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A microchip electrophoresis device with on-line microdialysis sampling and on-chip sample derivatization by naphthalene 2,3-dicarboxaldehyde/2-mercaptoethanol for amino acid and peptide analysis

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Abstract

The integration of rapid on-chip sample derivatization employing naphthalene 2,3-dicarboxaldehyde and 2-mercaptoethanol (NDA/2ME) with an easily assembled microdialysis/microchip electrophoresis device was carried out. The microchip device consisted of a glass layer with etched microfluidic channels that was sealed with a layer of poly(dimethylsiloxane) (PDMS) via plasma oxidation. This simple sealing procedure alleviated the need for glass thermal bonding and allowed the device to be re-sealed in the event of blockages within the channels. The device was used for analysis of a mixture of amino acids and peptides derivatized on-chip with NDA/2ME for laser-induced fluorescence (LIF) detection. A 0.6 mM NDA/1.2 mM 2ME mixture was simply added into the buffer reservoir for dynamic on-column derivatization of sample mixtures introduced at a flow rate of 1.0 μl/min. Using this scheme, sample injection plugs were derivatized and separated simultaneously. Injections of *ca.* 12 fmol of 5 mM amino acid and peptide samples were conducted using the system. Finally, a three-component mixture of Arg, Gly–Pro, and Asp was sampled from a vial using microdialysis, derivatized, separated and detected with the system. The ultimate goal of this effort is the creation of a micro-total analysis system for high-temporal resolution monitoring of primary amines in biological systems.
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1. Introduction

Microdialysis is a continuous sampling technique that has been used for various applications including pharmacokinetic [1–3], neurochemical [4,5], and biotechnology investigations [6]. Analytes of neurochemical importance monitored using this technique include aspartate (Asp) and glutamate (Glu) [7–9], catecholamines [10–12], methionine-enkephalin, and neurotensin [13,14]. *In vivo* or *in vitro* samples obtained by microdialysis are usually analyzed using separation techniques such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and, more recently, microchip electrophoresis.

Previously, we described a microchip electrophoresis system coupled to on-line microdialysis sampling [15]. Coupling micro-

dialysis directly to microchip electrophoresis systems facilitates fast analysis times (subsecond-to-second timescale) [16,17] and high-temporal resolution. Both the interface and injection components can be fabricated on a single chip. The small footprint of the device also reduces the amount of connective tubing and lab bench space needed compared to conventional systems [18,19]. This on-line system exhibited a high degree of integration and incorporated: (1) sample preparation and recovery (from the dialysis process); (2) direct sample delivery to the microchip device; (3) on-chip sample gating and injection; (4) electrophoretic separation and laser-induced fluorescence (LIF) detection. The device was connected to an external microdialysis probe and used to monitor an enzyme assay of the hydrolysis of a fluorescent substrate, fluorescein mono-β-D-galactoside, to its fluorescent product, fluorescein.

The use of fluorescent dyes and substrates is necessary for device optimization and characterization; however, for most real-world biological applications, the analyte of interest does

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not exhibit native fluorescence and, therefore, incorporation of a sample derivatization step is necessary for detection employing LIF. Pre- and postcolumn sample derivatization strategies for microchip devices have been reported [20–25]. In these studies, the sample is usually combined with a derivatization reagent stream prior to or after the separation channel for pre- and post-column derivatization, respectively.

Derivatization reactions conducted on microchip electrophoresis devices must occur very rapidly, on the order of seconds. A commonly employed reagent for on-chip derivatization of analytes containing a primary amine group (*e.g.*, amino acids and peptides) is the ortho-phthalaldehyde/2-mercaptoethanol (OPA/2ME) system [22,23]. Recently, Kennedy and coworkers reported a microfluidic chip coupled to microdialysis that incorporated a reactor channel for precolumn derivatization of amino acids with OPA [26].

