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Journal of Chromatography A, 1071 (2005) 213–222
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Integrated microfabricated systems including a purification module and an on-chip nano electrospray ionization interface for biological analysis

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Available online 10 February 2005

Abstract

We report here on an integrated microfabricated device dedicated to the preparation of biological samples prior to their on-line analysis by electrospray ionization-mass spectrometry (ESI-MS). This microfluidic device is fabricated using the negative photoresist SU-8 by microtechnology techniques. The device includes a chromatographic module plus an ESI interface for MS. The chromatographic module is dedicated to sample purification and is based on a polymer monolithic phase which includes hydrophobic moieties. The ESI interface is integrated onto the chip and is based on a capillary slot. We present here the integration of these different modules onto a single system that is fabricated via a SU-8-based microtechnology route. We present also their testing for the purification of peptide samples. This started with a partial integration step with the combination of at least two of the modules (microsystem + monolith; microsystem + nib) and their test before the fabrication and testing of fully integrated microsystems.

Keywords: Microfluidics; Integrated microsystems; Sample preparation; Mass spectrometry ESI-MS; Monolithic phase; Protein analysis

1. Introduction

Novel analytical tools named as microTAS (micro-total analysis systems) are currently emerging [1–4]. These devices integrate a whole analytical or reaction process, from the sample extraction to final analysis and characterization (and even quantification) step. Their development is due to the current need for high-throughput and automated systems. This microfluidic trend is particularly noticeable in the field of biological analysis and protein studies [5–7], where a need for high-throughput operation results from the high number of molecular species, especially proteins to be identified, characterized and sequenced so as to "know" proteomes. Such microTAS devices are also developed as diagnostic tools for medical applications.

As microsystems become smaller, operations become much faster. In addition, as they are highly integrated, there is no need for an extensive handling of the samples which can be time-consuming and causes sample loss and contamination. The former point is of particular interest for biological samples which often contain femtomolar amounts of material. Consequently, using microsystems not only provides lower analysis times but also enhances analysis conditions and sample treatment. There are numerous examples of microsystems dedicated to biological purposes both described in the literature and commercially available [6,8–10]. Microfluidic systems are mostly based on an electrically-driven pumping. Figeys and Aebersold [11] reported the use of a cartridge external to the system and inserted in the microsystem-MS connection for sample preparation. This treatment step can also be achieved using an electrically driven separation technique such as CEC, CE, MEKC (respectively capillary electrochromatography,

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doi:10.1016/j.chroma.2004.12.037

capillary electrophoresis, micellar electrokinetic chromatography). Detection relies on optical techniques or on MS techniques [12]. In the case of proteomic analysis, the last step of the protocol consists of an MS-based characterization, identification and sequencing as the latter techniques are currently the most powerful methods for protein studies [13]. Consequently, microfluidic devices dedicated to proteomic applications are mostly interfaced to MS techniques. They include an outlet connection to MS, either with a matrix-assisted laser desorption ionization (MALDI) [10] or ESI ionization mode [14,15]. The latter connection usually consists of a transfer capillary inserted in the outlet microchannel [16] and of a standard ionization emitter tip.

Other alternatives may be preferred in order to develop and fabricate a microfluidic system to be connected to ESI-MS. Firstly, in the case of an ESI-MS-based analysis an electroosmotic flow (EOF)-based pumping technique is not optimal as this implies the addition of charges in the solution present as salts. Such salts can deteriorate the ionization process of the analytes [17]. Thus, it is preferable to achieve the sample preparation on a real stationary phase and by using another pumping system (e.g. external, centrifugal. . .). Therefore, the sample treatment modules may be based on a polymer stationary phase [18–20] and an external pumping system such as a syringe-pump is used to make solutions flow through the microfluidic device. In addition, the integration of the ionization source and its fabrication using microtechnology techniques allow for improving the MS coupling quality and consequently the analysis conditions. It ensures source reproducibility and the control of fabrication as well as the suppression of dead volume [21,22] and gluing [16] problems resulting from the use of a transfer capillary inserted in the outlet microchannel.

We report here on the development of such an integrated microfluidic system for protein sample preparation before their on-line analysis by ESI-MS (Fig. 1). The system is fabricated using the negative photoresist SU-8 [23]. The system includes a chromatographic module based on a polymer monolithic phase [24] as well as an integrated interface to MS for sample ionization and having the shape of a nib [25–30]. Starting form these technological choices, the modules to be integrated on the microsystem have been first studied, developed and tested in a separate way. Their fabrication and testing have been described elsewhere. A reliable process based on the use of the negative photoresist SU-8 has also been developed for the fabrication of the microsystems [23]. We report here the integration of the modules onto a single SU-8-based system. This started with a partial integration step that consists of combining two of the modules together. Thus, monolithic phases were prepared in SU-8-based microchannels and tested for the purification of peptide samples. Then, the microsystem fabrication process was changed so that the microchannel outlet was coupled to an integrated ESI source based on a capillary slot. The latter systems were finally tested for their ability to perform on-line analysis using ESI-MS techniques of standard peptide samples that had flown through SU-8-based microchannels.

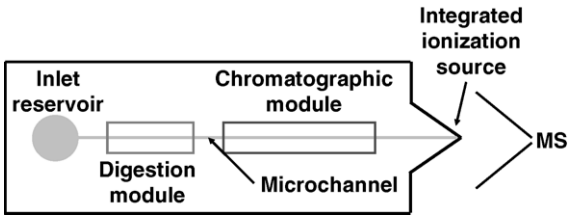

Fig. 1. Schematic representation of the microfluidic system which includes an enzymatic micro-reactor, a chromatographic device and an integrated ionization emitter tip having the shape of a nib.

