Development of online microdialysis-microchip system for *in vivo* monitoring

By

Pradyot Nandi

M.S., Industrial Pharmacy, 2003, The University of Toledo, Toledo, Ohio.

M.S., Pharmaceutical Chemistry, 2006, The University of Kansas, Lawrence, Kansas.

Submitted to the Department of Pharmaceutical Chemistry and the faculty of the Graduate School of The University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dissertation Committee

Susan Lunte, Chair

Jeffrey Krise

John Stobaugh

Cory Berkland

Karen Nordheden

Nathan Lacher

Dissertation Defense: August 14, 2009

The Dissertation Committee for Pradyot Nandi certifies that this is the approved version of the following dissertation:

Development of online microdialysis-microchip system for *in vivo* monitoring

Dissertation Committee

Susan Lunte, Chair

Jeffrey Krise

John Stobaugh

Cory Berkland

Karen Nordheden

Nathan Lacher

Dissertation Defense: August 14, 2009

Acknowledgements

I would like to sincerely thank my advisor, Dr. Susan Lunte for her guidance and patience throughout the last few years. Working in her group helped me to develop my own independent scientific thoughts and ideas as well as improve my personality skills. She encouraged me to write the AHA proposal which was a very helpful experience for me. Also, I appreciate her kindness for offering me a position when the next career move did not look very bright or promising.

Thanks to Dr. Karen Nordheden for reading my dissertation and suggesting the corrections. Thanks to Drs. John Stobaugh, Jeff Krise and Cory Berkland for agreeing to be a part of my dissertation committee.

Sincere thanks to Dhara Desai for her constant support and help during the extremely challenging *in vivo* (animal) microchip experiments. Without her assistance, it would have been impossible to acquire some of the microchip data and achieve the end goals of this dissertation. Our detailed deliberations helped me to keep things in perspective regarding what is feasible and practical.

A special word of thanks to Lunte group members especially David Fischer, Courtney Kuhnline and Ryan Grigsby for our discussions on CE, fabrication, microchips, as well everything else that becomes important in the day-to-day life of a (microfluidic) scientist. The humor and fun in the lab were definitely helpful in dealing with the stressful situations when microchips did not co-operate. Also, the inclination of Dave and Courtney towards spicy and exotic cuisine positively contributed to the well-being of the stomach which is directly linked (scientifically or not) to attaining stable mental health during distressful times in the lab.

Thanks to Dr. Matt Hulvey for some very helpful suggestions and discussions regarding on-chip mixing strategies. I also want to express sincere gratitude to Dr. Nathan Lacher who was instrumental in setting up my internship at Pfizer-St. Louis, which was an excellent and valuable experience for me. Also really appreciate his eagerness to serve as one of my committee members and reading my thesis very closely and thoroughly and coming up with the suggestions and corrections. Thanks

to Dr. Yan He, Steven Cook and Rachel Roberts at Pfizer-St. Louis, for their readiness to help me any time I had questions regarding my experiments. I would like to thank Drs. Bryan Huynh, Barbara Fogarty and Celeste Frankenfeld for their help and training after I joined the group. Thanks to Dr. Nobuyuki Suzuki (Nobu-san) for keeping us all engaged and entertained with his stories from Japan as well as cross country trips in USA during our "BIG" lab move in 2006. Also, thanks to members of Craig Lunte group for allowing us to use their animal surgery facility.

I would like to thank the department of Pharmaceutical Chemistry for providing me the opportunity to be a part of the PhD program. Thanks to Nancy Helm for patiently answering all my questions. Thanks to the faculty of the department for teaching some extremely relevant courses which will always be useful.

Last but not the least, I would like to thank my wife Pallabi. I could have never come to this point without her care, support, encouragement, patience and contribution. Being with her undeniably helped me a great deal during challenging and trying times of graduate school through our discussions about various issues of life. Also, I would like to thank both Pallabi's family and my family for their love, concern and well wishes.

