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Integrated microchip-device for the digestion, separation and postcolumn labeling of proteins and peptides

Norbert Gottschlich, Christopher T. Culbertson, Timothy E. McKnight, Stephen C. Jacobson, J. Michael Ramsey*

Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6142, USA

Abstract

A microchip device was demonstrated that integrated enzymatic reactions, electrophoretic separation of the reactants from the products and post-separation labeling of proteins and peptides prior to detection. A tryptic digestion of oxidized insulin B-chain was performed in 15 min under stopped flow conditions in a heated channel, and the separation was completed in 1 min. Localized thermal control of the reaction channel was achieved using a resistive heating element. The separated reaction products were then labeled with naphthalene-2,3-dicarboxaldehyde (NDA) and detected by laser-induced fluorescence. A second reaction at elevated temperatures was also demonstrated for the on-chip reduction of disulfide bridges using insulin as a model protein. This device represents one of the highest levels, to date, of monolithic integration of chemical processes on a microchip. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Microchip device; Proteins; Peptides

1. Introduction

Interest in microfabricated instrumentation for chemical sensing and analysis has grown exponentially over the past decade primarily because these miniature instruments may provide information rapidly and reliably at low cost. Microfabricated fluidic devices (microchips) constructed on planar substrates are advantageous for manipulating small sample volumes, rapidly processing materials, and integrating sample pretreatment, and separation strategies. The dexterity with which materials can be manipulated and the ability to machine structures with interconnecting channels with essentially zero dead volume contribute to the high-performance of these devices. To carry out a complete assay, functional elements can be serially integrated on these devices and include filters, valves, pumps, mixers, reactors, separators, cytometers, and detectors. Coupling these elements together at the photolithographic mask level using monolithic microfabrication processes and operating them under computer control will enable the development of a wide range of microchip-based assays.

To date, a variety of functional elements have been demonstrated. Two or more fluids can be electrokinetically proportioned in different ratios by controlling the potentials applied to the fluidic reservoirs to effect dilution [1], reactions [2,3], and solvent programming [4,5]. Samples and reagents can be dispensed with basic valve designs including a T-intersection [6], double T-intersection [7], and

*Corresponding author.

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cross intersection [8,9]. The cross intersection has been used for both constant [8] and variable volume [9] valving. These valving techniques have enabled high-performance, electrokinetically driven separations to be performed including capillary electrophoresis [1,8], electrochromatography [10], micellar electrokinetic chromatography [4] and capillary gel electrophoresis [11,7]. Some simple yet demonstratively powerful integrated devices have been developed for precolumn [9] and postcolumn [12,13] labeling reactions in conjunction with electrophoretic separations. Also, reactions carried out on-chip have been analyzed using capillary electrophoresis for competitive immunoassays [14] and restriction fragment analysis [15]. Polymerase chain reactions (PCRs) have been performed on-chip by thermal cycling a reaction well [16] and by flowing the reagents through different temperature zones [17]. In addition, PCR amplification has been coupled to electrophoretic separations for product sizing in monolithic [18] and hybridized [19] configurations.

For protein analyses on a reduced scale, capillary systems have been used for enzymatic or chemical cleavage of proteins and separation of the resulting peptides. Capillary microreactors with immobilized enzymes have been used for protein digestion, followed by separation of the products in a second capillary coupled to the first [20,21]. Separation of the peptide fragments has been accomplished by both capillary electrophoresis (CE) and microcolumn high-performance liquid chromatography (HPLC) [21]. Also, the digestion and separation of the peptide fragments have been performed on the same column [22]. With the ease of automating all fluidic manipulations, microchips are excellent candidates for configuring various protein processing and separation strategies.

In this paper we describe a microchip that monolithically integrates three separate biochemical processing steps; protein and peptide reactions, electrophoretic separation of the reactants from the products, and postseparation derivatization prior to fluorescence detection. To accomplish this, a reactor, an injector, a separator, and a second reactor were integrated for the first time on a monolithic device. Electrokinetically controlled fluidic manipulations were used to mix the sample and reagents in the reaction channel, dispense aliquots of the reactor effluent onto the separation channel, and mix separated zones with the derivatizing reagent. A small, detachable heater offers a simple and effective way to elevate the temperature in the reaction channel where the digestion or reduction occurs while leaving the fluid reservoirs, separation channel, and labeling reactor at room temperature. Tryptic digestion of insulin B-chain and reduction of the sulfide bridges of insulin were used to test the microchip, and naphthalene-2,3-dicarboxaldehyde (NDA) [23] was used as the labeling reagent.

