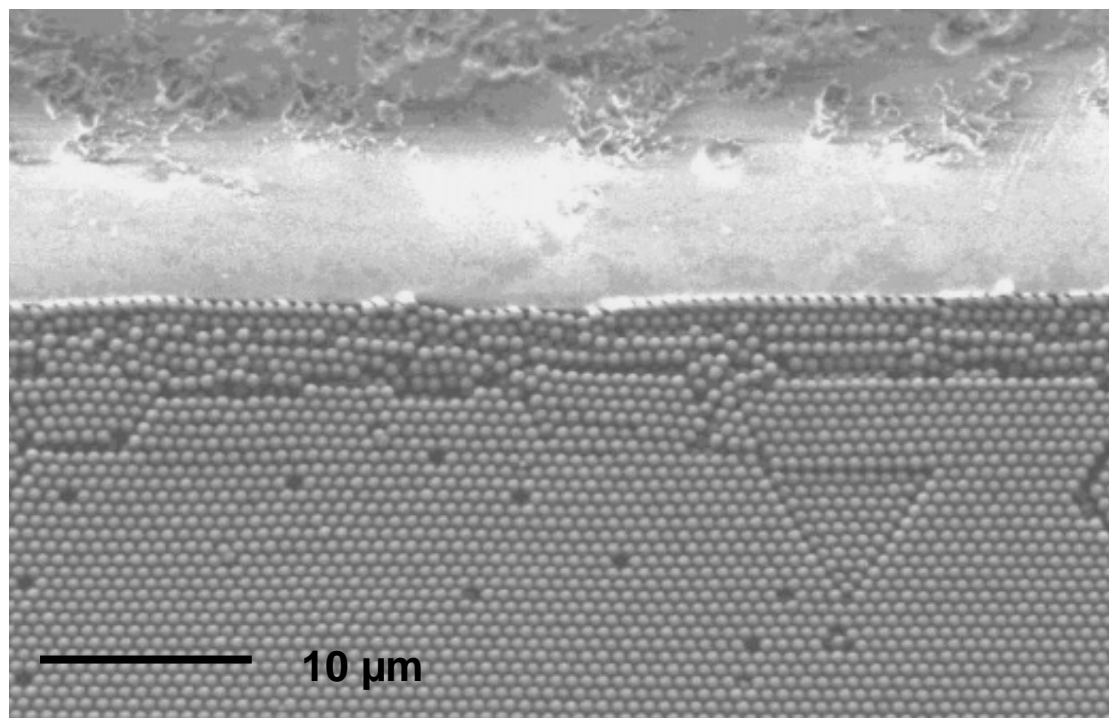


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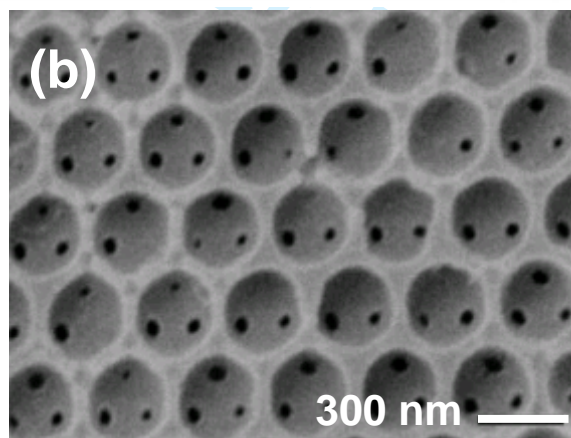