Abstract

Microdialysis is a sampling technique that can be employed to monitor biological events *in vivo* and chemical reactions *in vitro.* When it is coupled to an analytical system, microdialysis can provide near real time information on the time dependent concentration changes of analytes in the extracellular space or other aqueous environments. Online systems for the analysis of microdialysis samples enable fast, selective and sensitive analysis while preserving the temporal information. Analytical methods employed for on-line analysis include liquid chromatography (LC), capillary (CE) and microchip electrophoresis and flow-through biosensor devices. This dissertation is focused on the development of microchip systems coupled to microdialysis sampling for online near-real time monitoring with high temporal resolution for the analysis of amino acid neurotransmitters *in vivo*. Fluorescence detection was utilized for all the studies. Also, naphthalene-2,3-dicarboxaldehyde (NDA) and β-mercaptoethanol (β-ME) or CN^- are used as the derivatization reagents for rendering the amino acids fluorescent.

Initial studies were performed to evaluate a twin-t design with a 3 cm separation channel that incorporated on-column derivatization with NDA and β-ME. Biogenic amines and peptides were separated on chip demonstrating the feasibility of online microdialysis sampling with online derivatization and analysis *in vitro*.

Subsequently, the chip was further modified by incorporating a 20 cm serpentine separation channel and excitatory amino acid neurotransmitters (glutamate, aspartate) were separated from other analytes in rat brain microdialysis samples.

Next, precolumn derivatization was incorporated into the chip and online *in vivo* experiments were performed for the continuous analysis of glutamate from rat brain microdialysis sample. Also, fluorescein was included in the experiment for the possibility to simultaneously monitor the permeability of the blood brain barrier (BBB) along with changes in levels of excitatory neurotransmitters during online analysis applied to the rat stroke model. The chip was further modified and optimized for experiments that require fast injections and separations thereby attaining high temporal resolution. This work described an online microdialysis-microchip system that can be used for monitoring neurochemical events that involve fast changes in analyte concentration in the brain.

Table of Contents:

List of figures and tables:

Chapter 1

Introduction

("Recent trends in microdialysis sampling integrated with conventional and microanalytical systems for monitoring biological events: A review", Pradyot Nandi, Susan Lunte, Analytica Chimica Acta, 2009, accepted)

1.1 Introduction

There are very few tools that can be used to continuously monitor biological events with high temporal resolution. Microdialysis is a powerful sampling technique that makes it possible to continuously monitor the concentrations of biological molecules and other substances both *in vivo* and *in vitro*. Although the primary applications of microdialysis sampling have been in the area of neuroscience research [1], it has also been extensively employed for other pharmaceutical applications. These include the investigation of the transdermal delivery of drugs [2], tissue pharmacokinetics [3], and regional metabolism of drugs in tissues [4,5].

Microdialysis probes have been placed in virtually every tissue and organ in the body including the liver [6,7], heart [8,9], skin [4,10], blood [11,12], placenta [13], stomach [14,15] and ear [16]. **Fig. 1.1** shows the microdialysis sampling process. These probes act in a manner similar to a blood vessel, allowing substances to be removed or delivered to a specific site through diffusion with no net fluid loss. A probe containing a dialysis membrane with a specific molecular weight cutoff is implanted in the physiological region of interest. It is then perfused with a fluid that is similar in ionic strength and composition to the extracellular fluid being sampled. Small molecules in the extracellular fluid that are not present in the perfusate diffuse across the membrane based on their concentration gradient and are then transported to the analysis system. Likewise, compounds in the perfusate that are not present in the extracellular fluid can be delivered directly to the physiological site of interest. Therefore, it is possible both to deliver and recover compounds from a single tissue