2. Experimental

2.1. Materials and chemicals

The UV lamp (2×20 W, 365 nm, $I = 2100\ \mu W/cm^2$) was purchased from Elvetec (France). Lauryl methacrylate (LMA), butyl methacrylate (BMA), ethylene dimethacrylate (EDMA), 2,2′-azobisisobutyronitrile (AIBN), methanol (MeOH), ethylene glycol (EG), cyclohexanol (CyOH), acetone, sodium hydroxide (NaOH), formic acid (HCOOC), trifluoroacetic acid (TFA) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (L'isle d'Abeau, France). The commercial cytochrome *c* (Cyt C) digest was purchased from Dionex (Amsterdam, The Netherlands). Synthetic peptides having an around 20 wt.% salt content were purchased from Sigma-Aldrich. Deionized water (18.2 MΩ) was prepared using a Milli-Q system from Millipore (Billerica, MA, USA).

2.2. Microsystem fabrication

Microsystems were fabricated according to a procedure described in earlier publications [23,24]. It consists of a multilayer structure mainly based on the negative photoresist SU-8 but also pyrex and silicon. Fig. 2 illustrates the multi-layer structure.

More integrated microsystems that include a nib-shaped interface with MS were fabricated using a similar microtechnology process except that it included the fabrication of the nib in another SU-8 layer. The nanoESI interface was produced using a previously described procedure [27].

For both microsystems, accesses for the insertion of capillaries were fabricated in the main layer of SU-8. Capillaries were then glued into place using two-component epoxy glue. These capillaries allow for connecting the microsystem at its inlet and outlet to the real world.

2.3. Monolith preparation

Monolithic polymer phases were prepared using a widely described protocol based on a photopolymerization process [18]. The polymerization reaction mixture was first pre-

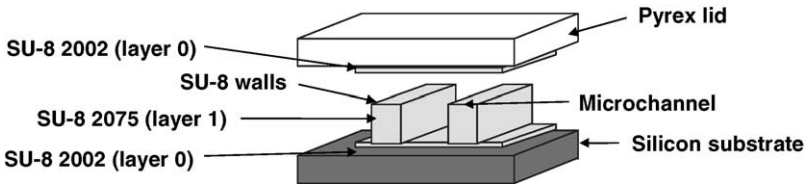

Fig. 2. Schematic representation of the multi-layer structure used for the fabrication of the SU-8-based microsystems.

pared using cross-linking (EDMA) and functional (LMA or BMA) monomers (Fig. 3), a suitable porogenic mixture and AIBN as radical initiator. The porogen consists either of a cyclohexanol–ethylene glycol (80:20, v/v) mixture in case of LMA-based monoliths or of butanediol-propan-1-ol (80:20, v/v) in case of BMA-based monoliths. The porogen and the monomeric mixtures were introduced in a 65/35 volume ratio in the polymerization mixture and AIBN as 1% (w/w) of monomers. The resulting mixture was sonicated and introduced in the microchannels. A photomask was used to define the zone to be irradiated and the system was placed under a UV lamp for around 2 h. The resulting polymer phase was washed using methanol before its use so as to remove any unreacted chemicals. These monolith preparation steps were carried out using a syringe-pump.

It should be noted that a preliminary step which consists of the treatment of the inner walls [18] so as to covalently anchor the monolithic phase in its support, was suppressed here for the in-channel polymerization. Firstly, the surface chemistry was changed between the fused-silica capillaries and the SU-8-based microsystems and secondly, we assumed that the recess in the channel geometry as well as the presence of capillaries would prevent the monolithic phase from escaping its support. We are fully aware that there may be through-pores between the channel walls and the monolithic phase that results in non-optimized conditions for the tests of the monolithic phases; nonetheless, this is not as crucial for purification steps as for separation processes, as observed here.

2.4. Off-line tests using MALDI-MS techniques

In-channel monolithic phases were first tested for the off-line purification of a peptide sample using MALDI-MS techniques (Voyager DE, Perspeptive Biosystems, Boston, MA, USA). The test protocol borrows much from the protocol used for desalting stages e.g. using a *ZipTip* material. Pumping of liquids through the monolithic material is based on the use of a syringe-pump placed upstream to the microsystem; the flow-rate was of 200 nL/min. Firstly, the column is equilibrated with two washing steps, one using the elution solution (ACN–water 50:50 (v/v), 1% TFA, 10 μL) and the other acidified water (deionized water, 1% TFA, 10 μL). The peptide solution is then loaded onto the monolithic material (3–4 μL). Following this, the monolithic phase is washed using acidified water (deionized water, 1% TFA) (10 μL). Finally, the peptides are eluted using ACN-water acidified with 1% of TFA (5 μL). Several stages of elution may then be carried out with increasing amounts of ACN in the elution solution. Solutions are collected at the tip of the outlet capillary and are later analyzed using MALDI-MS techniques. 1 μl of the solution is added to 1 μL of a matrix solution (dihydrobenzoic acid DHB, 10 mg/mL, water–MeOH 50:50 (v/v)). MALDI-MS analysis is made in the positive reflectron mode.

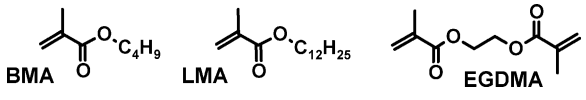

Fig. 3. Monomers used for the polymerization, LMA or lauryl methacrylate and BMA butyl methacrylate as functional monomers and EDMA or ethylene dimethacrylate as cross-linking agent.