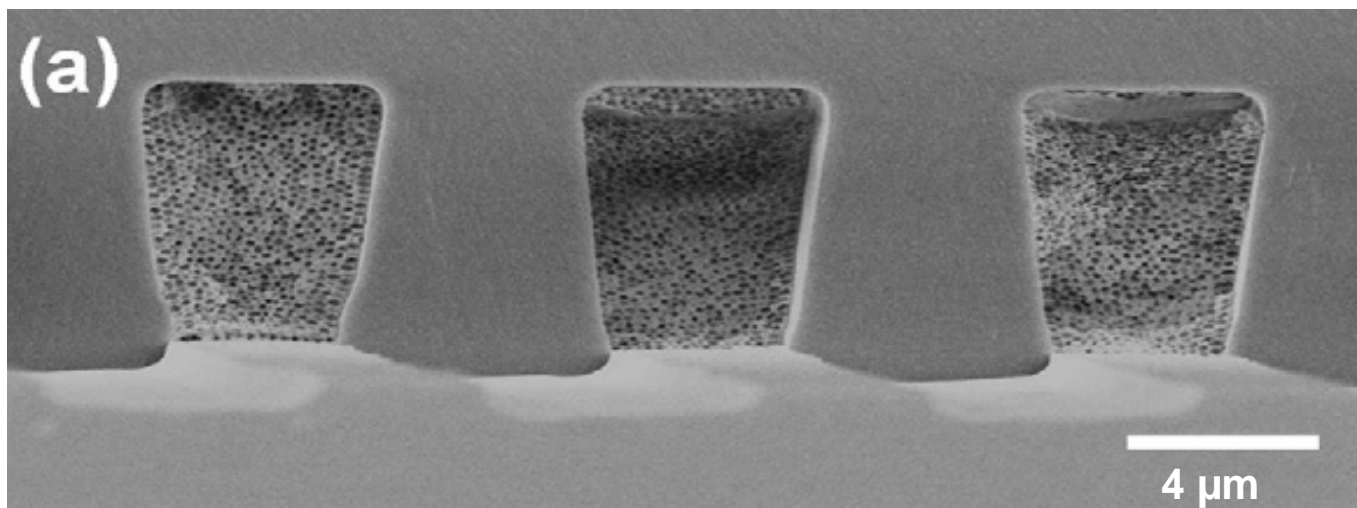


Figure 9

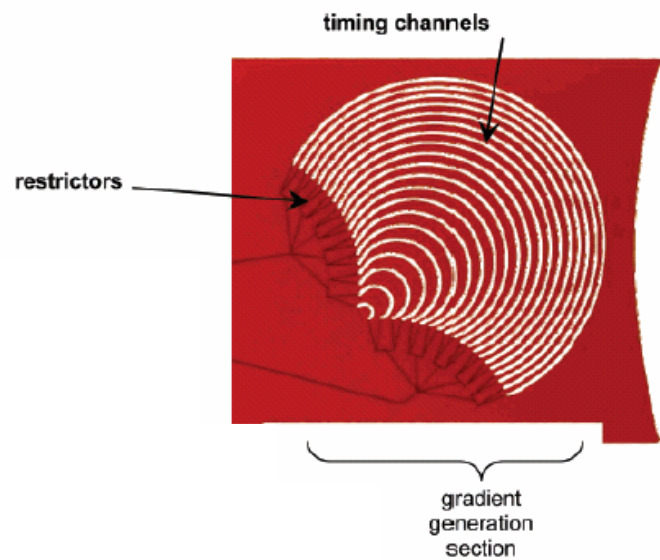
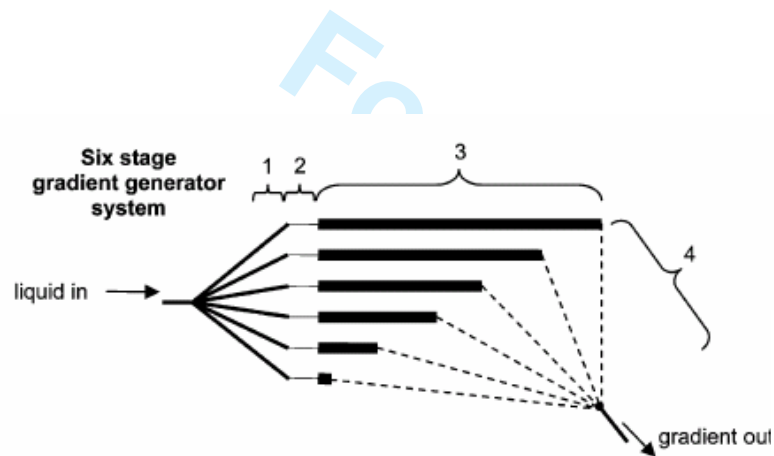


