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Microchip HPLC of Peptides and Proteins

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Rapid microchip reversed-phase HPLC of peptides and proteins at pressure gradients of 12 bar/cm (180 psi/cm) has been performed using a microdevice that integrates subnanoliter on-chip injection and separation with a miniaturized fluorescence detector. Proteins and peptides were separated on a C18 side-chain porous polymer monolith defined by contact lithography, and injection was achieved via a pressure-switchable fluoropolymer valve defined using projection lithography. Preliminary separations of peptide standards and protein mixtures were performed in 40−200 s, and switching between samples with no detectible sample carryover has been performed. The injections and separations were reproducible; the relative standard deviation (RSD) for retention time was 0.03%, and peak area RSD was 3.8%. Sample volumes ranging from 220 to 800 pL could be linearly metered by controlling the pressure injection pulse duration with conventional timing and valving. The current prototype system shows the potential for rapid and autonomous HPLC separations with varying modalities and the potential for direct connection to mass spectrometers at nanospray flow rates.

High-performance liquid chromatography (HPLC) is an important and ubiquitous separation technique with numerous implementations in both preparative and analytical systems.[1,2] HPLC results are robust and reproducible, and the wide variety of chromatographic media available affords the separations community a great deal of flexibility.

While HPLC to date has been implemented in a macroscopic format, in columns with typical capacities and internal diameters of 1 mL and 4.6 mm, respectively, miniaturization presents the potential for several advantages. Improvements have been realized by the advent of microbore and capillary-based HPLC columns, with internal diameters of 100 μm to 2 mm, although injection and detection methods have remained largely unchanged. Reducing the size of injections and separation columns reduces the necessary sample size, a critical advantage when samples are expensive or difficult to generate or when the scientific question requires minimization of the volume (e.g., single-cell analysis). Reduction of column dimensions also reduces the system flow rate, which leads to improved SNR when chromatographic separations are connected to concentration-sensitive detectors such as electrospray injection mass spectrometry.[3] Reduction of solvent flow rates allows the possibility of autonomous or portable HPLC-based environmental sensors.[4] Finally, HPLC-based separations in a microchip format makes more sophisticated low-volume analyses possible, as a variety of microchip topologies can be designed to allow for chemical reaction, mixing, and multidimensional separations.

The tools historically used in miniaturized chromatographic systems have often been chosen based on the ease with which they can be integrated. For example, electrokinetic techniques (including CEC and MEKC) have been common for separations due to the relative ease with which high-voltage control can be incorporated into a microscale system.[5,6] However, electrokinetic techniques are generally less reproducible than HPLC separations due to the dependence on surface electrochemical properties. When pressure actuation is used to drive fluids, low-pressure (open-tubular) techniques have been used owing to the modest integration challenges associated with low-pressure operation, but these techniques are less efficient than HPLC.[7,8]

While its implementation is more challenging, high-pressure fluid actuation is ideal for chromatographic separation due to its excellent elution time reproducibility and the ease with which separation modes may be defined by surface chemistry. High-pressure flow places difficult demands on the substrate mechanical and chemical properties, flow control techniques, and world-to-chip interfacing; however, high-pressure actuation allows the use of high-surface-area media, and strict pressure actuation eliminates uncertainties associated with poorly specified or spatially variable electroosmotic mobilities. Prior work toward the implementation of LC in a microchip format focused on the ability to create separation columns within a silica or silicon chip.[9,10]

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Recent microchip HPLC efforts have focused on control of pumping pressure and sample injections in polymer[11−13] and glass[14] microsystems. Here we present miniaturized HPLC results with injection and separation integrated at the microchip level and show repeated rapid analysis of ~500 pL samples. Rapid sample switching with undetectable sample carryover is demonstrated.

