Anal. Chem. **2002,** *74,* 784−789

Electrochromatography in Microchips: Reversed-Phase Separation of Peptides and Amino Acids Using Photopatterned Rigid Polymer Monoliths

Daniel J. Throckmorton, Timothy J. Shepodd, and Anup K. Singh*

Chemical & Radiation Detection Laboratories, Sandia National Laboratories, Livermore, California 94551

A microfabricated glass chip containing fluidic channels filled with polymer monolith has been developed for reversed-phase electrochromatography. Acrylate-based porous polymer monoliths were cast in the channels by photopolymerization to serve as a robust and uniform stationary phase. UV light-initiated polymerization allows for patterning of polymer stationary phase in the microchip, analogous to photolithography, using a mask and a UV lamp for optimal design of injection, separation, and detection manifolds. The monoliths are cast in situ in less than 10 min, are very reproducible with respect to separation characteristics, and allow easy manipulation of separation parameters such as charge, hydrophobicity, and pore size. Moreover, the solvent used to cast the polymer enables electroosmotic flow, allowing the separation channel to be conditioned without need for high-pressure pumps. The microchip was used for separation of bioactive peptides and amino acids labeled with a fluorogenic dye (naphthalene-2,3-dicarboxaldehyde) followed by laser-induced fluorescence detection using a Kr^+ ion laser. The microchip-based separations were fast (six peptides in 45 s), efficient (up to 600 000 plates/m), and outperformed the capillary-based separations in both speed and efficiency. We have also developed a method for complete removal of polymer from the channels by thermal incineration to regenerate the glass chips.

Separation of biological molecules using microfluidic chips has attracted significant attention recently, as microchips offer a number of advantages including speed of analysis, portability, ability to multiplex, and compatibility with integration allowing development of "micro-total-analysis systems (μTAS)". Numerous separation processes have been adapted to microchips for analysis of proteins and peptides. These include zone electrophoresis,[1−4] sieving gel electrophoresis,[5,6] isoelectric focusing,[7,8] micellar electrokinetic chromatography,[9] and electrochromatography.[10] The majority of these separations are performed in an open or surface-modified microfluidic channel and predominantly use electrophoresis as the basis of separation.

Chromatography (e.g., HPLC) has become the most versatile and reliable method for separating proteins and peptides; consequently, efforts have been directed toward miniaturizing chromatography in chips. The major challenge in performing chip chromatography is the difficulty of placing the stationary phase in channels. Uniform packing of channels in a chip with particles and creation of retaining frits are practically impossible, especially in complex architectures comprising curved and intersecting channels. A chip with channels packed with 5-μm C18 beads has been developed,[11] but separation was poor due to nonuniform packing. Microchips with a two-dimensional array of posts in a channel were fabricated to mimic a packed bed and separation of dyes and peptides was demonstrated.[12,13] However, the post array has much lower surface area than typical packed beds and extra steps were needed to chemically modify the posts. Chips for open tubular chromatography have been developed using surface polymerization of channel walls,[4] but these chips suffer from low surface area and hence lower loading capacity. Recently, casting of soft polymers in chips for electrochromatography and pressure-driven chromatography has been reported.[10] The results of chip separation were good and compared well with the capillary-based separations. However, in this approach, chemical initiation is used for polymerization, and the polymer cannot be localized. A microchip with channels completely filled with polymer may not be optimum as it may be desired to have open segments for detection and injection purposes.

* Corresponding author: (phone) 925-294-1260; (fax) 925-294-3020; (e-mail) aksingh@sandia.gov.

(1) Chiem, N.; Harrison, D. *Anal. Chem.* **1997**, *69*, 373−378.
(2) Colyer, C.; Mangru, S.; Harrison, D. *J. Chromatogr., A* **1997**, *781*, 271−276.
(3) Liu, Y.; Foote, R.; Jacobson, S.; Ramsey, R.; Ramsey, J. *Anal. Chem.* **2000**, *72*, 4608−4613.
(4) Gottschlich, N.; Jacobsen, S.; Culbertson, C.; Ramsey, J. *Anal. Chem.* **2001**, *73*, 2669−2674.
(5) Yao, S.; Anex, D.; Caldwell, W.; Arnold, D.; Smith, K.; Schultz, P. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5372−5377.
(6) Bousse, L.; Mouradian, S.; Minalla, A.; Yee, H.; Williams, K.; Dudrow, R. *Anal. Chem.* **2001**, *73*, 1207−1212.
(7) Hofmann, O.; Che, D.; Cruickshank, K.; Muller, U. *Anal. Chem.* **1999**, *71*, 678−686.
(8) Macounova, K.; Cabrera, C.; Yager, P. *Anal. Chem.* **2001**, *73*, 1627−1633.
(9) Rocklin, R.; Ramsey, R.; Ramsey, J. *Anal. Chem.* **2000**, *72*, 5244−5249.
(10) Ericson, C.; Holm, J.; Ericson, T.; Hjerten, S. *Anal. Chem.* **2000**, *72*, 81−87.
(11) Ocvirk, G.; Verpoorte, E.; Manz, A.; Grasserbauer, M.; Widmer, H. M. *Anal. Methods Instrum.* **1995**, *2*, 74−82.
(12) He, B.; Regnier, F. *J. Pharm. Biomed. Anal.* **1998**, *17*, 925−932.
(13) He, B.; Ji, J.; Regnier, F. E. *J. Chromatogr., A* **1999**, *853*, 257−262.