2.5. On-line tests using ESI-MS techniques

On-line tests of the microsystems were carried out using a nanoLC–MS–MS set-up. The experiment was monitored from the nanoLC (LC Packings-Dionex, The Netherlands) for the injection of 1 μL of sample (Famos injection system, LC Packings-Dionex) and the elution in gradient conditions at a flow-rate of 200 nL/min (Ultimate Micro Pump and Accurate stream splitter, LC Packings-Dionex). The elution was performed using a gradient of solvent A (100% water, 0.1% TFA) and solvent B (95% ACN, 5% water, 0.1% TFA). This gradient corresponds to a linear increase in the ACN content from 5 to 80% in 30 min. The monolithic column was allowed to re-equilibrate for approximately 40 min before another test was run. The pressure drop during the nanoLC runs was measured using the Ultimate Micro Pump system and recorded by *Chromeleon* software (LC Packings-Dionex). Detection was achieved on QqTOF mass spectrometer, API QStar Pulsar (Applied Biosystems, MA, USA). The microsystem was connected at its inlet and outlet to a fused-silica capillary tubing (20 μm i.d.; 280 μm o.d.) using Teflon butt-to-butt connections (LC Packings-Dionex). Thus, the system was connected to the nanoLC system and at its output to a fused-silica PicoTip source (20 μm i.d.; 360 μm o.d.; 15 μm i.d. at its tip; New Objective, Cambridge, MA, USA) using the same butt-to-butt Teflon connection as previously. The tip position was adjusted to 1–2 mm in front of the MS inlet. Detection was carried out in positive mode with a 2.2–2.5 kV HV (high voltage) and spectra were acquired on a *m/z* 300–2000 range. Data acquisition was controlled by software *Bioanalyst* (Applied Biosystems) in IDA mode with a detection cycle time of 10 s. Fragmentation was triggered for any doubly charged

species reaching an intensity threshold of 30 counts with a relative collision energy that had been calculated taking account of the charge state of the selected species and with Ar as collision gas. Once selected and fragmented, eluted species were excluded from the MS–MS selection (±0.5 U mass window exclusion around the ion *m*/*z* value) for 1 min.

2.6. On-line tests of the fully integrated microsystems

Fully integrated microsystems were tested on an ion trap mass spectrometer (LCQ Deca XP+, Thermo Finnigan, San Jose, CA, USA) so as to assess the ionization performances of the integrated nib tips and the ability of the systems for on-line analysis using ESI-MS techniques. A channel of the microsystem was continuously fed at a given flow-rate (100–400 nL/min) using a syringe-pump. HV was applied on the silicon support of the microsystem, in the same way as for the stand-alone nib tips; electrical contact was achieved at the nib level where the liquid sample is in close contact with silicon. HV supply was of 2.0 kV for this series of tests on integrated microsystems. The temperature of the inlet transfer capillary of the ion trap mass spectrometer was of 100 °C. The test sample consisted of the same mixture of standard peptides as for the cleaning tests (100% water, 0.1% HCOOH; each peptide being at 1 pmol/μL). Nonetheless, here, this solution was diluted with methanol so as to give a MeOH–water 50:50 (v/v) solution, the final concentration of the peptides being then of 0.5 pmol/ μL. The mass spectra were plotted as an average over a 2 min period of signal acquisition.

3. Results and discussion

3.1. Microsystem description

We aim at developing a microsystem dedicated to protein sample preparation before their on-line analysis using ESI-MS techniques. This integrated microsystem would then be used for the treatment steps required before an MS analysis to optimize this latter, such as protein digestion, sample purification and salt removal. Typically, protein samples are injected into the microsystem inlet, proteins are digested on an enzymatic microreactor on the chip [31], the resulting peptide mixture is then cleaned, purified and eventually separated using a hydrophobic stationary phase. Finally, the resulting cleaned peptide solution is analyzed on-line using ESI-MS techniques. Fig. 1 is a schematic representation of such a microsystem. The microsystems are fabricated using the negative photoresist SU-8 by employing standard photolithography techniques [23]. We designed a multi-layered structure for the microsystem as discussed in former papers [23,24] so as to ensure the robustness of the bonding as well as its resistance to a wide variety of conditions, acidic as well as organic conditions. Firstly, the microsystem is fabricated on a silicon wafer and the cover wafer consists of Pyrex so that the system is transparent. The microchannels are comprised of two thick walls of the resist SU-8; this allows for decreasing the surface area between the bottom of the microsystem and its Pyrex cover (Fig. 2). A large surface area would result in the warping of the Pyrex lid and would thus compromise the system bonding. Lastly, two thin layers of SU-8 are deposited on the silicon wafer and on the Pyrex lid so as to promote the adhesion of the main SU-8 structure onto the top and the bottom wafers. The microchannels are linear and have a length of 3 cm a depth of 100 μm. Various width values are available onto a single wafer (from 100 to 250 μm). Due to the dimensions of the microchannels, it was seen that no separation step was conceivable using the technology we are developing and a hydraulic pumping system [24]. The channel section is too large and the channels are too short. We thus decided, using these microsystems, to focus on a simple purification step or desalting stage using a hydrophobic phase before the MS analysis of the samples.

3.2. Modules to be integrated on the microsystem

3.2.1. NanoESI interface

A first choice to be made concerning the microsystem dealt with its coupling to ESI-MS. We decided to integrate the ionization source as part of the microsystem in order to optimize the coupling conditions. Thus, any dead volume [21,22] or glue [16] problems are avoided. In addition, using a microtechnology route for the fabrication of the ionization sources should ensure their robustness and reproducibility.

In this context, we came to a nib-like design for the microfabricated sources. The emitter tip is composed of a reservoir feature, a capillary slot and a point-like structure where electrospraying occurs [25–30]. The nib prototypes were fabricated using the negative photoresist SU-8 and they were tested in stand-alone conditions so as to validate their functioning and to assess their ionization performances. We were thus able to determine the critical (geometrical) parameters in order to reach optimal analysis conditions [25], these critical parameters being the nib dimensions at its tip, both the slot width and the height of material used to fabricate the nib point structure. The ESI-MS tests were carried out first using standard peptide samples at various concentrations and HV values in the nanoESI range, and then using more complex solutions, such as protein digests [29]. It was thereby demonstrated that this alternative ionization tip having the shape of a nib is suitable for fabricating nanoESI sources and that the nib performances were very good and could even outstand those of standard capillary-based ionization tips [29].