An analog of OPA that exhibits very similar reaction chemistry is naphthalene 2,3-dicarboxaldehyde (NDA); derivatization products of NDA have been found to be more stable than those of OPA, particularly in the case of peptides [27]. It has also been shown that replacement of cyanide (CN) with a thiol increases the reaction rate, making NDA/2ME more suitable for on-column and postcolumn derivatization. NDA/2ME has been employed for rapid amino acid and peptide analysis following liquid chromatography [28,29] and capillary electrophoresis [30]. More recently, NDA was employed for on-column derivatization of reduced glutathione on a microchip electrophoresis device [31].

The devices used for the studies described in this paper consist of a glass layer containing etched microfluidic channels that is plasma sealed with a layer of poly(dimethylsiloxane) (PDMS) [32]. This fabrication approach is a quick and easy alternative to the production of similar devices previously reported by our laboratory that were composed exclusively of glass. Specifically, the hybrid device obviates the very time-consuming and delicate high-temperature bonding process that is necessary to produce glass microchip devices. The hybrid glass–PDMS devices constructed here are easily sealed and can also be taken apart and re-sealed in the event of a channel blockage.

In these studies, hybrid microchip electrophoresis devices are constructed for continuous on-line sampling from a syringe or microdialysis. The combination of this device with dynamic on-column sample derivatization using NDA/2ME was evaluated for peptide and amino acid analysis. Such work demonstrates the integration of on-chip sample derivatization to an already highly integrated device. This separation-based sensor can ultimately be employed for high-temporal resolution monitoring of primary amines of biological significance.

2. Experimental

2.1. Materials and reagents

All chemicals and materials were used as received: boric acid, sodium hydroxide, 2-mercaptoethanol, amino acids, and peptides were purchased from Sigma (St. Louis, MO). Hydrochloric acid, sulfuric acid, 30% hydrogen peroxide, ammonium hydrox-

ide, acetone, and isopropyl alcohol were from Fisher Scientific (Fair Lawn, NJ). Naphthalene 2,3-dicarboxaldehyde, microdialysis probes, 1000 μl syringes, 250 μm i.d. (1/16 in. o.d.) FEP microdialysis tubing, and tubing connectors were purchased from Bioanalytical Systems (BAS, West Lafayette, IN). The syringe pump was purchased from CMA Microdialysis (North Chelmsford, MA). PEEK tubing (250 μm i.d., 510 μm o.d.), tubing fittings, and low dead-volume unions were obtained from Upchurch Scientific (Oak Harbor, WA). Soda lime glass with pre-deposited layers of chrome and positive photoresist was purchased from Telic (Santa Monica, CA). Sylgard 184 with curing agent was from Ellsworth Adhesives (Germantown, WI). Positive photoresist developer was from Clarion (Sommerville, NJ) and chrome stripper was from Cyantek Corp. (Fremont, CA). Buffered oxide etchant containing 10% hydrofluoric acid was from Fox Scientific (Alvarado, TX). The syringes, syringe pumps, probes, and Teflon tubing (0.65 mm o.d. × 0.12 mm i.d.) used for the experimental setup were obtained from Bioanalytical Systems.

2.2. Construction of the microchip devices

Microchip devices were fabricated in-house using standard photolithographic techniques. The microchip design was drawn using Microsoft Freehand 8.0 software and then a negative mask transparency was produced. For the production of the glass layer containing the separation channel [15], the negative mask transparency was placed on top of a piece of soda lime glass with the pre-deposited layers of chrome and AZ1500 photoresist. The glass was exposed to UV light for 15 s then placed in AZ developer, rinsed with Nanopure water, and baked at 95 °C for 10 min. The exposed layer of chrome was then removed using chrome stripper. A temperature-controlled circulating etch bath (Modutek, San Jose, CA) of buffered oxide etchant was used to etch the channels in the glass. (*Caution*: Buffered oxide etchant contains hydrofluoric acid and should be handled with extreme care.) The total etching time was 40 min. The glass was removed from the bath intermittently and dipped into a solution of 1 M hydrochloric acid, rinsed with water, and dried with a filtered stream of N_2 gas to minimize particulate build-up in the channels. Once the etching was complete, the protective photoresist and chrome layers were removed using acetone and chrome stripper, respectively.