2. Experimental

2.1. Chemicals

The buffer used in all experiments was 10 m*M* sodium tetraborate (EM Science, Gibbstown, NY). Sodium cyanide (NaCN) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ). Appropriate precautions should be observed when working with NaCN as it is poisonous. Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Molecular Probes, Inc. (Eugene, OR). A 1 m*M* solution was prepared by dissolving NDA in DMSO at 1 mg/ml and diluting this stock solution with buffer. Oxidized chain B from bovine insulin (B-chain), oxidized chain A from bovine insulin (A-chain), bovine insulin, bovine trypsin (EC 3.4.21.4), dithiothreitol (DTT) and benzylamine were obtained from Sigma (St. Louis, MO).

2.2. Microchip device

The microchips were fabricated using standard photolithographic, wet chemical etching and bonding techniques as described previously [8]. The channels on the microchip were 11.4 μm deep and 43.6 μm wide (measured at half-depth), and the channel surfaces were not modified. Channel access holes were drilled in the glass slide, and cylindrical glass reservoirs were attached using epoxy (Epoxy Technologies, Inc., Billerica, MA).

High voltage was applied to the microchip with a high-voltage supply (UltraVolt, Ronkonkoma, NY) through a voltage divider and high voltage relays (Kilovac, Santa Barbara, CA). The waste reservoir

was grounded. Electrical contact between the solutions in the fluid reservoirs and the high-voltage leads was achieved using platinum wires. A computer controlled system was used to switch the potentials at the microchip reservoirs. The program was written in LabVIEW (National Instruments, Austin, TX).

A thin Thermofoil™ Heater (Minco Products, Inc., Minneapolis, MN) was used to control the temperature in the reaction channel. The heater was attached to the glass chip using a C-clamp. Thermal contact between the heater and the microchip was provided using a 2.5 cm×1.4 cm piece of thermally conductive silicone elastomer (Therm-A-Gap A274, Chomerics, Woburn, MA) as shown in Fig. 1. The heater temperature and the glass temperature on top of the microchip were measured using type K thermocouples. Temperature control was achieved using an indicating controller (UT350, Yokogawa, Newnan, GA) and a DC power supply (6205C, Hewlett-Packard, Palo Alto, CA).

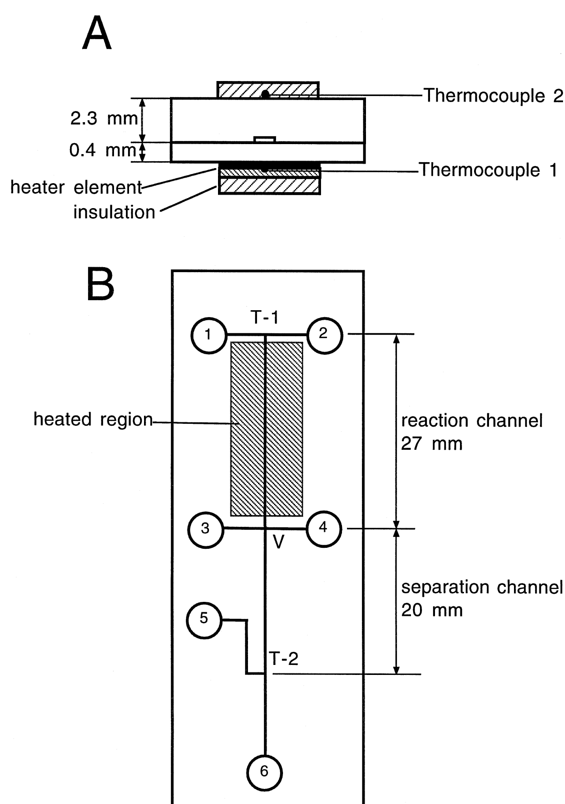

Fig. 1. (A) Cross-sectional view of the microchip, heating element, and the thermocouples. (B) Schematic of the microchip used for on-chip reactions, separations and postcolumn labeling. The fluid reservoirs are: (1) substrate, (2) enzyme or DTT, (3) buffer, (4) sample waste, (5) NDA, and (6) waste.