10.1021/ac011077o CCC: $22.00
Published on Web 01/19/2002

Recently, researchers at University of California, Berkeley[14,15] and in our group[16,17] have developed capillaries filled with photoinitiated hydrophobic polymer monoliths for reversed-phase capillary electrochromatography. A few reports have appeared in past few months that discuss application of microchips containing polymer monoliths for preconcentration and solid-phase extraction[18] and for separation of polyaromatic hydrocarbons.[19] The present work reports on development of microchips containing channels filled with porous polymer monoliths for separation of peptides and amino acids. These microchips are miniaturized chromatographic systems that contain cast-in-place rigid surface-attached polymers, can withstand up to 3000 psi of pressure (provided chip itself does not start leaking), and can be used or stored in liquid for months. Moreover, because polymerization is initiated by UV light, the channels can be photolithographically patterned. Using a mask, the polymerization is restricted to UV-exposed regions and monomers from the unexposed regions are flushed after the irradiation step. This allows polymer to be cast selectively in separation channels, while injection channels and the detection window remain open. This allows for rapid and repeatable injection, easy cleanup of injection arms, and more sensitive detection. The ability to photopattern will also facilitate multidimensional separation by enabling multiple separate stationary phases in a single chip. Electrolytes are incorporated into the monomer mix, allowing for generation of electroosmotic flow (EOF) immediately after polymerization. This obviates the need of pumps to condition the channels by removal of excess solvent and monomers.

EXPERIMENTAL SECTION

Chemicals. Acrylate monomers, 2,2′-azobisisobutyronitrile (AIBN), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), the adhesion promoter trimethoxysilylpropyl acrylate, and HPLC grade acetonitrile and methyl alcohol were obtained from Aldrich. Naphthalene-2,3-dicarboxaldehyde (NDA) and potassium cyanide were purchased from Molecular Probes (Eugene, OR). Amino acids, peptides (papain inhibitor, proctolin, Opioid peptide (α-casein fragment 90−95), Ile-angiotensin III, angiotensin III, and GGG), and buffer salts (sodium tetraborate and sodium phosphate) were purchased from Sigma. Deionized water (18.2 MΩ) was obtained using an Ultrapure water system from Millipore (Milford, MA).

Chip Fabrication. The microchip was fabricated from Schott D263 glass wafers (4-in. diameter, 1.1-mm thickness; S. I. Howard Glass Co., Worcester, MA) using standard photolithography, wet etch, and bonding techniques. The photomask for the offset double-T chip (Figure 1) was designed using AutoCAD 2000 (Autodesk Inc., San Rafael, CA) and the mask was generated by PhotoSciences (San Jose, CA). Glass wafers were sputtered with chrome (200 nm) which served as the hard mask. A 1.2-μm-thick layer of OCG 825 (Arch Chemical Co., Columbus, OH) positive photoresist was spin-coated and soft-baked (90 °C, 5 min). The mask pattern was transferred to the photoresist by exposing it to UV light in a contact mask aligner at 80 mJ/cm^2. Exposure time varied depending on flux intensity (MA6, Karl Suss America Inc., Waterbury Center, VA). After exposure, the photoresist was developed with OCG 934 2:1 developer and hard-baked (120 °C, 30 min). Exposed chrome was etched with CEN 300 Microchrome ecthant (Microchrome Technologies Inc., San Jose, CA) and the subsequently exposed glass was etched with a 25% HF solution (Shape Products Co., Oakland, CA). Access holes were drilled in the cover plate (D263) with diamond-tipped drill bits (Olympic Mountain Gems, Port Orchard, WA). The etched wafers and cover plates were cleaned with 4:1 H_2SO_4/H_2O_2 (100 °C) and 1:5:1 $NH_4OH/H_2O/H_2O_2$ (75 °C), rinsed in a cascade bath, followed by a spin rinse dry, aligned for contacting, and thermally bonded at 610 °C in an N_2-purged programmable furnace (Thermolyne, Dubuque, IA). The standard chips contained offset double-T junctions, where the length of the main channel was 7 cm, the injection arms were 1 cm each, and the distance from the junction to the waste reservoir was 6 cm. The channels were 25 or 40 μm deep and 90−130 μm wide.