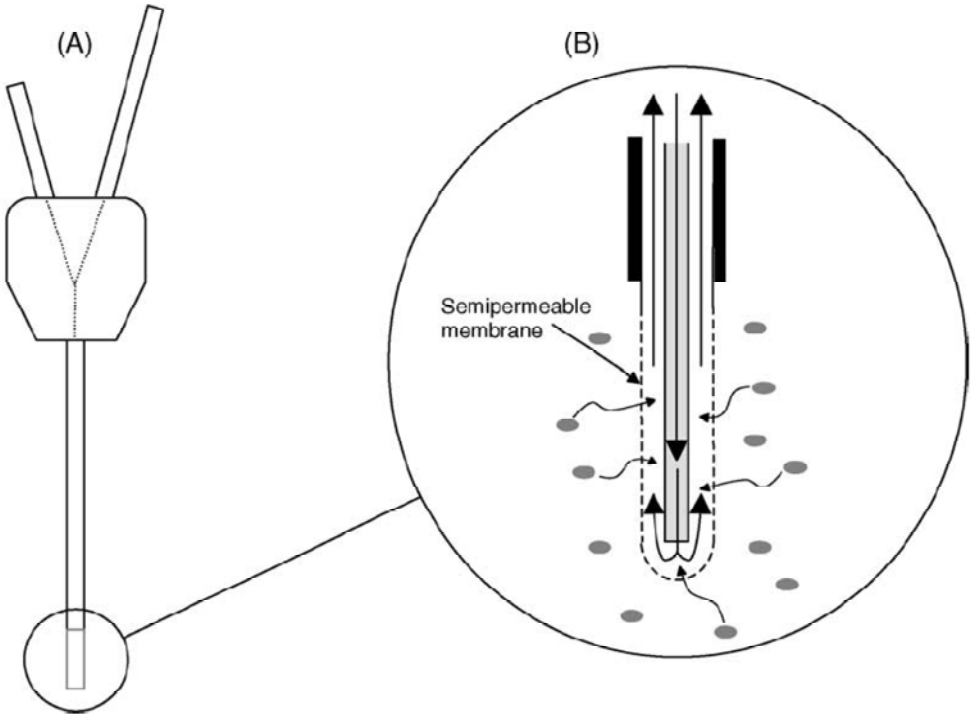


Fig. 1.1: Sampling process across microdialysis probe membrane (from Plock *et al*. [107])

site. This feature can be very useful for looking at site-specific release of neurotransmitters, observing regional metabolism of neuropeptides [17,18], or comparing the metabolism of antineoplastic agents in tumor versus healthy tissue [19].

Over the past decade, a considerable amount of research has been conducted on the development of online analytical systems capable of analyzing microdialysis samples. The direct coupling of the analytical system to the microdialysis sampling system allows continuous near real-time monitoring of biological and other processes in vivo and *in vitro*. Online methods usually provide better temporal resolution and precision than offline analyses. This chapter addresses some recent advances in the development of hyphenated systems incorporating microdialysis with microanalytical systems with particular emphasis on microchip based devices.

1.1.1. Microdialysis probes

Fig. 1.2 shows some typical microdialysis probe configurations. A concentric cannula design (**Fig. 1.2A**) is normally employed for neurochemical studies. These probes are composed of stainless steel and are implanted into the specific brain region of interest using a guide cannula. A typical probe used for rat brain studies is 15 mm long with an outer diameter between 200 and 500 microns. The dialysis membrane is located at the end of the concentric cannula and is usually from 1 to 4 mm in length. Probes are also available for brain sampling in mice. The mouse probes employ shorter shafts (7–12 mm) and have outer diameters between 220 and 380 microns.

For sampling soft tissue, a linear probe can be employed (**Fig. 1.2B**). These probes consist of a microdialysis membrane (4–10 mm) placed between two pieces of flexible tubing. Soft tissue is more homogeneous than brain tissue and, therefore, spatial resolution is not as important. Thus, the surface area of these probes is generally larger than that of the brain probe. This type of probe has been used for sampling from liver, muscle, heart and skin.