The etch profiles were determined using a Tencor Alpha Step 200 profilometer (San Jose, CA); the channel dimensions were found to be 90 μm wide at the top of the channel and 35 μm wide at the bottom of the channel with a depth of 20 μm. For the wider sampling channel, the width was 500 μm and the depth was 20 μm. Channel lengths for all microchips were as follows (see Fig. 1A): the sampling channel was 23 mm; the segment connecting the sampling channel to the injection T was 7 mm; the arms connecting the injection T to the buffer and buffer waste reservoirs were both 10 mm; the separation channel was 25 mm total with fluorescence detection carried out 20 mm downstream from the injection T.

Access holes were drilled into the glass layer and the fluidic connector was adhered to the glass layer using J.B. Weld (Sul-
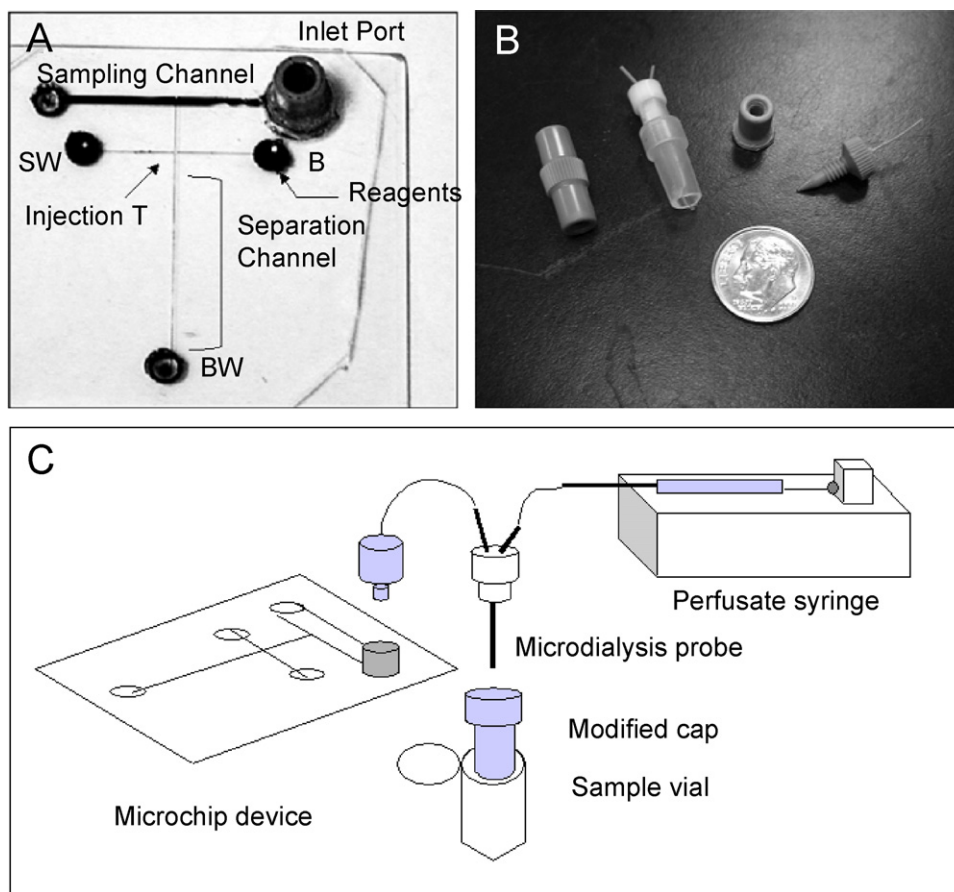
Fig. 1. (A) Picture of PDMS–glass hybrid device used in initial studies. Device is constructed by etching channels into the glass layer and irreversibly sealing with a layer of PDMS. *Reservoirs*: B, buffer; SW, sample waste; BW, buffer waste. Derivatization reagents were added into the buffer reservoir. (B) Inlet sample port was modified from a high-pressure union, into which a male fitting could be fastened. Also shown is a microdialysis brain probe with modified cap that could be slotted into a vial for *in vitro* sampling. (C) Schematic of the setup for microdialysis sampling to the microchip.