3.2.2. Monolith

A second choice was to determine which kind of stationary phases or solid supports would be appropriate for microfluidic applications. We chose to use a polymer-based monolithic support to prepare the sample treatment modules. The proteolytic enzyme (tryspin) can be anchored in a covalent way on a monolithic support and the same type of materials can be used for the preparation of a hydrophobic stationary phase

[32,33]. In addition, these monolithic materials present a series of advantages that make them suitable for microfluidic applications. They have a double porosity, small pores which are involved in molecular interactions between the analytes and the stationary phase as well as large through-pores which facilitate the flowing through the monolithic phase [34–37]. Consequently, the resistance to mass transfer of monolithic phases is lower than those of conventional packed phases. Secondly, their preparation in situ in microchannels is much easier than the packing of porous particles for example. Finally, the monolith physical and chemical properties are easy to adjust depending on the application field. By changing the polymerization mixture composition, the porosity properties, the morphology and the functionality of the monolith can be modulated. Furthermore, the monolithic support functionalization can be performed once the monolith is formed.

We first made preliminary studies in a capillary format of different types of monolithic phases for various applications. In particular, we developed hydrophobic phases for desalting or separation purposes. We thus determined reliable conditions for the polymerization as well as suitable reaction parameters, such as the nature of the porogen, which is the solvent to be used for the polymerization [24]. For example, a binary porogen composed of cyclohexanol and ethylene glycol was seen to be appropriate for the preparation of phases based on LMA, giving a good balance between micro- and macro-pores. The scanning electron microscopy (SEM) photograph of the phase (Fig. 4) shows that the monolith is composed of particles having a regular size and a homogeneous repartition along the capillary section. In addition, no large through-pores which prevent the analytes from interacting with the stationary phase were observed, as was the case for other monolithic phases that we prepared [24]. This LMA-based phase demonstrated good separation capabilities even for very low amounts of biological material, down to 0.8 fmol of a Cyt C digest [24].

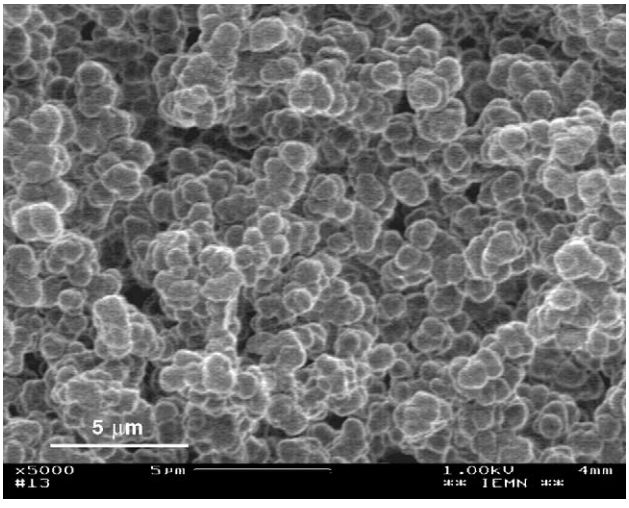

Fig. 4. Scanning electron microscopy (SEM) photograph of a section of a monolithic phase prepared from LMA/EDMA and a binary porogenic mixture.

3.2.3. Partial integration

Once every module as well as the fabrication route for the microsystem had been determined and carefully designed and studied, we moved to the integration of the different parts towards the production of fully integrated microsystems.

3.3. On-line tests of nanoESI interfaces

The first step consisted in validating on-line analysis using nib tips. There were indeed still some doubts left concerning the tip consumption and the sample flow-rate in the capillary slot during the electrospray ionization.

Thus, a nib structure (slot width of 10 μm) was tested for on-line analysis using a nanoLC separation step. A standard capillary (i.d. 10 μm; o.d. 280 μm) was glued in place on a nib structure using a two-component epoxy glue (Fig. 5). This was connected to the outlet of a nanocolumn (PepMap, i.d. 75 μm) for the ionization and analysis of the eluted solution from the column. The MS detection was carried out using an ion trap mass spectrometer, LCQ Deca XP+. The analysis conditions in ESI-MS were not changed compared to usual analysis: the flow-rate through the column was maintained at 100 nL/min and the ionization voltage was of 1.5 kV, as used with a PicoTip ionization emitter tip. The separation test was carried out on 800 fmol of a commercial Cyt C digest using routine conditions.

The spray was seen to be stable for several hours (several nanoLC runs) as expected with a standard tip. The analysis of the solution eluted from the column was possible and the same as using routine conditions. Fig. 6 presents the base peak trace of one separation run; the mass spectrum of the Fragment 92–99 which is eluted at 23.8 min is placed in insert. This preliminary test for on-line analysis using a nib tip shows that this kind of tips is entirely reliable for on-line analysis, the sample being continuously fed into the capillary

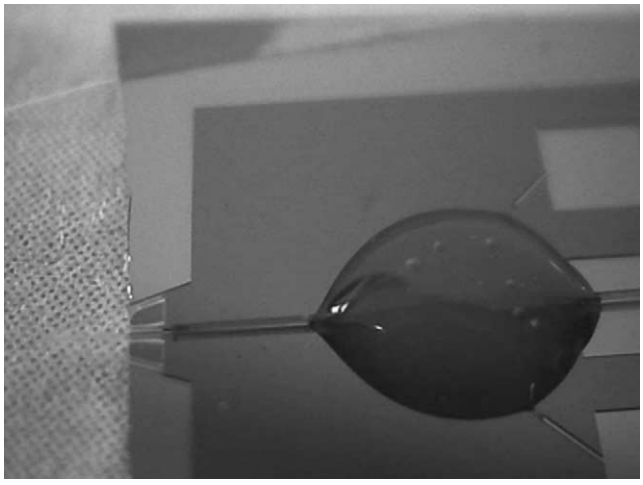

Fig. 5. Photograph of a nib-shape nanoESI interface used for on-line tests. The nib structure is fabricated using the negative photoresist SU-8 and its capillary slot is coupled to standard capillary tubing (i.d. 10 μm) for on-line MS analysis.