2.3. On-chip reactions and separation of reactants and products

Reservoirs 1 and 2 in Fig. 1 were filled with insulin B-chain (1 mg/ml) and trypsin (0.1 mg/ml) solutions, respectively. Reservoir 3 contained buffer with 1 m*M* sodium cyanide. NDA was dissolved in DMSO (1 mg/ml), diluted to 1 m*M* with buffer and placed in reservoir 5.

A gated injection scheme [12] was used to introduce sample into the separation channel. In the run mode, sample flowed from reservoirs 1 and 2 to reservoir 4 and buffer was electroosmotically pumped from reservoir 3 to reservoir 6. 1.5 kV was applied to the microchip for these experiments. The relative potentials at reservoirs 1, 2, 3, 4, 5, and 6 were 1, 1, 0.4, 0, 0.2, and 0, respectively. With these voltage settings fluid from reservoir 1 and reservoir 2 were mixed at a 1:1 ratio in the reaction channel. A sample plug was injected into the separation column at position V (Fig. 1) by lowering the potential at reservoir 3 to 0.2 and raising the potential at reservoir 4 to 0.2 for 0.8 s. After injection the run conditions were restored.

For the on-chip reduction of the disulfide bridges of bovine insulin the protein was dissolved in buffer, filtered (0.2 μm) and then placed in reservoir 1. The concentration of insulin was determined to be 0.55 mg/ml by measuring the absorbance at 280 nm. DTT (10 m*M*) was added to reservoir 2. The chip was operated in a continuous flow mode. The injection time in this experiment was 0.2 s.

2.4. Off-chip digestion and analysis of oxidized insulin B-chain

Off-chip digestion and derivatization with NDA was done by mixing 100 μl of the B-chain solution and the trypsin solution. After incubation for 15 min, 100 μl of sample was mixed with 300 μl of buffer, 2.5 μl of sodium cyanide solution (0.2 *M*) and 50 μl of NDA stock solution. The labeling reaction was

allowed to proceed for 10 min. The labeled peptides were separated on a simple cross microchip (see inset of Fig. 3). The length of the separation channel was 31 mm. 2.5 kV was applied to the chip, and a gated injection scheme was used as described above. The injection time was 0.2 s.

2.5. Detection

Separations were monitored using laser-induced fluorescence (LIF). A krypton laser (Innova 300; Coherent, Santa Clara, CA) was utilized for excitation and focused onto the microchip using a 200 mm (focal length) lens. The fluorescence signal from the analytes was collected using a 40× (numerical aperture 0.45) objective. After passing through a spatial filter (1 mm diameter pinhole), the signal was spectrally filtered using both a 413.1 nm holographic notch filter and a 470 nm bandpass filter (65 nm bandwidth; Kaiser Optical Systems). The signal was then measured using a photomultiplier tube (77348; Oriel, Stratford, CT), amplified (SR570; Stanford Research Systems, Sunnyvale, CA) and read using a multifunction I/O card (National Instruments, Austin, TX). The data acquisition program was written in LabVIEW.

3. Results and discussion

Fig. 1 depicts the microchip used for on-chip reactions, separations and postcolumn labeling. The reaction channel was heated using a resistive heater. The heater temperature and the glass temperature measured on top of the microchip differed not more than 2°C in the temperature range from 30°C to 80°C down the length of the reaction channel. Simplified calculations have shown that less than 2 mm of the reaction channel are necessary to heat the fluid to the desired reaction temperature.

Fig. 2a shows the electrophoretic separations following on-chip tryptic digestion of the B-chain at 37°C. Although optimal conditions for the enzyme reaction are between pH 7 and 9, a slightly more basic pH (9.2) was chosen to assure rapid derivatization of the resulting peptides with NDA [23]. The total voltage applied to the reservoirs was 1.5 kV. This corresponds to electric field strengths in the reaction channel and the separation channel of 380 V/cm and 103 V/cm, respectively. The residence time for the oxidized insulin B-chain in the reaction channel was about 16 s at this field strength. The field strength in the separation channel and the side channel delivering the NDA reagent were 103 and 78 V/cm, respectively, resulting in a 1:0.76 mixing ratio of the separation effluent to NDA solution. Only a small fraction of the peptide was digested in the continuous flow mode. To allow the enzymatic reaction to proceed toward completion, the flow in the reaction channel was stopped by opening the electrical connection at reservoirs 1 and 2 while maintaining the potentials at reservoirs 3–6. Buffer, therefore, still flows from reservoir 3 to reservoirs 4 and 6 and prevents sample from diffusing into the separation channel.