fur Springs, TX). The fluidic connector was a nanoport modified from a low dead-volume union (Fig. 1B). To produce the PDMS layer, a 7:1 (w/w) mixture of Sylgard 184 and curing agent was poured onto a silicon wafer and allowed to bake in a 70 °C oven for at least 3 h. The layer was then carefully peeled off of the wafer master and used to plasma-seal the glass layer. Both the glass and PDMS layers were placed into a plasma oxidizer (Harrick Scientific, Ithaca, NY) for 90 s and put in contact with one another immediately to complete construction of the device. The flexible PDMS layer could easily be removed using a razor blade to allow the glass layer to be cleaned and re-sealed in the case of blockages.

2.3. Device coupling to syringe and microdialysis sampling

The general setup for these experiments has been described previously [15], and a schematic representation is shown in Fig. 1C. Briefly, the modified nanoports (Fig. 1B) were cut in half using a dremel tool (Ace Hardware, Lawrence, KS) and adhered to the devices as described above. Tubing could then be inserted into a male fitting and easily screwed into the nanoport attached to the microchip. This setup served as a fluidic connection to the microchip, and the other end of the tubing was connected to either a syringe or microdialysis probe. A flow rate of 1.0 μl/min

was used in the experiments. Microdialysis probes received from the manufacturer (BAS) were packaged with a plastic protective cap; this cap was cut using a razor blade to expose the probe tip (Fig. 1B). This fixture could then be easily slotted into a 1.5 ml Eppendorf tube (Fisher Scientific) that contained sample. The perfusion syringe filled with boric acid running buffer was connected to the inlet of the 4-mm microdialysis brain probe (BAS).

2.4. Device operation conditions

A solution of 25 mM boric acid buffer adjusted to pH 9.2 with 1N NaOH was used as the electrophoresis buffer. The device was conditioned by flushing the microchip with 0.1N NaOH for 15 min prior to filling with boric acid buffer. Platinum electrodes were inserted into the reservoirs of the microchip, and a voltage of 2300 V was applied to the buffer reservoir (B) while keeping the buffer waste (BW) and sample waste (SW) reservoirs at ground (Fig. 1A). The voltage was applied to the buffer reservoir using a four-channel bipolar high voltage system (Jenway, Essex, UK) that was controlled by a Toshiba laptop computer. During device operation, voltage was applied to hydrodynamically pump sample away from the separation channel of the microchip. When the voltage was turned off (1 s), the injection

T was filled with sample and reapplication of voltage allowed a small plug to be injected down the separation channel [15,33].

For sample derivatization experiments, stock solutions of 10 mM NDA and 60 mM 2ME were prepared in methanol and in a 1:1 solution of methanol and run buffer, respectively. The following stock solutions of amino acids and peptides were prepared in Nanopure water: 50 mM Phe, 25 mM Glu, 26.5 mM Asp, 15.2 mM Leu–Enk, 50 mM Gly–Pro, and 50 mM Arg. Sample mixtures were prepared in buffer from the stock solutions and used to fill a syringe for direct sampling or placed into a vial for on-line microdialysis. A derivatization mixture of 0.6 mM NDA/1.2 mM 2ME in buffer was placed in the buffer reservoir of the device. This derivatization mixture was determined to be optimum for device operation as it was the maximum concentration of agents that could be placed in the buffer reservoir while maintaining proper injection gating. This determination was carried out by hydrodynamically pumping a syringe of 200 μM disodium fluorescein at 1.0 μl/min and imaging the injector T using an Axioskop fluorescence microscope (Zeiss USA, Thornwood, NY) with different mixtures of various NDA/2ME concentrations (data not shown). This was carried out under device operating conditions of 2000 V applied voltage and an injection time of 1 s.