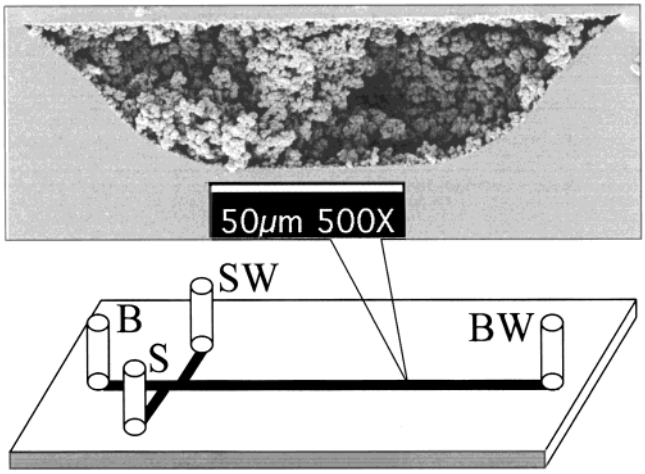

Figure 1. Schematic of the microchip used for electrochromatography. B, S, BW, and SW denote reservoirs containing buffer, sample, buffer waste, and sample waste, respectively. The inset shows a scanning electron micrograph of a channel cross section filled with photoinitiated acrylate polmer monolith. The mean pore diameter is 1 μm (higher magnification images are provided in refs 16 and 17).

Photopatterning of Rigid Polymer in Microchips. After fabrication and bonding, microchips were examined under an optical microscope to check for defects or blockage in the channels. Channels were extensively flushed with deionized water and methanol using a syringe. Homemade PEEK fittings were attached to the holes in the cover plate using a UV-cure epoxy. Care was taken to avoid insertion of epoxy in channels. Homemade 100−250-μL PEEK reservoirs were then screwed on to the fittings. The channels were then rinsed with the casting solvent (1:3:1, EtOH/ACN/5 mM PB, pH 6.8). The polymerization procedure is described in detail elsewhere.[16] Briefly, reservoir SW was filled with the monomer mixture in casting solvent (1:2 monomer/solvent) that contained by volume 30%1,3-butanediol diacrylate, 0.5% AMPS, 0.3% trimethoxysilylpropyl acrylate, 6.92% lauryl acrylate, 62.28% butyl acrylate, and 0.5 wt % of the initiator

(14) Viklund, C.; Ponten, E.; Glad, B.; Irgum, K.; Horstedt, P.; Svec, F. *Chem. Mater.* **1997**, *9*, 463−471.
(15) Yu, C.; Svec, F.; Frechet, J. M. J. *Electrophoresis* **2000**, *21*, 120−127.
(16) Ngola, S.; Fintschenko, Y.; Choi, W. Y.; Shepodd, T. J. *Anal. Chem.* **2001**, *73*, 849−856.
(17) Shediac, R.; Ngola, S. M.; Throckmorton, D. J.; Anex, D. S.; Shepodd, T. J.; Singh, A. K. *J. Chromatogr., A* **2001**, *925*, 251−263.
(18) Yu, C.; Davey, M. H.; Svec, F.; and Fréchet, J. M. J. *Anal. Chem.* **2001**, *73*, 5088−5096.
(19) Fintschenko, Y.; Choi, W. Y.; Ngola, S.; Shepodd, T. J. *Fresenius J. Anal. Chem.* **2001**, *371*, 174−181.

AIBN. Channels were allowed to fill by capillary action and different channels (or portions of a single channel) were selectively exposed to UV light from a 100-W mercury lamp using either a mask made in glass (Photosciences, San Jose, CA) for patterning high-resolution features or a homemade mask for low-resolution features. The homemade mask was easily and inexpensively made using thin black cardboard or a Teflon sheet using a milling machine. The mask was placed directly underneath the chip and aligned using a flashlight. The mask was then secured to the chip using clips. The chip was then affixed on a stand and the UV lamp was placed 15 cm directly underneath. The relatively large gap between the lamp and the mask ensures that the incident light is collimated. A fan was used to prevent heating caused by the lamp. The entire setup was encased in a dark chamber to avoid exposure to the ambient light and to protect the experimenter from the intense UV light.

Fluorescent Labeling of Peptides and Amino Acids. Peptides and amino acids were labeled off-chip prior to separation with the fluorogenic dye NDA in the presence of KCN. A 40-μL sample of 1 mM bioactive peptide was added to 1 mL of 50 mM borate buffer (pH 9.5) followed by 40 μL of 20 mM KCN and 40 μL of 10 mM NDA. The reaction mixture contained 10-fold excess of NDA and a 20-fold excess of KCN with respect to the peptide. The reaction was carried out in the dark at ambient temperature for 15 min. Slow addition of the NDA is critical in order to keep the NDA in solution. Amino acid derivatization was carried out by adding a 40-μL aliquot of 0.3 mM amino acid stock solution to 1 mL of 50 mM borate (pH 9.5) followed by aliquots of 40 μL of 4.8 mM KCN and 40 μL of 2.4 mM NDA. An 8-fold excess of NDA and a 16-fold excess of KCN were added over the total amino acid concentration. Chemicals were added in the order described to avoid benzoin condensation.[20] Prior to injection, labeled peptides and amino acids were diluted in the separation buffer.