MATERIALS AND METHODS

Chemicals. 2,2,3,3-Tetrafluoro-1,4-butanediol diacrylate was purchased from Monomer-Polymer & Dajac Laboratories Inc. (Feasterville, PA). Glacial acetic acid, 3-(trimethoxysilyl)propyl acrylate, 1,4-dioxane, 2, 2′-azobisisobutyronitrile (AIBN), acetonitrile (ACN), stearyl acrylate, 1,6 hexandiol diacrylate, tetrahydrofurfural acrylate, 1,2 dichloroethane, and 2-methoxyethanol were purchased from Sigma-Aldrich (St. Louis, MO). A mixture of peptides (0.125 mg of glycine−tyrosine; 0.5 mg each of valine−tyrosine−valine, methionine enkephalin, leucine enkephalin, and angiotensin II) was also purchased from Sigma-Aldrich and labeled using fluorescamine. Proteins were purchased from Sigma and labeled with fluorescein isothiocyanate (FITC) using standard procedures. Fluorescamine and FITC were purchased from Molecular Probes (Eugene, OR). Rhodamine Cl 560 was purchased from Exciton (Dayton, OH). Tridecafluoro-1,1,2,2,-tetrahydrooctyltriethoxysilane (TDFTES) was purchased from Gelest, Inc. (Tullytown, PA), and heptafluorobutyric acid (HFBA) was obtained from Pierce Biotechnology (Rockford, IL).

Microchip and Injection Valve Fabrication. The integrated injection and separation microchip was fabricated from fused-silica substrates using previously described multilevel wet etch[15] and polymer photopatterning[14] techniques, which are briefly summarized here.

Silica microchips were fabricated from Corning 7980 fused-silica wafers of 100 mm diameter and 0.75 mm thickness (Sensor Prep Services, Inc., Elburn, IL) using standard photolithography, wet etch, and bonding techniques. Nominal microchannel depths of 20 and 5 μm were used. In the valve regions, both the bottom and top wafers the microchip were etched, creating an approximately circular cross section. The surface friction coefficient was reduced by functionalizing the internal surfaces of the chip with a fluoroalkyl coating (TDFTES) via incubation at 70 °C for 45 min with a solution of 30:5:4:1 1,4-dioxane, glacial acetic acid, TDFTES, and deionized water.

A microvalve injector was fabricated by laser-polymerizing a cylindrical fluoropolymer element[14] to serve as a switchable valve seat at the intersection between the two hemicylindrical input channels and the output (separation) channel.

In Situ Fabrication of Separation Media. Following the fabrication of the microvalve injector, the microchip surface was functionalized to facilitate covalent attachment of the polymer separation media. The surface was functionalized with 3-(trimethoxysilyl)propyl acrylate by incubation at room temperature with a solution consisting of 2:2:1 glacial acetic acid, deionized water, and 3-(trimethoxysilyl)propyl acrylate for 30 min. The chip was then flushed with acetonitrile.

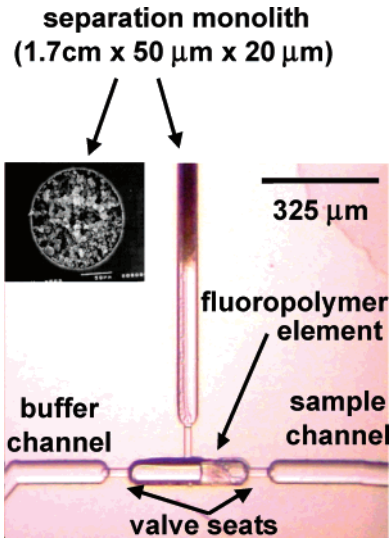

Figure 1. Micrograph of injector valve and beginning of separation media. Inset is scanning electron micrograph of separation media polymerized inside 150-μm-i.d. glass capillary.

The 1.7-cm-long porous polymer monolith separation medium was polymerized downstream of the injection valve, patterned in situ using offset contact lithography.[16−19] The column area was loaded with the monomer solution (400 μL of stearyl acrylate, 400 μL of 1,6-hexanediol diacrylate, 150 μL of tetrahydrofurfural acrylate, 150 μL of 10 mM acetate (pH 5.0), 1.85 μL of methoxyethanol, and 6 mg of AIBN), and masking tape was used to define the column length (Figure 1). The microchip was exposed for ~15 min to UV light (30 W, Cole-Parmer, Chicago, IL) until polymerization was observed. After polymerization, the chip was flushed with acetonitrile.