As the stop flow reaction time increased, the growth of one major product peak was observed while the B-chain peak decreased. After stopping the flow in the reaction channel for 15 min, the enzymatic reaction was close to completion. Fig. 2b shows the areas of the product and oxidized insulin B-chain peaks normalized with the total peak area. As expected, the relative area of the B-chain peak decreases with increasing reaction times, and the product peaks though unresolved increase accordingly. Control runs without the B-chain were performed in both continuous flow mode and stopped flow mode. In the stopped flow mode the reaction time was 6 min. No significant background due to trypsin autolysis was detected. The arrows in Fig. 2a indicate the migration time of benzylamine, which was added as an electroosmotic mobility marker. The migration times of the marker showed good reproducibility for the entire experiment with a relative standard deviation of 1.8%. However, decreased peak heights were observed after stop times of 10 and 15 min. This could be compensated for by increasing the injection time. The reduced amount of injected sample was probably due to dilution of the reactor contents at the injection cross by diffusion or adsorption onto the channel walls in the reaction channel. This would reduce the electroosmotic flow in this channel, and, therefore, the amount of sample injected into the separation channel.

From the amino acid sequence of insulin B-chain, the possible cleavage sites for trypsin can be easily

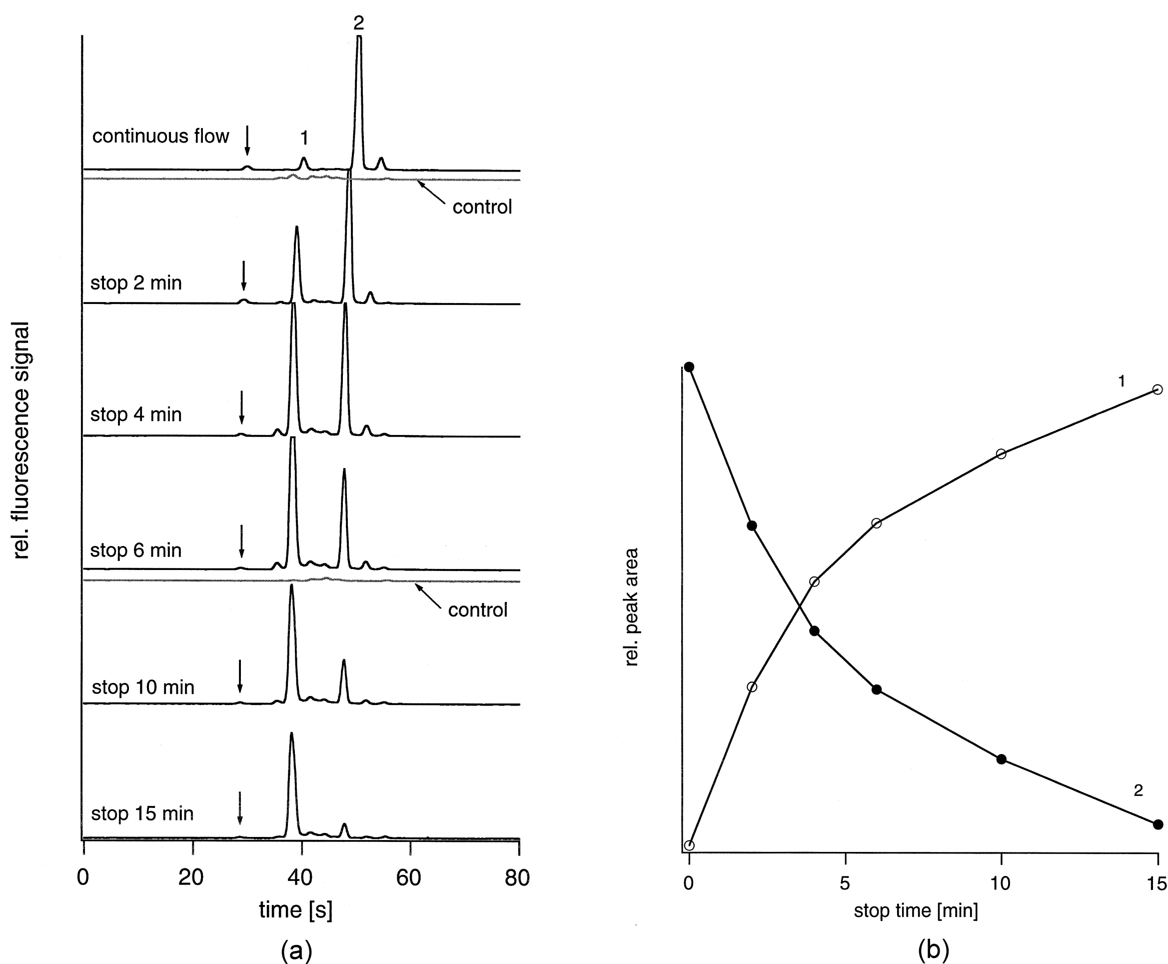

Fig. 2. (a) Electropherograms of the products following on-chip hydrolysis of oxidized insulin B-chain at 37°C. The flow in the reaction channel was stopped for different times. Control runs without insulin B-chain were performed in the continuous flow mode and with a stop time of 6 min. The arrows indicate the migration time of benzylamine, which was added as a electroosmotic mobility marker. All curves are plotted on the same scale with an offset for clarity. (b) Relative areas of the product (1) and B-chain peak (2) as a function of the reaction time. Prior to integration the peaks were approximated by a Gaussian function and the areas are normalized to the total peak area.