Laser-Induced Fluorescence Detection. An epifluorescence format was used to detect NDA-labeled peptides and amino acids after separation in a microchip. The 413-nm light from a Kr ion laser is filtered through a chopper spinning at 220 Hz. A 20× microscope objective focuses the laser onto the channel of the microchip. The microchip is secured in an in-house-designed holder mounted on a *XYZ* translation stage to allow focusing of laser beam on the separation channel. The fluorescence from the channel is collected by the microscope objective and passes through a dichroic mirror and a 430-nm long-pass filter to reject scattered light. An adjustable iris spatially filters the emitted light before it enters a photomultiplier tube (PMT). A home-built power supply is used to provide high voltage for the PMT, and the signal from the PMT is demodulated using a lock-in amplifier. The output signal from the amplifier is collected by a personal computer via a data acquisition interface comprising a PCI-1200 DAQ card (National Instruments, Austin, TX). The data are collected using a computer program written in LABVIEW and processed (background subtraction and smoothing) using an in-house algorithm.

Chip-Based CEC of Peptides and Amino Acids. High voltage to different reservoirs in the chip is provided via platinum wires by a home-built voltage divider circuit connected to a 5-kV dc power supply (SRS model PS 350, Stanford Research, Sunnyvale, CA). The reservoirs are filled with the separation buffer and the channels in the chip sequentially conditioned at 100−200 V/cm until the current and fluorescence background signal is stabilized (∼1−2 h). A switch box connected to the HV supply is used to switch between injection and separation modes. Application of countervoltages is required for sharp injection plugs and to avoid leakage of sample during the run.[19,20] The injection and run voltages are optimized using imaging of fluorescein in the channels.[21] Injection and separation voltages for different separations are listed in the figure captions. A typical injection is done by applying 100%:80%:70%:0% of the high voltage to sample (S)/buffer (B)/buffer waste (BW)/sample waste (SW), respectively; while a typical run is carried out at 100%:80%:70%:0% (B/S/SW/BW). Injections and runs are performed at 200−800 V/cm. Detection is performed at a point ∼5 cm from the injection tee. The final concentration of peptide and amino acid in each run is between 10^{-7} and 10^{-9} M.

RESULTS AND DISCUSSION

Porous Polymer Monoliths. Figure 1 shows a schematic of the microchip used and scanning electron micrograph (SEM) of a channel in the microchip containing photopolymerized porous polymer monolith. The characteristic shape of the glass channel is due to isotropic etching. The polymer is cast uniformly over the cross section of the channel and is covalently bonded to the wall. A well-defined porous structure is discernible; however, it should be noted that the SEM was performed on the air-dried sample, and hence, the porous structure of the wet polymer may be slightly different. It is remarkable that even upon drying the polymer is not detached from the wall of the chip, probably because of the presence of a silane (trimethoxysilylpropyl acrylate) in the monomer mixture. We have noticed detachment of polymer from the wall if silane is omitted. The BET measurements on these polymers yield a mean pore diameter of 1 μm. Detailed characterization of the porous polymer monoliths is provided elsewhere.[16,17] The polymer monoliths possess several properties that are well-suited for chromatographic media for microchip separations. They are cast-in-place, continuous rigid structures and hence do not require packing or retaining frits. They can be made rapidly (10 min) by UV irradiation and can be photopatterned. They can be made from a wide variety of monomers, enabling the charge and hydrophobicity of the stationary phase to be easily tuned. Since the fluid flow in the column relies on EOF, it is imperative to have charged functionalities present in the polymer monolith. For neutral- and high-pH separations, a negatively charged polymer was synthesized by incorporating a sulfonic acid monomer. For low-pH (or neutral) separations, positively charged porous polymer monoliths can be prepared by incorporation of a tetraalkylammonium compound as the charged functionality.

Photopatterning of Polymer in Channels. The ability to use UV light for initiation of polymerization allows patterning of the chromatographic phase in the channels of a microfluidic chip for optimization of specific functions in the microchip. Figure 2a shows an offset double-T injection manifold in which the injection arms have no polymer, allowing for a faster and uniform transport of sample and for easy and fast switching between samples. The

(20) Roach, M. C.; Harmony, M. D. *Anal. Chem.* **1987**, *59*, 411−414.