2.5. LIF detection

A home-built LIF detection setup similar to that previously described was employed [15]. Briefly, laser light was directed into an optics cube using three mirrors (Thor Labs, Newton, NJ). The optics cube contained a dichroic mirror that directed the beam 90° into a long working distance objective (N.A. = 0.6, 40× LWD Plan Fluorite LCP LFL; Olympus America, Melville, NY), which was translatable in the *X–Y* planes. This objective focused the beam to a spot onto the separation channel of the microchip, which was secured on an *X–Y–Z* translatable stage. Fluorescence was collected with the same microscope objective, passed through the dichroic mirror, and reflected 90° with a mirror toward a R1477 photomultiplier tube (Hamamatsu, Bridgewater, NJ) contained in a tube housing (Thermo Oriel, Stratford, CT). Appropriate excitation and emission spectral filtering were used to decrease background. However, spatial filtering with a pinhole was not used in this setup. The 442-nm line of a HeCd laser (Kimmon, Englewood, CA) with 450DRLP dichroic was employed for the experiments. Analog signal filtering was accomplished with the use of a Standford Research Systems pre-amplifier (Sunnyvale, CA) and converted into digital output using a DA-5 unit from BAS.

3. Results and discussion

3.1. Hybrid microchip device

A hybrid microchip device was employed for the studies described in this paper. Plasma sealing of a PDMS layer to a glass layer with etched fluidic channels is a convenient method for labs that lack facilities for high-temperature glass bonding. Devices were quickly and easily assembled for use. In cases where chan-
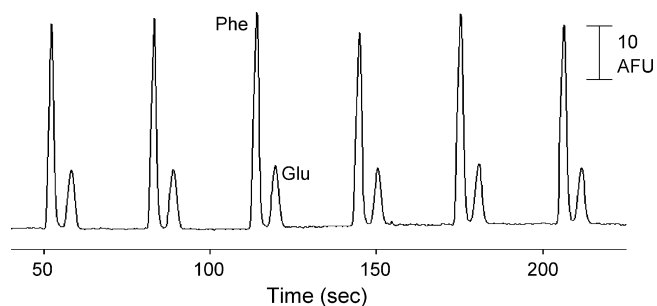

Fig. 2. Sequential injections and separations of 5 mM concentrations of Phe and Glu, derivatized on-chip with a mixture of 0.6 mM NDA and 1.2 mM 2ME. Conditions were as follows: applied voltage of 2300 V was used with a 1 s sample fill time and a flow rate of 1.0 ml/min. LIF detection was conducted 20 mm from the injector T.

nel blockages were seen, the PDMS layer of the device was removed with a razor blade and the glass layer was cleaned for re-use. In this case, the channels are composed of three walls of glass and one wall of PDMS. The presence of PDMS in the channel can have an effect on the electroosmotic flow as well as providing some sites with potential for interactions with hydrophobic analytes. However, for many applications, this is not an issue. A more thorough investigation involving a direct comparison of the hybrid and all-glass chips would better elucidate how the PDMS layer affects device operation and performance; however, such work is beyond the scope of this paper.