For blood sampling, a flexible probe has been developed (**Fig. 1.2C**). This type of probe was first described by Telting-Diaz *et al.* [20]. Its design is similar to that of the brain probe and consists of two pieces of fused silica tubing attached to the dialysis membrane. The flexible probe can bend when the animal moves, minimizing any damage to blood vessels. In general, analyte recoveries are higher from blood than from the stagnant interstitial fluid [21].

A shunt probe has also been developed for sampling from moving fluids *in vivo* or *in vitro* (**Fig. 1.2D**) [22]. This probe has been used to sample bile in awake, freely moving animals. For these experiments, the bile duct is cannulated and bile is sampled by a second dialysis membrane filled with fluid of similar ionic composition running in the opposite direction. This probe has also found use for desalting protein samples prior to introduction into a mass spectrometer [23].

Most microdialysis probes have molecular weight cut-offs between 20,000 and 60,000 Da. Recoveries decrease dramatically for most compounds of about 10,000 Da molecular weight. In cases of extremely fast sampling, diffusion across the dialysis membrane can be the limiting factor for temporal resolution [24]. To

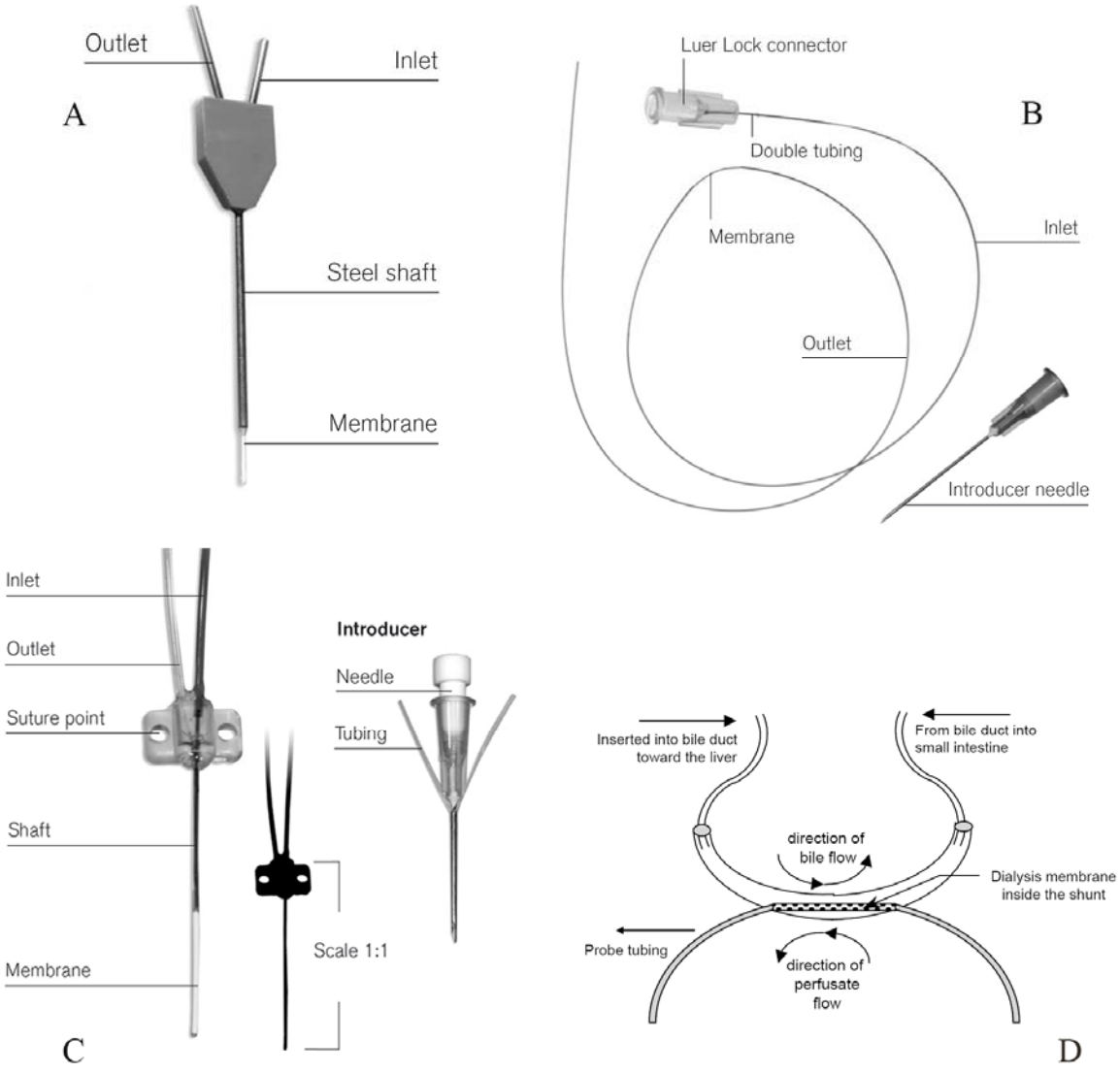


Fig. 1.2: Microdialysis probes. A. Cylindrical probe for brain. B. Linear probe for peripheral tissues. C. Flexible probe for intravenous use. D. Shunt probe for bile duct sampling (Fig. 2A–2C: CMA product catalogue; 2D: Huff *et al*. [22])

circumvent these problems and make it possible to sample larger molecules as well as obtain higher recoveries of small molecules, Shippy's group has developed a low-flow, push-pull perfusion device [25]. The push-pull perfusion probe consists of a 27-gauge stainless steel cannula that is perfused with saline. A fused silica capillary acts as the inner cannula and is used to withdraw fluid from the extracellular space; the fluid is then transferred to the analysis system. Flow rates using this method are from 10 to 50 nL/min and analyte recovery is 100%.

1.2. Microdialysis sampling