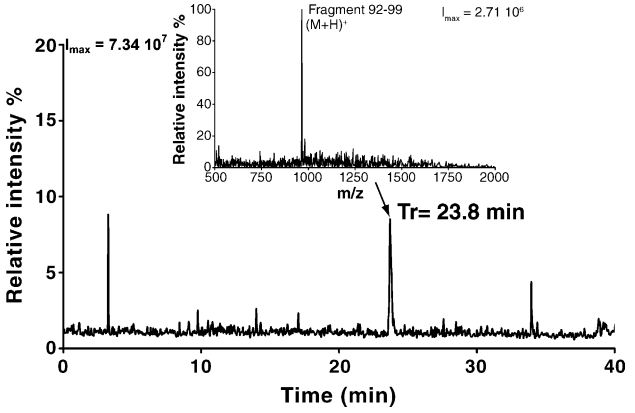

Fig. 6. Base peak trace obtained for the separation of 800 fmol of a Cyt C digest using a nanoLC set-up coupled to a nib-shaped nanoESI interface. In insert, mass spectrum corresponding to the Fragment 92–99 of Cyt C, which is eluted at 23.8 min. (flow-rate 100 nL/min, HV of 1.5 kV).

slot, and not only for stand-alone analysis, the sample being in this latter case simply dropped in the reservoir feature of the nib. Thus, nib tips can be used as the outlet interface of microfluidic systems for the on-line analysis of samples by ESI-MS techniques.

3.4. In-channel monolithic column

Another step towards the integration was to polymerize monolithic materials in microchannels. The microfluidic systems that we use were fabricated according to the procedure previously described. The system consists of linear microchannels built using the negative photoresist SU-8 between two walls of the resist. The polymerization of monolithic phases had already been validated in SU-8 microchannels as previously described by us [24]; the presence of a thin layer of SU-8 on the top wafer did not hinder the photopolymerization process. A photomask was placed above the microchannel for the spatial control of the polymerization reaction. Thus, the monolithic phase was prepared only in the irradiated part of the microchannel. The monolithic phase can be visualized at the end of the polymerization process as a white trace in the microchannel [24]. As we aimed here at preparing a phase dedicated to sample cleaning and desalting, we use less hydrophobic monomers, such as butyl methacrylate mixed with ethylene dimethacrylate. Nonetheless, the molar ratio was changed to a 50:50 mixture of BMA/EDMA to give a polymer with a higher level of cross-linkage.

It should be noted that using a whole microsystem having several microchannels, the contact surface area was large enough so that the bonding was able to resist the use of various chemicals required for the preparation of the monolithic column as well as the high pressure observed when solutions flow through the column. Nonetheless, it was not too large and prevents the cover wafer from warping. This pressure drop was measured to be of around 50 bar using *Chromeleon* software on the nanoLC set-up.

3.5. Off-line tests

We first decided to test the in-channel monolithic columns for the purification of peptide samples. The test sample consisted of a series of commercial peptides having a high level of salts (up to 20 wt.% of salt for each peptide). The protocol of the tests borrows much from a standard *ZipTip* protocol. The sample is first loaded onto the monolithic column. This is then washed using acidic water (water, 1% TFA) so as to remove species that are not specifically trapped onto the phase. Then, the peptides are eluted using ACN–water mixtures acidified with 1% TFA. The solution flow was monitored using a syringe-pump and the eluted solutions were collected at the outlet of the column in microtubes. The solutions to be pumped through the column were changed at almost the same time as the microtubes for the collection of eluted solutions. Finally, the collected solutions were analyzed off-line using MALDI-MS techniques (DHB matrix).

Fig. 7 presents the mass spectra obtained for the peptide test sample (Fig. 7A) as well as for the eluted solution (Fig. 7B). It was first seen that the test sample contained a high level of impurities, salts as well as polymers. After passing through the monolithic module, the peptide solution was seen to be cleaned; no salt adduct was detected and the con-

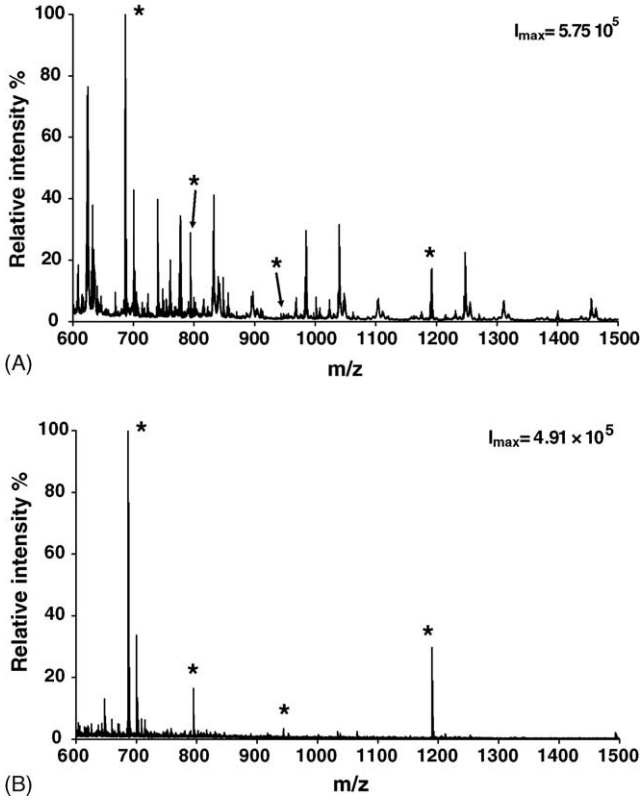

Fig. 7. MALDI-MS mass spectra obtained for the off-line tests of an in-channel monolithic phase using a test solution composed of standard peptides and impurities. Peptides are labelled with stars (matrix DHB). (A) Native peptide solution. (B) Eluted solution after passing through the monolithic column.

tamination had entirely disappeared. The peptides could be easily identified.