Figure 10

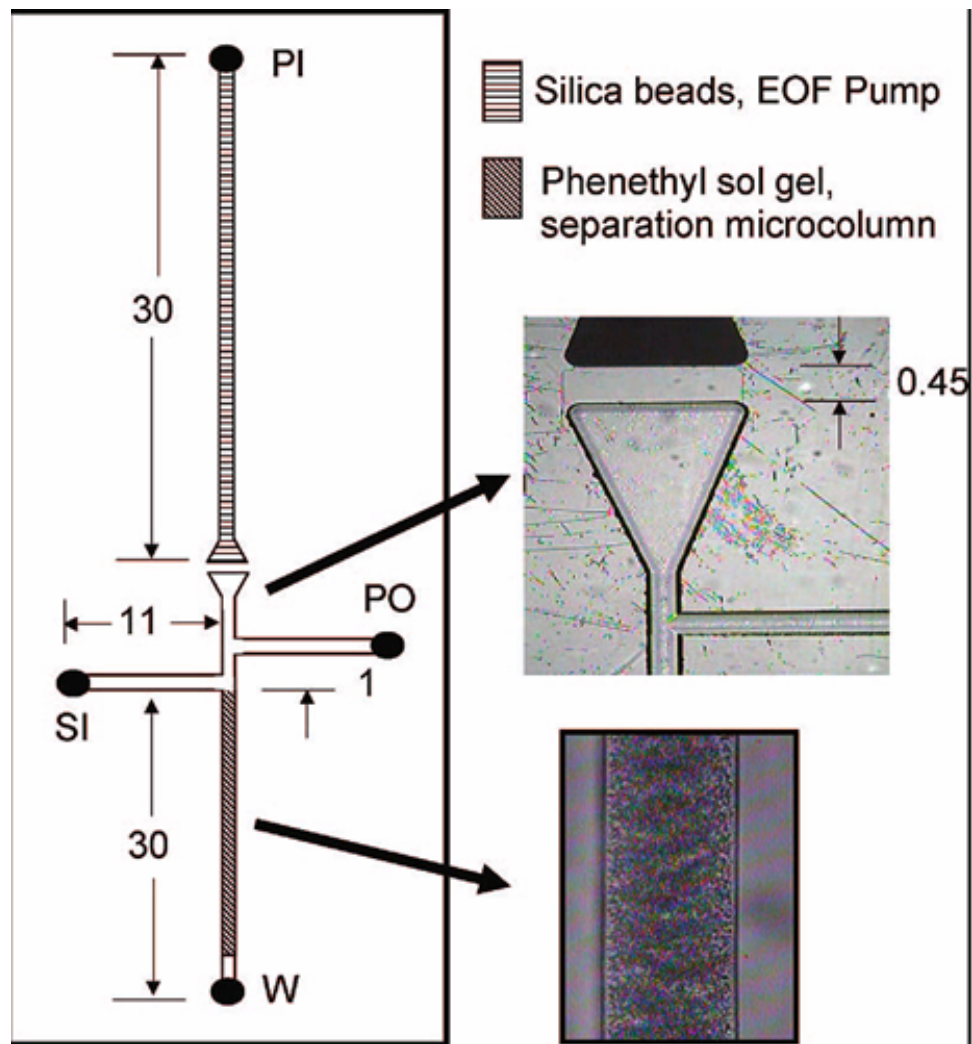


Figure 11