(21) Jacobson, S.; Hergenroder, R.; Koutny, L.; Warmack, R.; Ramsey, J. *Anal. Chem.* **1994**, *66*, 1107−1113.

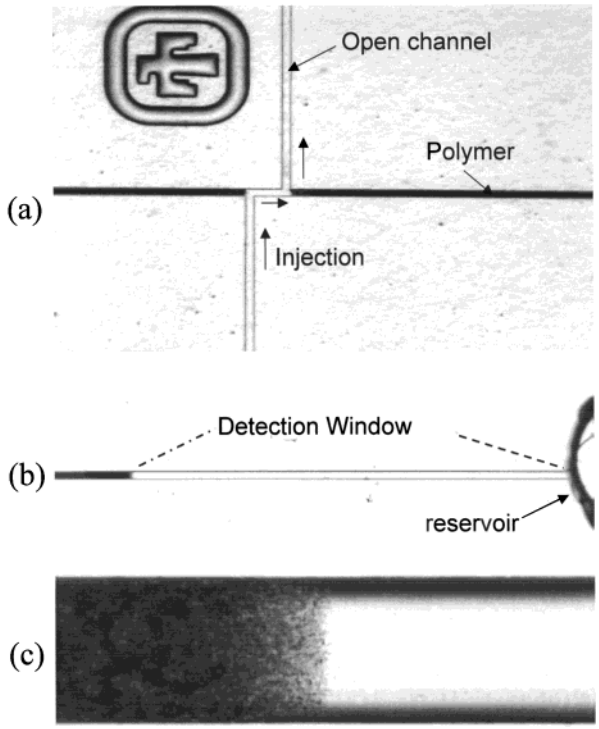

Figure 2. Photopatterning of polymer in channels for optimization of different functions. The polymer was patterned using a mask and a UV lamp. (a) Patterning of monolith to keep injection arms void of polymer in an offset double-T chip to allow for fast injection and for rapid switching between samples. Open injection path also allows for hydrodynamic injection. (b) Section of channel after the separation region kept free of polymer to allow for lower background and, hence, more sensitive detection. (c) Top view of the edge of polymer monolith. The sharpness of the interface indicates good resolution of the patterning process.

presence of polymer in the injection arms will require that sample be injected for a sufficiently long time to achieve uniform concentration of the entire sample at the junction of injection and separation channels. This duration, dependent on the electric field strength and the length of the injection arm, can be up to 5 min for the first injection and 15−60 s for subsequent injections. Moreover, before switching to a different sample, the injection arms need to be completely cleaned of prior sample and that can easily take several minutes. The design shown in Figure 2a can prevent these potential problems, as the injection path contains no separation matrix, allowing for a faster injection. Moreover, open injection arms allow for hydrodynamic as well as electrokinetic injection. Figure 2b shows creation of a window that is kept void of polymer for allowing sensitive detection using laser-induced fluorescence. Most of the organic polymers fluoresce to some extent and, combined with scattering, lead to a higher fluorescence background. However, there is one inherent problem in creating fluidic channels that contain both open and polymer-filled segments for EOF-driven separations—difference in ζ potential (and tortuosity) in the open and polymer-filled segments. This problem is frequently encountered in capillary electrochromatography using packed capillaries where detection is performed on an open segment. Mismatch of ζ potential leads to a pressure-driven parabolic flow in the open segment that can severely reduce plate numbers.[24,25] In chip architectures shown in Figure 2a and b, the mismatch of ζ potential was substantial enough to provide lower separation efficiency than a chip where all channels are completely filled with polymer. Therefore, in this study, all results reported are obtained with microchips where the separation and injection channels are filled with polymer, leaving only the reservoirs void of polymer.[24,25] We are actively exploring ways of matching ζ potential in open and polymer-filled sections by tuning the charge in each section to avoid generation of pressure-driven flow in open sections.

Figure 2c shows the top view of the edge of polymer cast in a channel; the sharp interface underscores the good control over photopatterning. The resolution of photopatterned polymer in channels is determined by two factors: (1) photolithographic resolution and (2) diffusion of free radical from exposed region to unexposed areas. The photolithographic resolution is limited by the wavelength of light used as well as the thickness of glass wafer as given by[26]

$$2b_{\min} = 3\sqrt{\lambda(s + z/2)}$$

where $b_{\min}$ is resolution limit, λ is the wavelength, s is the distance between mask and polymer surface, and z is the polymer thickness. For photopatterning of acrylate polymer in chips, λ is 365 nm, s is 1.1 mm (since the mask is in contact with the bottom of the chip, s is the thickness of bottom glass wafer), and z is the depth of channel (typically 20−40 μm). This leads to a theoretical resolution limit of 30 μm. In practice, we observe ~200 μm for the resolution limit indicating that diffusion of free radical is the limiting factor. Use of an intense UV light source in close proximity to the chip can also lead to heating and, consequently, to heat-induced polymerization even in the unexposed regions. A cooling fan was used to maintain a constant temperature, thereby minimizing heat-induced polymerization.