Microdialysis has several advantages for sampling biological tissues and fluids, especially when combined with separation-based analytical methods. The resulting samples are most commonly composed of an aqueous salt solution that contains only small molecular weight analytes. Cells and macromolecules are excluded by the dialysis probe so centrifugation or protein precipitation steps are not required prior to analysis. In addition, because sampling is based on diffusion of substances across the dialysis membrane, no fluid is removed. Therefore, it is possible to continuously monitor substances in the extracellular fluid of the brain and other tissues for several months [26,27]. The animal can also serve as its own control. This latter advantage is particularly important for tissue distribution studies where the normal protocol is to sacrifice several animals at each time point to obtain enough data to make statistically valid conclusions.

Another significant advantage of microdialysis is that studies can be performed in awake, freely moving animals. This is especially important in studies that correlate brain drug concentration and/or neurotransmitters with behavior. Because of the small size and relatively noninvasive nature of the microdialysis probes, it is also possible to put multiple probes in a single animal. It is therefore possible to measure blood, brain, and tissue concentrations of drugs or endogenous substances simultaneously. If the animal is awake, these measurements can be correlated temporally with behavior.

A very nice example of the use of multiple probes in a single animal is shown in **Fig. 1.3** [28]. In this experiment, the rat was given an i.v. injection of methylphenidate (Ritalin). Brain and blood sampling was accomplished using a concentric cannula probe and flexible probe, respectively. Dialysates were collected offline, and the concentrations of methylphenidate and dopamine in both the brain and blood were determined using liquid chromatography with electrochemical detection. In this manner, it was possible to measure the transport of Ritalin across the blood-brain barrier as well as its effect on catecholamine release. Lastly, by using a "RatTurn," the extracellular concentration of these substances could be directly correlated with the overall activity level of the rat. For long-term-microdialysis studies, careful consideration should be made regarding the tissue damage associated with probe implantation. Fibrosis or gliosis can occur after several days of probe implantation [29]. Grabb *et al*. compared the effects of acute (2–4 hours) and chronic (24 hours) implantation of microdialysis probes. They reported the occurrence of