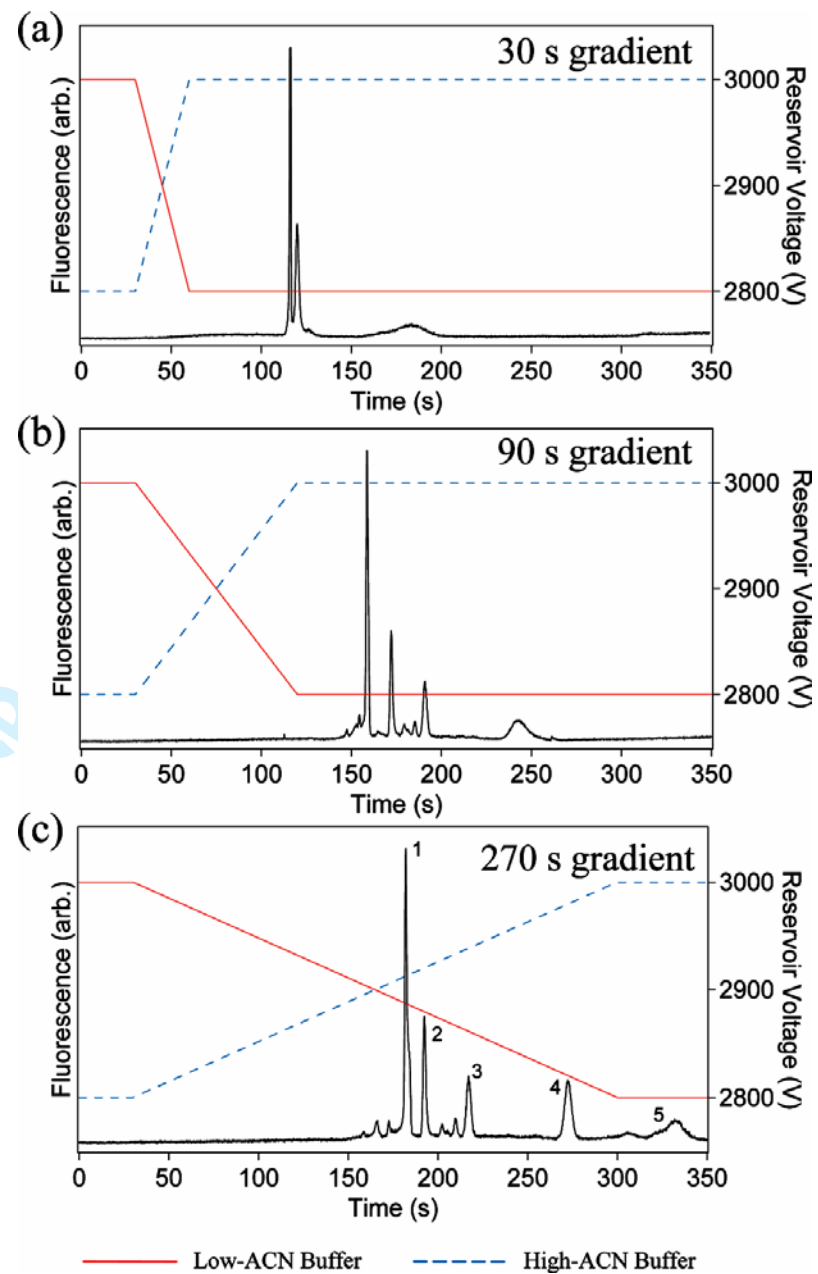
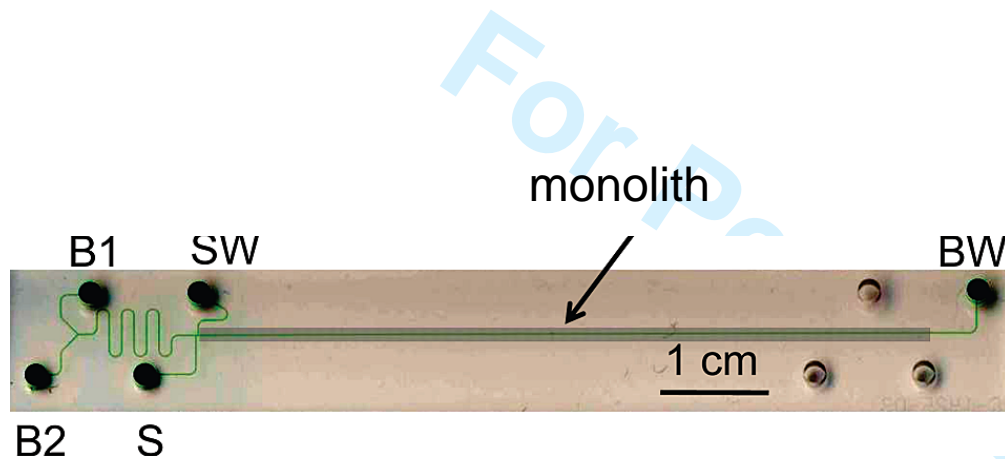