Nonetheless, this protocol was not appropriate as we did not have a continuous picture of the elution from the monolithic column. In other terms, the changes of the microtubes were more or less arbitrary and the results were not as relevant as those obtained using on-line analysis.

3.6. On-line tests

The microsystem including a monolithic phase was thus also used for on-line tests. As already discussed, this is more relevant as the former tests as the precise time for the peptide elution can be determined. The microsystem was thus connected to a nanoLC set-up upstream to it and to a standard ionization source downstream to it for an on-line analysis using ESI-MS techniques.

The experiment was monitored from the nanoLC set-up for the injection of the test sample and its elution using a gradient based on deionized water and acetonitrile. Thus, 1 μL of the same peptide sample as before having a high contamination level and each peptide being at around 1 pmol/μL was injected into the system. The elution was then made using a gradient between solvent A (100% water, 0.1% TFA) and solvent B (95% ACN, 5% water, 0.1% TFA), with a one-step linear increase in solvent B in 30 min. The MS analysis was carried out in MS and MS–MS modes. Every time MS detects ion having an intensity higher than a 30 counts threshold, this ion is fragmented.

The MS and MS–MS traces (Fig. 8A and B) of the experiment showed that the peptides were eluted in a 4-min duration period between 17 and 21 min, which corresponds to an amount of ACN of around 50%. All the peptides injected in the test sample are detected on the mass spectrum corresponding to this 4 min duration. Peaks corresponding to peptides are labelled with a sign on this mass spectrum (Fig. 9). A close look at each peak shows that the peptides are pretty well desalted and that the monolithic column was efficient for the sample cleaning (Fig. 10A–D).

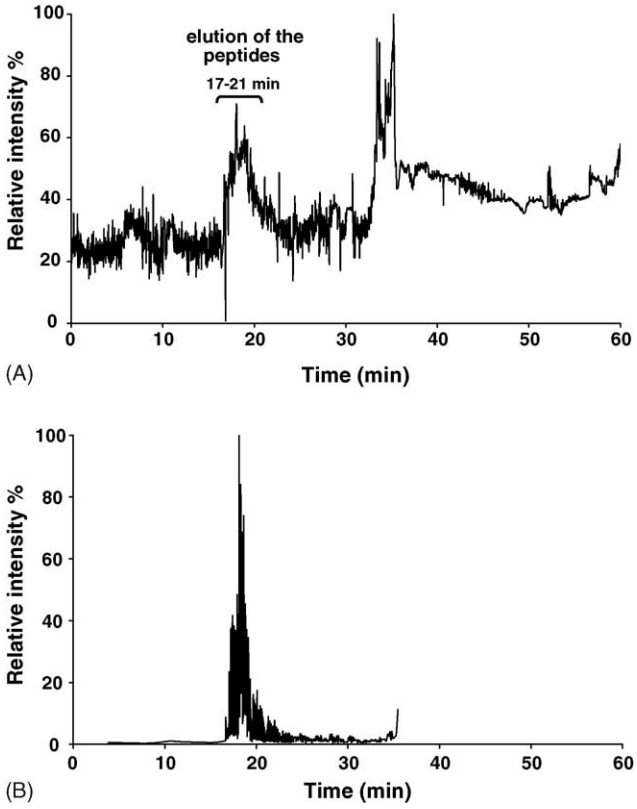

Fig. 8. MS (A) and MS–MS (B) traces obtained for on-line tests of an in-channel monolithic column. Peptides are eluted between 17 and 21 min (flow-rate of 200 nL/min, HV of 2.2–2.5 kV).

3.7. Fully integrated microsystems

3.7.1. Description

The last step in the development of our systems was the integration of the different modules, the monolithic column and the ionization nib tip, onto a single SU-8-based microsystem. The first prototype which we designed is shown in Fig. 1. It is fabricated using the negative photoresist SU-8 and it includes a monolith-based purification module and an on-chip ionization emitter tip having the shape of a nib. Firstly, the microtechnology process to produce the microsystems was modified to include the fabrication of the nib tip. The fabrication of the integrated nib tip is basically the same as this of isolated nib tips; the nib structure is produced using photolithography techniques on a Si wafer, which is cleaved at the end of the fabrication process so as to release the point of the nib. This is achieved in a first layer of SU-8 which is directly deposited onto the Si wafer. Photographs of the resulting fabricated microsystem are presented in Fig. 11, with an enlarged view corresponding to the integrated nib tip (Fig. 11, lower panel).

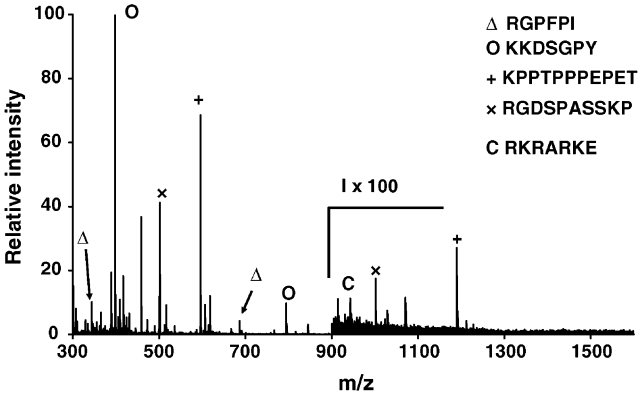

Fig. 9. On-line tests of an in-channel monolithic column: mass spectrum corresponding to the 17–21 min duration time. Peptides are labelled with signs (flow-rate of 200 nL/min, HV of 2.2–2.5 kV).