Sample Injection. The microchip injector was used to meter controlled amounts of sample by allowing external pressure-actuated switching of the inlet lines for controlled durations.[14] The photopatterned fluoropolymer element forms a high-pressure seal between the sample and buffer lines, switching in response to pressure differentials to connect either the buffer or sample line (but never both) to the separation channel.[14] Manually controlled syringe pumps with 1 mL syringes were used as constant pressure sources for both injection and separation. Injections were performed by applying pressure pulses to the sample line while the buffer line pressure remained constant within the range 150−300 psi. The pressure to the sample channel was pressurized to 150 psi above the buffer line pressure for durations of 165−1000 ms to switch the injector valve and inject a metered sample of fluid. External pressure switching components (electronically controlled valve; C1-2006,VICI, Houston, TX; gate/delay generator; DG-535, Stanford Research Systems, Sunnyvale, CA) were used to select pulse width and frequency.

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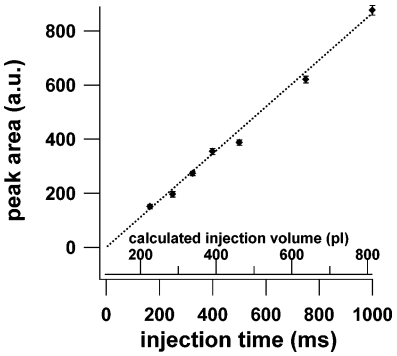

Figure 2. Linear dependency of peak area on injection duration. Rhodamine 560 injections were performed at 450 psi; buffer flow was constant at 300 psi. Error bars are standard deviation, with at minimum nine replicates at each injection duration.

Miniaturized Fluorescence Detector. Elution of fluorescently labeled compounds was detected at 10 Hz using a custom miniaturized (2 in. × 3 in. footprint) LIF detector by a PC running Labview 5 (National Instruments, Austin, TX). A miniaturized GaN laser diode (405 nm, 5 mW, Nichia, Southfield, MI) and epifluorescent unit was used to measure the fluorescence at >450 nm.[20,21] The laser diode illuminates a 100 μm × 70 μm area of the channel. The raw data were smoothed using an 11-point moving average by use of a Matlab v6.1 script (The Mathworks Inc., Natick, MA).

RESULTS AND DISCUSSION

Elution Repeatability and Detector Linearity. Elution repeatability and detector linearity were tested by injecting single-component samples and detecting the injections with the fluorescence detector. The sample consisted of a solution of 253 ng/mL Rhodamine 560 dye in 30% ACN and 70% deionized water. The fluorescent signal was measured in the open channel immediately following the separation media (postcolumn), so that the sample injection performance of the valve/column chip could be determined by measuring the elution of the dye. The injections were performed using a 450 psi pressure pulse at 0.025 Hz with constant duration (between 165 and 1000 ms). The buffer channel was filled with 30% ACN and 70% 5 mM sodium phosphate buffer at pH 6.8. The buffer channel was held at 300 psi both during and between injections.

The separation system successfully demonstrates that the injection volumes may be straightforwardly metered by controlling the pressure pulse duration. The chromatograms for the repeated injections show the injections result in reproducible peak retention time and area, and peak area is linearly dependent on the injection duration (Figure 2). Using the results of 104 injections, the overall relative standard deviation (RSD) for peak area was 3.9% and 3.6% for retention time. The measured peak area is linear with injection duration ($r^2 = 0.993$).