Figure 12

Liquid Chromatography on chip

Karine Faure

Laboratoire des Sciences Analytiques,
UMR UCBL - CNRS 5180, Université de Lyon
Université Claude Bernard Lyon 1
43 boulevard du 11 Novembre 1918
69622 Villeurbanne, France
karine.faure@univ-lyon1.fr

Figure captions

Figure 1. SEM images of RP-HPLC beads (Supersphere, 60 RP-8 endcapped) embedded in trimethylol-propane-trimethacrylate polymer on a 2 mm wide, 10 μm deep circular glass channel [13]

Figure 2. SEM image of microfabricated collocated monolith support structures produced in quartz by deep reactive ion etching [18]

Figure 3. SEM image of COMOSS structures imprinted from a deep reactive ion etched silicon master onto COC material [21]

Figure 4. Van Deemter curves of C440 (unretained compound - circle) and C480 (retained compound - square) in mobile phase compositions of 50/50 and 30/70 (v/v) methanol/water, respectively, obtained by pressure-driven chromatography on unmodified COC pillars [22]

Figure 5. SEM images showing porous layers generated at the surface of porous silicon pillars [23]

Figure 6. SEM images of carbon nanotubes grown on silicon COMOSS structures [24]

Figure 7. SEM image of vertically aligned carbon nanotubes directly grown in microchannel. Chemical structure of 1-pyrenedodecanoic acid modified carbon nanotube [26]

Figure 8. SEM image of colloidal silica packing in a microchannel [39]

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3 **Figure 9.** (a) SEM image of SU8 porous nanostructures produced in glass microchannel via
4 “sacrificial nanoparticle” method [62]. (b) Close-up view of a nanostructure made from 300
5 nm silica nanoparticles.
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10 **Figure 10.** Left - Schematic of gradient generation concept with 1- inlet, 2- restrictors, 3-
11 timing channels, 4- outlet. Right - photograph of a 20-stage Agilent gradient generation chip.
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17 **Figure 11.** Diagram of liquid chromatography microfluidic device including closeups of the
18 weir structure and the sol-gel stationary phase. Units are in millimeters. PI) pump inlet, PO)
19 pump outlet, SI) sample inlet, and W) waste. [72]
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24 **Figure 12.** Left - Picture of the Chip-EC device combining two buffer inlets, a serpentine
25 mixer, a cross injection region and a separation channel filled with monolith. Right -
26 Electrochromatograms of FITC-labeled peptide standards (1) bradykinin, 2) oxytocin, 3)
27 angiotensin I, 4)angiotensin IV, and 5) leucine enkephalin). Reservoir voltages on the low-
28 ACN (2%) (red, solid) and high-ACN (60 %) (blue, dashed) buffer inlets were varied as
29 indicated on gradient programming scheme. [49]
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Laboratoire des Sciences Analytiques,
UMR UCBL - CNRS 5180, Université de Lyon
Université Claude Bernard Lyon 1
43 boulevard du 11 Novembre 1918
69622 Villeurbanne, France
karine.faure@univ-lyon1.fr

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Karine Faure

Laboratoire des Sciences Analytiques,
UMR UCBL - CNRS 5180, Université de Lyon
Université Claude Bernard Lyon 1
43 boulevard du 11 Novembre 1918
69622 Villeurbanne, France
karine.faure@univ-lyon1.fr

Abbreviation list

AMPS, 2-Acrylamido-2-methylpropane sulfonic acid; **CD**, cyclodextrin;
Chip-LC, pressure-driven liquid chromatography on chip; **Chip-EC**, liquid
electrochromatography on chip; **COC**, cyclic olefin copolymer; **COMOSS**, collocated monolith
support structure; **Dns-AAs**, dansylated amino acids; **EDMA**, ethylene dimethacrylate; **EOF**,
electroosmotic flow; **ESI**, electrospray ionization; **PDMS**, polydimethylsiloxane; **TEOS**,
tetraethyl orthosilicate.