determined. The peptide consists of 30 amino acids with 2 cleavage sites for trypsin, one between amino acids 22 and 23 and one between amino acids 29 and 30 with residue 30 being alanine. Trypsin, however, preferably hydrolyzes peptide bonds in a polypeptide chain. The cleavage of peptides with charged carboxy groups at the C-terminus is inhibited due to the negatively charged active site of the enzyme [24,25]. Only two major peaks, therefore, can be expected for a complete digestion. These peaks were not resolved in this experiment using capillary electrophoresis with postcolumn labeling. Separation of these fragments by free solution electrophoresis is difficult but has been shown using a high efficiency separation capillary with mass spectrometry detection [20]. Microfluidic devices have been successfully interfaced with a time-of-flight mass spectrometer [26] and coupling the chip described in this work with time-of-flight mass spectrometry detection could improve the analysis of complex mixtures. However, by labeling the digestion products prior to the separation these peptides could be resolved as shown in Fig. 3. For this experiment, oxidized insulin B-chain was incubated off-chip with trypsin for 15 min

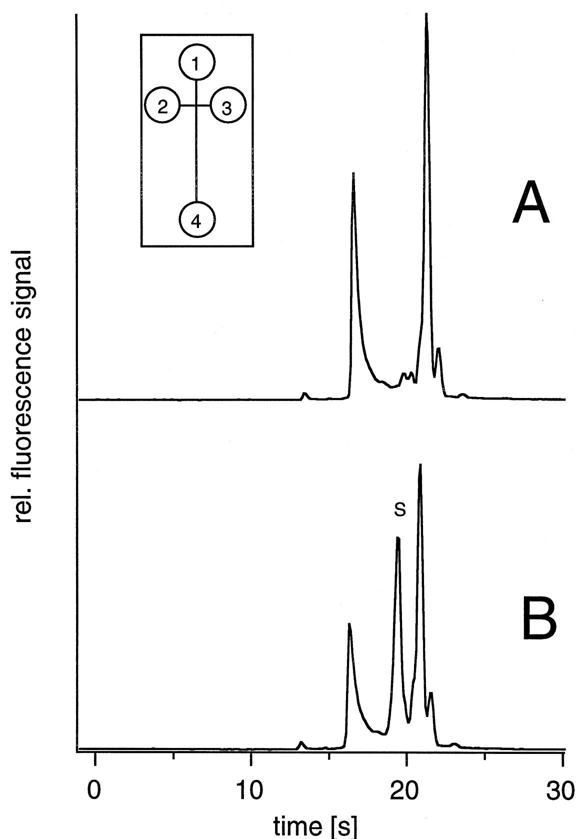

Fig. 3. (A) Electropherogram of insulin B-chain after off-chip tryptic digestion and off-chip labeling. The reaction was allowed to proceed for 15 min at room temperature. The reaction products were labeled with NDA/NaCN. (B) Electropherogram of the same digest with labeled B-chain (S) added. The inset shows the cross microchip layout used in these experiments. The separation length was 3.1 cm.

and then labeled off-chip with NDA. Two major peaks were detected as expected. NDA labeled insulin B-chain was added to confirm that neither of the peaks was due to undigested insulin B-chain. The off-chip labeled products have different mobilities than the unlabeled peptides and in this case result in a better separation. It has been verified in a separate experiment that neither of these peaks represent alanine.