On-Chip RP-HPLC Separations. Rapid isocratic RP-HPLC separations using protein and peptide mixtures have been performed using the aforementioned fluoropolymer injector and porous polymer monolith separation media. Injections were performed at 450 psi, and the mobile-phase pressure was held constant at 300 psi. For the peptide standard mixture, the sample channel was filled with the 319 μg/mL peptide mixture, and the buffer channel contained 30% ACN with 0.1% TFA in 5 mM phosphate buffer at pH 2.2. Repeated separations, 50 s in duration, were performed (Figure 3). While the peaks are clearly not fully resolved, injections of peptides have repeatable total peak area (RSD of 3.8%) and peak retention times (RSD of 0.03%, based on peak maximum). Using a similar technique, fluorescently labeled proteins were also successfully separated in ~100 s (Figure 4) in 24% ACN + 0.16% HFBA in 5 mM phosphate buffer (pH 2.0). Using the full width at half-maximum method and the α-lactalbumin peak, the theoretical peak height is 45 μm and the column efficiency is 25 000 plates/m. All separations were performed at a flow rate of 30 ± 10 nL/min. The uncertainty in the reported flow rate is due to the difficulty in measuring the absolute flow rate. The stability of the retention times indicates little variation in the mobile-phase flow rate.

The fluoropolymer injector allows repeated and rapid sample injection and switching, while the fritless separation medium facilitates system integration. The porous polymer monolith

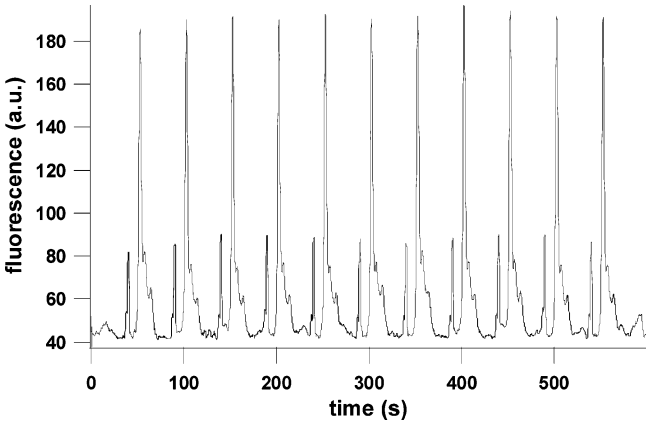

Figure 3. Repeated 470-pL injections of a peptide mixture. Isocratic separation using 30% ACN with 0.1% TFA in 5 mM phosphate buffer (pH 2.2) at 300 psi.

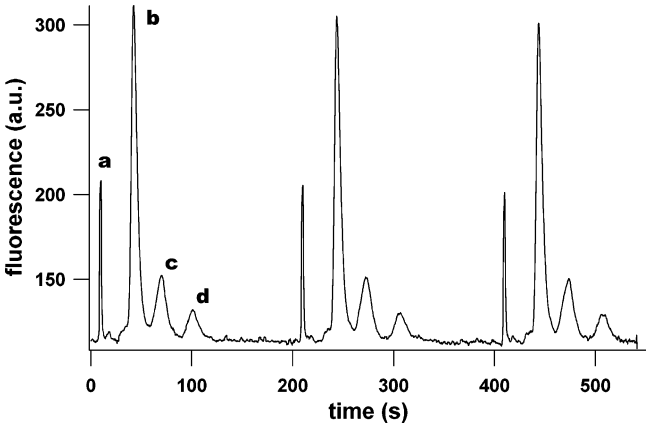

Figure 4. Repeated 6400pL, 750-ms injections of a protein mixture. Isocratic separation was performed using 24% ACN + 0.16% HFBA in 5 mM phosphate buffer (pH 2.0) at 300 psi. Peak identities and retention factors: a, free dye; b, insulin (3.2); c, anti-biotin (6.0); d, α-lactalbumin (9.1).

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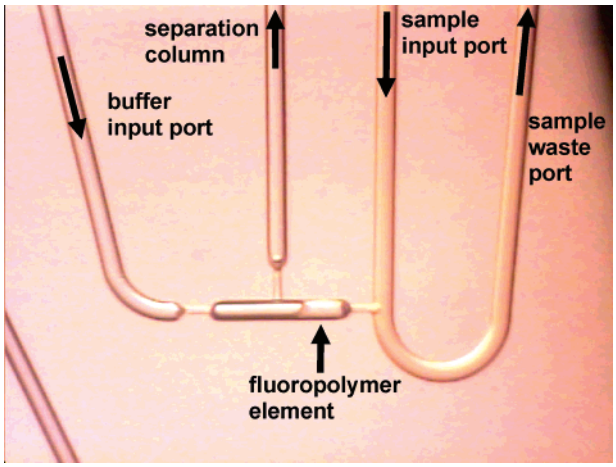

Figure 5. Four-port valve allowing rapid sample changes. Sample is changed by flushing at low pressure when the sample waste port is open. Sample is injected by closing sample waste port and pressurizing the sample line. Valve size same as Figure 1.