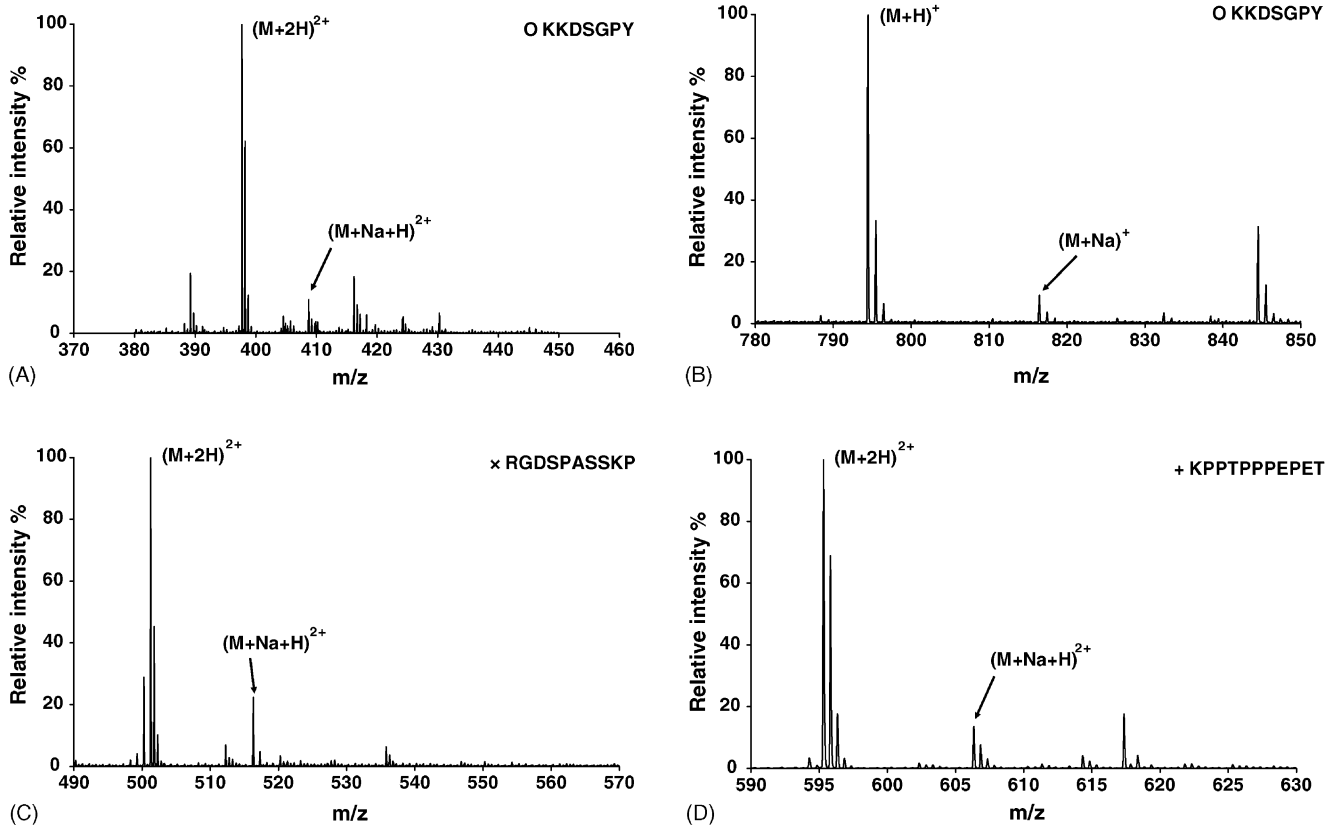

Fig. 10. Zooms on the peptide peaks in the mass spectrum corresponding to the 17–21 min elution time. (A) Peak corresponding to the $(M + 2H)^{2+}$ species of KKDSGPY (MW = 793.40). (B) Peak corresponding to the $(M + H)^{+}$ species of KKDSGPY (MW = 793.40). (C) Peak corresponding to the $(M + 2H)^{2+}$ species of RGDSPASSKP (MW = 1000.49). (D) Peak corresponding to the $(M + 2H)^{2+}$ species of KPPTPPPEPET (MW = 1188.60). (Flow-rate of 200 nL/min, HV of 2.2–2.5 kV.)

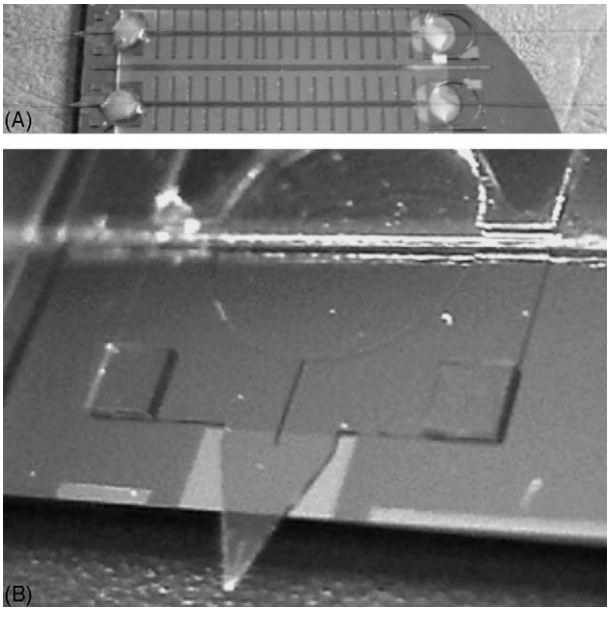

Fig. 11. Photographs of an integrated microsystem fabricated using the negative photoresist SU-8. (A) Views of two independent analysis devices composed of microchannels and nanoESI interfaces having the shape of nib. (B) Enlarged view of the interface for MS based on a capillary slot.