Separation in Chips. One of the biggest advantages microchips offer over conventional separation devices is significant improvement in speed of analysis. Figure 3 shows chip electrochromatographic separation of six bioactive peptides in less than 45 s using a negatively charged lauryl acrylate monolith. The six peptides are separated with good resolution at a field strength of 770 V/cm in 30:70 (v/v) acetonitrile/25 mM borate, pH 8.2. These peptides, when separated in a capillary (100-μm i.d, 303 V/cm, 23.5 cm to detection window) filled with the same acrylate monolith, took over 9 min for separation (data not shown). Hence, use of microchips allows for an order of magnitude increase in speed of separation over capillary electrochromatography. Similar improvements in analysis time have also been reported by a number of other researchers.[5,27] The major cause of this improvement is the fact that chips allow separation to be performed at higher electric field strengths than capillary-based systems. Chips, due to the rectangular shape of channels (hence, higher surface area-to-volume ratio) and the presence of a relatively large mass of glass surrounding a channel, allow for more effective Joule heat

(22) Harrison, D.; Fluri, K.; Seiler, K.; Fan, Z.; Effenhauser, C.; Manz, A. *Science* **1993**, *261*, 895−897.
(23) Wallenborg, S. R.; Bailey, C. G. *Anal. Chem.* **2000**, *72*, 1872−1878.
(24) Rathore, A.; Wen, E.; Horvath, C. *Anal. Chem.* **1999**, *71*, 2633−2641.
(25) Rathore, A.; Horvath, C. *Anal. Chem.* **1998**, *70*, 3069−3077.
(26) Madou, M. *Fundamentals of Microfabrication*; CRC Press: Boca Raton, 1997.
(27) Schmalzing, D.; Koutny, L.; Adourian, A.; Belgrader, P.; Matsudaira, P.; Ehrlich, D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10273−10278.

Table 1. Efficiency of Chip-Based Electrochromatography of Peptides[a]

	peak 1		peak 2		peak 3		peak 4		peak 5	
kV	N/m (10^5)	N/s	N/m (10^5)	N/s	N/m (10^5)	N/s	N/m (10^5)	N/s	N/m (10^5)	N/s
1	2.49	48.6	3.82	66.2	3.2	45.2	2.4	31.9	1.51	18
2	4.53	191	5.9	218	6.09	180	2.16	60	1.27	31.3
3	3.93	262	2.58	149	3.1	140	1.33	56.1	0.894	32.8
4	2.97	299	1.8	149	2.2	133	1	55.7	0.73	34.4

[a] Peaks are as listed in caption of Figure 3.

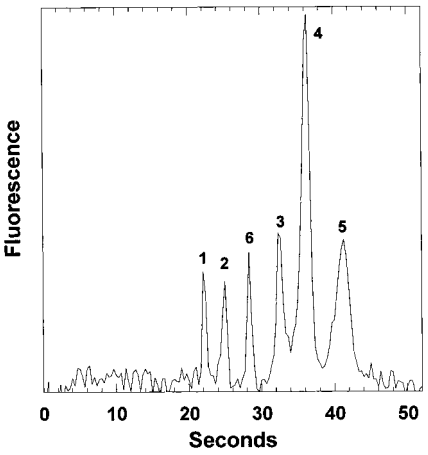

Figure 3. Reversed-phase electrochromatography of peptides in a microchip. The polymer was negatively charged lauryl acrylate monolith, peptides were labeled with NDA, and LIF detection was performed using the 413-nm line of a Kr ion laser. Field strength 770 V/cm (5 kV). Mobile phase: 30:70, ACN/25 mM borate, pH 8.2, containing 10 mM octane sulfonate. Offset-T microchip dimensions: 40 μm deep, 120 μm wide. The peptides are (1) papain inhibitor, (2) proctolin, (3) Opioid peptide (α-casein fragment 90−95), (4) Ile-angiotensin III, (5) angiotensin III, and (6) GGG.