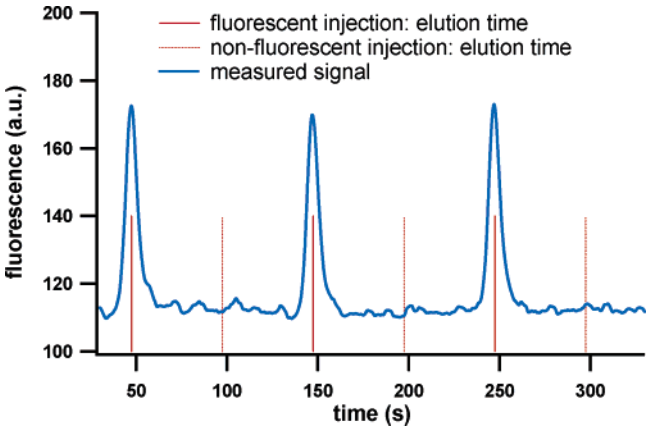

Figure 6. Rapid sample switching performed without sample carryover. Injections at 50-s spacings, alternating between a sample containing rhodamine 560 and a blank sample of run buffer. Separation conditions same as Figure 2. Expected elution times of fluorescent (solid line) and nonfluorescent (dotted line) injections are marked.

imitates the separation behavior of reversed-phase particles (confirmed by elution time measurements as a function of ACN concentration; data not shown) by presenting pendant stearyl groups on a polyacrylate backbone cross-linked to provide the mechanical integrity required to withstand the 180 psi/cm pressure gradients used in this work.

Rapid Sample Switching and Sample Carryover. The sample carryover of a rapidly switched picoliter-injection HPLC system was measured by using a four-port microchip (Figure 5) to rapidly inject alternating samples of fluorescent and nonfluorescent liquid (Figure 6). Any sample carryover, if present, would be observed as a false peak or a baseline shift observed when the nonfluorescent liquid sample is eluted. To within the SNR of this detection system for a single peak with known elution time (SNR = 55), no measurable baseline shift or false peak is detected—clear peaks are observed at the elution time of the fluorescent sample, while no measurable carryover is detected at the elution time of the subsequent nonfluorescent sample. While ambiguity in the figure is minimized by injecting single components and allowing 50-s spacings between injections, the fundamental switching limit of the system is ~0.2 Hz due to the time required to flush the chip to external tubing connections. This result points to the unique ability of the system to rapidly switch samples for high-throughput analysis.

CONCLUSIONS

Rapid microchip RP-HPLC of peptides and proteins at pressure gradients of 180 psi/cm has been performed using a microdevice that integrates on-chip injection, separation, and detection with a miniaturized LIF detector. Separation was achieved via definition of a C18 side-chain porous polymer monolith using contact lithography, and injection was achieved via definition of a pressure-switchable fluoropolymer valve using projection lithography. Preliminary separations of peptide standards and protein mixtures were performed in 40−200 s, and switching between samples with no detectible sample carryover has been performed at 72 injections/h. Sample volumes ranging from 220 to 800 pL could be linearly metered by controlling the pressure injection pulse duration with conventional timing and valving.

The current system shows the potential for rapid and autonomous HPLC separations with varying modalities. The small footprint and reagent requirements may lead to the development of HPLC devices for real-time environmental monitoring. In addition, the low flow rate (~30 nL/min) is typical of nanospray systems and should allow the microchip HPLC to be directly connected to nanospray mass spectrometry for sensitive quantitation and identification of analytes. Future work may involve a variety of separation modalities through use of different porous polymer monoliths or packed particle-based columns and an increase in separation efficiency by the use of gradient elution.

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