3.8. On-line tests of empty fully integrated microsystems

Fully integrated microsystems that are composed of microchannels as well as nib-like outlet interfaces were tested for on-line analysis before the preparation of any monolithic phase. This allowed us to check the reliability of the integrated ionization nib-like emitter tips and the relevancy of their dimensions for on-line analysis of peptide samples after their preparation onto the microsystem. On-line tests of empty microsystems were carried out on an ion trap mass spectrometer in similar conditions as for the stand-alone nib-like ionization emitter tips: the ionization voltage was directly applied onto the Si bottom wafer that supports the SU-8-based feature. The microsystem was continuously fed with a peptide solution (0.5 pmol/μL each, water–MeOH 50:50, 0.05% HCOOH) using a syringe-pump. The liquid flow-rate was in a 100–400 nL/min range. These were relatively high flow-rate values but they were linked to the comparatively high value of the nib tip slot width. The latter was determined to be of around 20 μm using an SEM photograph (enlarged view). The optimal HV supply to observe a stable spray was seen to be of 2.0 kV. This high value is again to be accounted by the large features of the nibs and the resulting high flow-rate to be used. It was also seen that the temperature of the inlet transfer capillary of the ion trap mass spectrometer had to

be changed and decreased to improve electrospray ionization phenomena. As a matter of fact, as the liquid flow-rate was high, the droplets that are formed at the nib tip were large and the charge concentration is relatively low. A too high temperature was observed to prevent from the peptide ionization as charges were killed due to the high temperature of the inlet capillary. Thus, the MS inlet transfer capillary was only heated at 100 °C for this series of experiments.

These tests showed that on-line analysis using integrated microsystems including a nib-like outlet interface was possible on a 100–400 nL/min range of flow-rate values. Mass spectra were plotted as an average over 2 min of signal acquisition. The mass spectra exhibit a high maximal intensity and all the peptides were detected and identified. Fig. 12 represents the mass spectrum obtained for the on-line test of a fully integrated microsystem using a 100 nL/min flow-rate. Peaks corresponding to peptide species are labeled with a star. Nonetheless, it should be noted that the signal-to-noise ratio (S/N) on these mass spectra was pretty low. The tested sample consisted of a mixture of peptides (0.5 pmol/μL) having a high amount of salts. This sample was first prepared and used for the test of in-channel monolithic phases to assess their ability to purify and desalt peptide samples (as described above). This high amount of salt hinders electrospray ionization phenomena. This results in a low S/N of the mass spectra as obtained in this series of experiments.

Nonetheless, the analysis performances of these integrated nib tips were outstood by those of stand-alone nibs that had already been tested and described [25,29]. For these integrated prototypes, the run conditions were not satisfying, the flow-rate to use was too high, HV values were in the 2.0 kV range and ionization conditions were seen not to be suitable for on-line analysis. As already mentioned, this is to be linked to the relatively large dimensions of the nib-like outlet interfaces. Thus, we decided to first change and improve the nib shape and dimensions so as to enhance the analysis conditions and

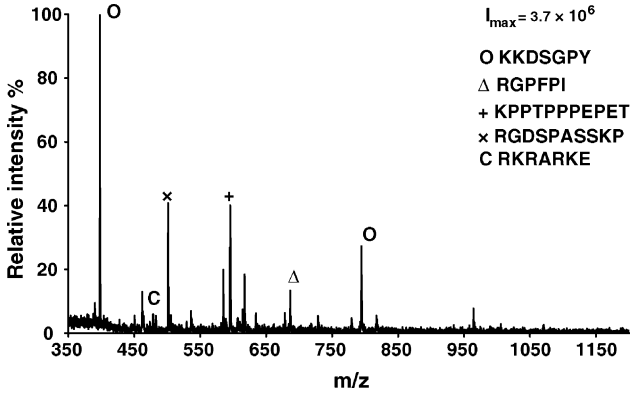

Fig. 12. Mass spectrum obtained for the on-line test of an empty integrated microsystem using its nib-like outlet interface. Flow-rate of 100 nL/min, HV supply of 2.0 kV, temperature of the MS inlet capillary of 100 °C, peptides (KKDSGPY MW = 793.40; RGDSPASSKP MW = 1000.49; KPPTPPPEPET MW = 1188.60; RGPFPI MW = 685.39; RKRARKE MW = 942.58) at 0.5 pmol/μL each (water–MeOH 50:50 (v/v), 0.05% HCOOH solution).

results before the preparation of any monolithic phase in a microchannel and its test for sample cleaning and desalting.

4. Conclusion

In this paper we described the development of integrated microsystems dedicated to on-line analysis of protein samples using ESI-MS techniques. We showed the separate development of the modules and their integration into a microsystem consisting of a chromatographic module, an enzymatic micro-reactor for the digestion of proteins and an interface for mass spectrometry that has the shape of a nib. The fabrication route for the microsystem used the epoxy-based negative photoresist SU-8. In-channel monolithic columns were successfully prepared and tested both on-line and off-line for the desalting of peptide samples. Integrated microsystems including a nib-shaped outlet interface were fabricated and tested for the characterization of the ionization interface.

Acknowledgements

The authors would like to thank the *GenHomme* research network *(BioChipLab* consortium). The Mass Spectrometry facility used for this study is funded by the European community (FEDER), the Région Nord-Pas de Calais (France), the CNRS and the Université des Sciences et Technologies de Lille. We also would like to thank Christophe Boyaval from IEMN for the SEM images as well as Adeline Page from the Mass Spectrometry facility for her kind help on the ion trap mass spectrometer. Finally, we would like to thank Dr. Christian Rolando for his help in supervising this work.

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