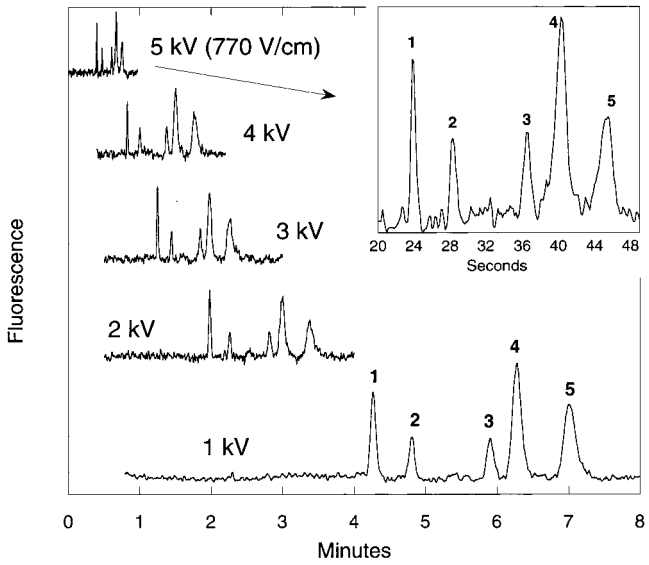

Figure 4. Effect of voltage on separation of peptides. The peptides are the same as listed in Figure 3 minus peptide 6. Other conditions same as Figure 3. The 1−4-kV runs were performed in a 25-μm-deep channel; the 5-kV (770V/cm) run was performed in a 40-μm-deep channel. The efficiency values are listed in Table 1.

dissipation than cylindrical capillaries typically used for CZE and CEC.[28,29] Separation in chips is primarily based on reversed-phase interaction as CZE of the six peptides resulted in only two peaks. The peptides 4 and 5, Ile-angiotensin III and angiotensin III, have the same p*I* (8.75), similar molecular weight (931 and 897, respectively), and the same number of amino acids and differ only in their terminal amino acid (Phe in angiotensin III and Ile in Ile-angiotensin III). Despite the small difference in properties, these two peaks are baseline resolved even at a high speed of separation, underlining the good resolving power of chip electrochromatography.

Figure 4 shows the effect of field strength on separation of the five bioactive peptides at varying field strengths from 1 to 5 kV. Increasing the voltage increases the speed concomitantly; the separation duration is lowered from 8 min at 1 kV to less than 50 s at 5 kV. Moreover, even at 5 kV, corresponding to 770 V/cm, all five peaks are baseline separated. Plate number (and plate height) is a function of the speed of separation, which in turn is a function of electric field strength in electrochromatography (other variables such as buffer conditions being constant). Although, separation is the fastest at the highest field strength, the highest separation efficiency is obtained at intermediate field strength (Table 1). Faster eluting peptides have the highest plate efficiencies at 2 kV (4.53×10^5, 5.9×10^5, and 6.09×10^5 m^{-1} for peptides 1, 2, and 3, respectively) while peptides 4 (2.4×10^5 m^{-1}) and 5 (1.51×10^5 m^{-1}) have the highest plate numbers at 1 kV. These values are approximately double the plate numbers obtained for the separation achieved in capillaries using polymer monoliths. For separation in miniaturized devices, speed is of utmost importance and hence we evaluated the time-based efficiency (N/t) of separation as a function of field strength as well. For peptides 1 and 5, the N/t increases with field strength attaining the maximum of 299 plates/s for peptide 1 at 4 kV, while for peptides 2−4, maximum in N/t is attained at 2 kV (218 plates/s for peptide 2).

Figure 5 shows separation of five NDA-labeled amino acids (10^{-7}−10^{-8} M) in a chip using a mobile phase of 10:90 ACN/20 mM phosphate buffer at a field strength of 770 V/cm. The five amino acids were selected as a representative sample containing one neutral (Arg), two hydrophilic (Ser, Gly), and two hydrophobic (Phe, Trp) amino acids. NDA reacts with a primary amine in an amino acid resulting in an uncharged adduct. Therefore, upon labeling, Arg is neutral, while Ser, Gly, Phe, and Trp carry −1 charge each. Separation of these amino acids by CZE at similar buffer conditions resulted in three poorly resolved peaks. Hence, chip electrochromatography yields higher resolution separation of the amino acids than CZE. The order of elution in CZE is Arg;

(28) Fan, Z.; Harrison, D. *Anal. Chem.* **1994**, *66*, 177−184.
(29) Seiler, K.; Harrison, D.; Manz, A. *Anal. Chem.* **1993**, *65*, 1481−1488.

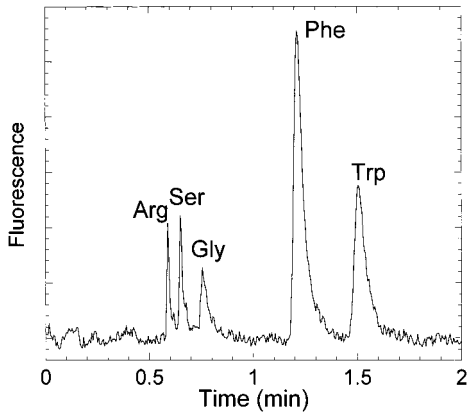

Figure 5. Electrochromatographic separation of five amino acids in a microchip. Mobile phase, 10:90, ACN/20 mM phosphate buffer. Stationary phase, lauryl acrylate polymer monolith. Electric field is 770 V/cm.