3.2. On-chip sample derivatization of peptides and amino acids using NDA/2ME

Device characterization and optimization had been carried out previously using a glass microchip and fluorescein-based analytes [15]. To increase the utility of the on-line microdialysis–microchip capillary electrophoresis system for detection of non-fluorescent samples of biological interest such as peptides and amino acids, a derivatization scheme was incorporated into the chip. The primary amine groups of amino acids and peptides were sites of interest for derivatization chemistry using naphthalene 2,3-carboxaldehyde/2-mercaptoethanol. Initially, a solution of 1 mM Glu was pumped into the device and reacted with a mixture of 0.6 mM NDA/1.2 mM 2ME in the buffer reservoir. Under these conditions, individual injection plugs of Glu were detected in less than 20 s using LIF detection (data not shown) at an excitation wavelength of 442 nm. Concentrations of less than 1 mM of this and other amino acids examined resulted in no detectable peaks under the conditions used.

Next, a simple mixture of two amino acids, 5 mM Phe and Glu, was pumped into the device using the same conditions described above, and sequential injections were carried out. Fig. 2 shows multiple baseline-resolved separations that were conducted every 30 s with good reproducibility (R.S.D. = 4.3 and 4.5% for Phe and Glu, respectively; *n* = 6). The migration times of the analytes can be viewed as the amount of time the analytes had to react with the derivatization agents. This was estimated
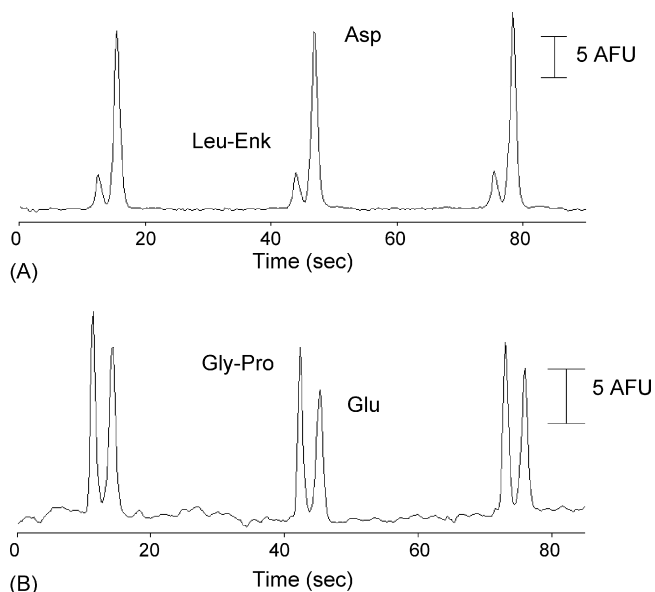
Fig. 3. Amino acid and peptide separations. Amino acids and peptides were derivatized on-chip using NDA/2ME. Consecutive injections of (A) Leu–Enk and Asp and (B) Gly–Pro and Glu. Conditions were similar to those used in Fig. 2.

to be 12 and 18 s for Phe and Glu, respectively. Separation of simple mixtures of peptides and amino acids introduced from a syringe was also evaluated using this system. These mixtures, which consisted of (A) leucine–enkephalin (Leu–Enk) and Asp and (B) glycine–proline (Gly–Pro) and Glu, were analyzed and the results are shown in Fig. 3. The concentrations of analytes in their respective mixtures were 5 mM. In these analyses, simultaneous separation and reaction of the analytes was accomplished in less than 20 s.

On-chip derivatization with simultaneous separation has been conducted for proteins and peptides in a microchip capillary gel electrophoresis format by Landers and coworkers [34,35]. In these studies, the investigators used NanoOrange, a commercially available dye, as a non-covalent fluorescent binding agent for protein–SDS complexes. Long separation channel lengths (75 mm) were employed to allow greater product yield and, thereby, decrease limits of detection (LOD) but at the cost of longer analysis times (300 s).