To evaluate the performance of this microchip design at elevated temperatures, we monitored the reduction of disulfide bonds using the microchip design in Fig. 1. Bovine insulin consists of two peptide chains cross-linked by two disulfide bonds. Treatment with a disulfide-reducing agent, such as DTT, breaks the protein down into its A and B subunits. Fig. 4 shows the electrophoretic separation of the products following on-chip reduction of the disulfide bridges of insulin. The chip was operated in a continuous flow mode. As the temperature in the reaction channel was increased, the insulin peak decreased. No insulin was detected at 55°C and only one product peak was detected at 55°C. In separate experiments the migration time of this peak corresponded with the migration time of the B-chain, and the A-chain migrated well before the B-chain. Due to

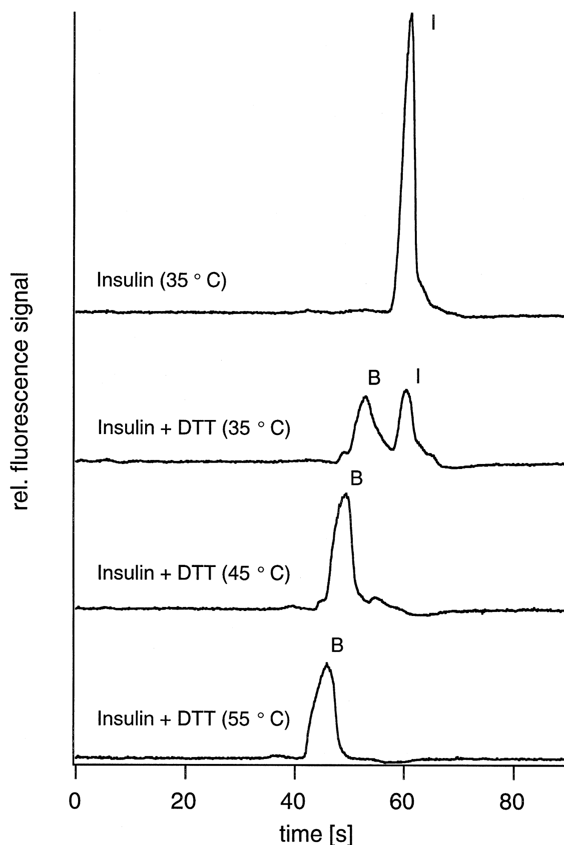

Fig. 4. Electropherograms of the product from the on-chip reduction of insulin's disulfide bonds with postcolumn labeling. Insulin (I) and DTT were reacted under continuous flow conditions at the listed temperatures. The migration time of the observed product peak (B) corresponds to the B-chain. The fluid reservoirs are: (1) sample, (2) buffer, (3) sample waste, and (4) waste.

its low solubility and poor labeling characteristics, however, the A-chain was not observed in this experiment. Also, the migration times decreased as the temperature in the reaction channel was increased. This indicated that the elevated temperature in the heated section of the chip influenced the migration time in the separation channel. The influence, however, is much smaller than would be expected if the separation channel itself was heated.

In conclusion, the incorporation of multiple functions (reagent mixing, separation and postcolumn labeling) enhances the advantages of microchip devices as biochemical analysis tools. The monolithic integration of chemical processing features essentially automates the biochemical procedure while enhancing the performance through near zero dead volume connections between the processes. Moreover, the miniaturization and device integration of the measurement process leads to reduced analysis times and reagent volumes. The low volumetric flow-rate through the channels and fast sample heating allowed reactions to be easily performed at elevated temperatures. Longer residence times in the reaction channel would enable the microchip to be operated in a continuous flow mode. Although the separative performance of the device demonstrated here was less than desirable, alternative designs are possible to greatly enhance ($>10^6$ plates) this performance feature [27]. The resolution of the products may also be increased by labeling the hydrolysis products prior to separation. Preliminary results using micellar electrokinetic chromatography showed improved resolution for pre-labeled tryptic digests.

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