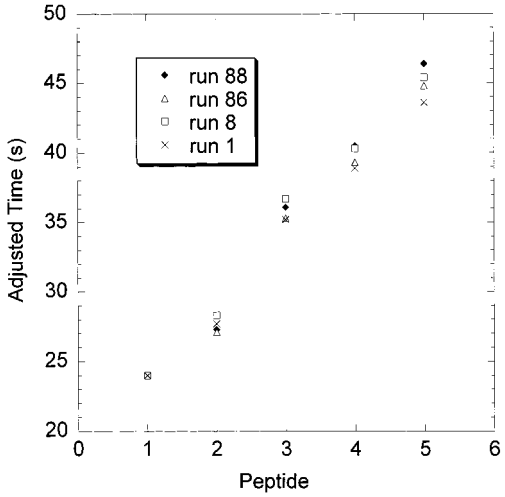

Figure 6. Reproducibilty of retention time for peptides. Field strength 770 V/cm. Offset-T microchip dimension: 40 μm deep, 120 μm wide. Other conditions and peptides are the same as in Figure 4.

Phe, Trp coelute; Ser; and Gly.[30] In chip electrochromatography (Figure 5), the order of elution is Arg, Ser, Gly, Phe, and Trp. Hence, as expected, separation of the amino acids involves both electrophoretic migration and chromatographic retention.

Reproducibility. Figure 6 shows the normalized retention times of peptides for run numbers 1, 8, 86, and 88 on the same chip. Retention times of peptides 2−5 have been normalized with respect to the retention time of peptide 1 to compensate for any changes in EOF. The RSD values for retention times of peptides 2−5 were 1.9%, 2%, 1.9%, and 2.5%, respectively, indicating good run-to-run reproducibility on a chip. These chips were used for over two months without significant change in separation performance as long as the monoliths were maintained wet. We did observe a loss in separation performance if polymer in the channels was allowed to dry, probably because of irreversible changes in the porous and surface properties.

Chip Recovery. A practical concern with the development of microchips is the longevity and reusability. Glass (or fused silica, quartz) microchips are rather expensive because of the requirement of specialized equipment and fabrication facilities. The component of the microchip developed here that has the shortest functional life is the separation matrix (the polymer monolith). It can degrade over time because of drying, irreversible adsorption of sample components, or impurities or can be blocked due to particulate impurities. Akin to a commercial HPLC column that can be emptied and repacked with separation matrix, we have developed a thermal decomposition protocol for complete removal of the polymer material from channels yielding a clean glass chip that can be readily reused. This involved flushing the chip thoroughly with deionized water, followed by incubating in an oven to obtain 550 °C at a ramp rate of 10 °C/min, holding at 550 °C for 2 h, and then cooling to 20 °C at 10 °C/min. The chip was then incubated in 0.2 N NaOH overnight. Figure 7 shows a SEM of a channel cross section after thermal decomposition, indicating complete removal of polymer. The recovery of chips allows for considerable saving in cost and time, especially for chips with complex architectures.

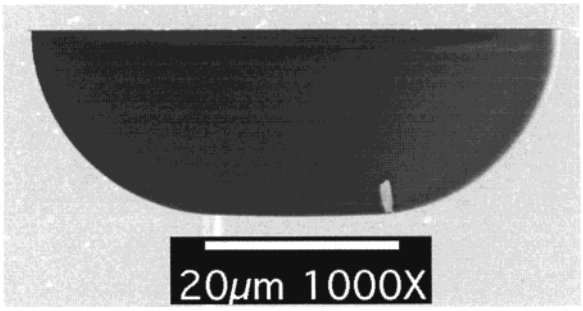

Figure 7. Scanning electron micrograph showing the cross section of a channel after removal of polymer monolith by thermal incineration. The regenerated microchip can be reused for patterning of polymer monolith and separation.

CONCLUSIONS

A simple, robust, and reproducible method has been developed to create glass microchips for chromatography employing photopolymerization for localized placement of separation matrix in channels. These chips provided high-resolution separation of peptides and amino acids and exceeded the efficiency and speed obtained in capillary electrochromatography. Spatial control over polymerization will allow one to create chips with multiple channels containing different separation matrixes to achieve multidimensional separation.

ACKNOWLEDGMENT

We thank George Sartor for microfabrication of chips, Dr. Isaac Shokair for developing the software for background subtraction and smoothing of chromatographic data, and Ron Renzi for design and fabrication of interconnects. This work was financially supported by Laboratory Directed Research and Development program at Sandia National Laboratories. Sandia is a multiprogram laboratory operated by Sandia Corp., a Lockheed Martin Co., for the United States Department of Energy under Contract DE-AC04-94AL85000.

Received for review October 10, 2001. Accepted December 4, 2001.

AC011077O

(30) Ueda, T.; Rosalind, M.; Fumito, K.; Metcalf, T. *J. Chromatogr.* **1992**, *593*, 265−274.