Estimated injection plug lengths of 20 μm correspond to approximately 12 fmol injection out of 5 mM amino acid and peptide samples. In comparing the present studies to earlier work utilizing precolumn OPA/2ME derivatization on microchip devices, detection limits of 0.55 and 0.83 fmol for arginine (Arg) and glycine (Gly), respectively, were reported by Jacobson et al. [22]. More recently, Kennedy and coworkers used this approach for precolumn derivatization of Arg, Glu, and Asp with OPA/2ME using on-line microdialysis/microchip electrophoresis. Their detection limits were 0.13, 0.08, and 0.09 fmol for Arg, Glu and Asp, respectively [26]. For postcolumn derivatization, detection limits were 200, 130, and 120 fmol for Arg, Gly, and Thr, respectively [23]. The limits of detection for this work employing on-column derivatization were better than those reported for a postcolumn scheme; however, precolumn deriva-
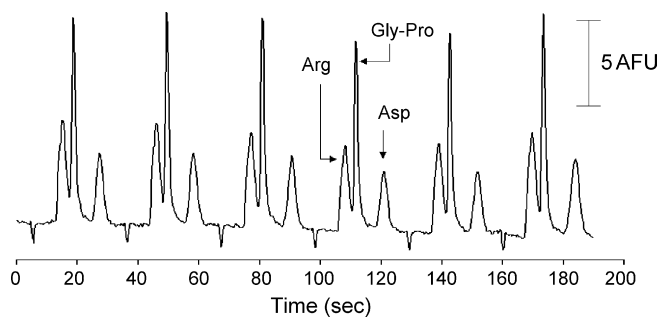

Fig. 4. Continuous separations of a three-component mixture sampled continuously through a 4-mm microdialysis probe at a flow rate of 1.0 ml/min. Analyte concentrations are as follows: 7.95 mM Arg, 10 mM Gly–Pro, and 10 mM Asp. Derivatization conditions were similar to those used in Fig. 2.

tization limits of detection were 2–3 orders of magnitude lower than the LOD values here.

Future improvements to the current design can be made by increasing device channel length for on-column derivatization or the investigation of precolumn derivatization to ensure complete reaction of reagents and analytes. Employing a pinhole filter in the LIF detection setup to reduce scattered light and fluorescence background will be considered to improve detector sensitivity. In addition, further optimization of buffer conditions and the use of cyclodextrin modifiers to reduce any background and increase fluorescence, respectively, can be carried out [36].

3.3. On-line microdialysis sampling with on-chip derivatization

The goals of this work were to: (1) establish a method for construction of an easily assembled, re-usable microdialysis–microchip device; (2) utilize on-column NDA/2ME derivatization for amino acids and peptides on-chip; (3) incorporate the additional feature of on-chip sample derivatization to our on-line system. The last of these was demonstrated by sampling a three-component mixture from a vial by using a 4-mm microdialysis brain probe. The probe was then coupled to the chip device for derivatization and analysis. Fig. 4 shows the results of this analysis. Concentrations of analytes were as follows: 7.95 mM Arg, 10 mM Gly–Pro, and 10 mM Asp. Injections were conducted at a very rapid rate (every 30 s), and the reproducibility was good (R.S.D. = 8.2, 5.9, and 11.8% for Arg, Gly–Pro, and Asp, respectively; n = 6). The longevity of the device was determined in part by the run buffer. Analyses could be carried out for approximately 35 min before needing to replenish reservoir B with buffer. Future investigations will focus on automating the buffer replenishing and channel conditioning steps to increase the overall run time of a chip.

4. Conclusion

The incorporation of on-chip sample derivatization was carried out using an easily assembled microchip CE device coupled on-line to microdialysis sampling. This miniaturized analysis system exhibits a high degree of process integration, including sample preparation, delivery, sample gating, dynamic derivati-

zation, separation, and detection for high-temporal resolution monitoring of biological systems. Future studies will involve improvement of LIF detector sensitivity by addition of a pinhole filter as well as lengthening the separation channel for on-column reaction and investigation of a precolumn derivatization scheme to increase fluorescent product yield. The ultimate goal of this work is the development of a micro-total analysis system for high-temporal resolution biological monitoring.

Acknowledgments

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