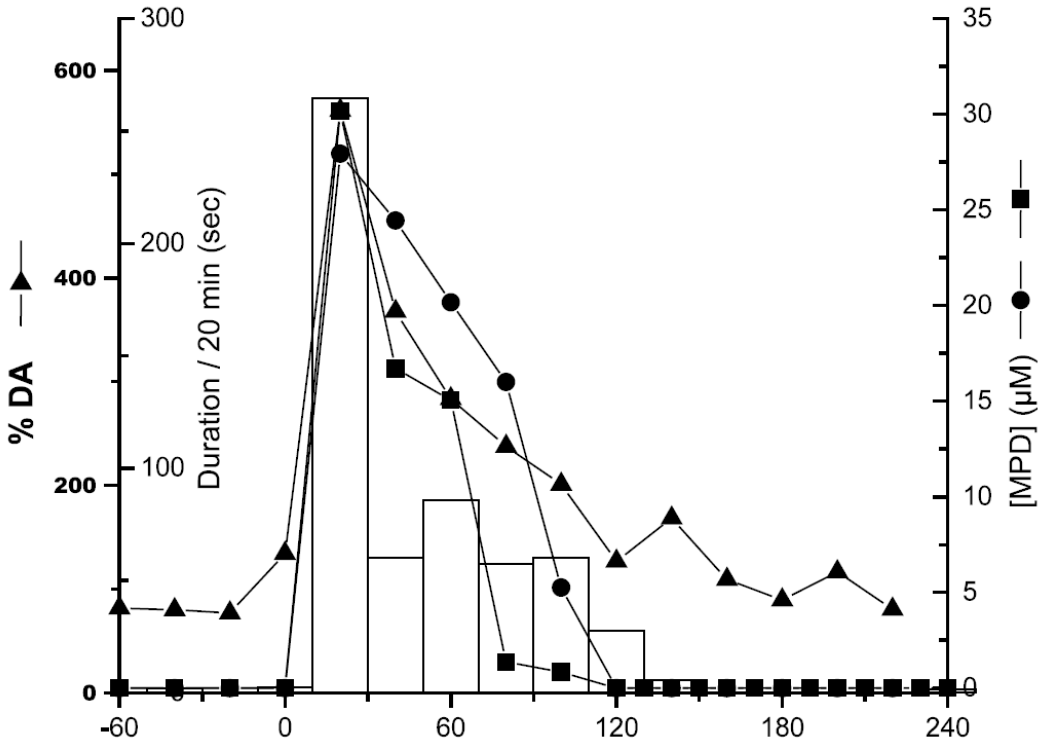


Fig. 1.3: Simultaneous monitoring of dopamine and methylphenidate (MPD) from multiple probes. The filled squares represent MPD from jugular dialysate, the filled circles represent MPD from brain dialysate, and the filled triangles are % dopamine with respect to basal. X-axis is the time from dosing in minutes. (From Huff *et al*. [28])

inflammation, hemorrhage and edema surrounding the microdialysis probe following 24 hours of implantation; they also observed the development of fibrin-like polymer around the probe (gliosis). The extracellular edema increases the diffusional distance between the probe and the extracellular fluid, and this fibrin-like polymer can create a physical barrier between the dialysis probe and the extracellular fluid surrounding the cells [30]. Both of these factors can adversely affect recovery of the probe. In a separate study based on local cerebral blood flow (LCBF) and local cerebral glucose metabolism (LCGM), Benveniste *et al.* recommended a 24-h period of recovery after probe implantation [31]

The integrity of the BBB following probe implantation is also a factor for discussion. Studies conducted using autoradiography with 14C-AIB (which does not cross the BBB under normal conditions) as well as transport characteristics of hydrophilic and moderately lipophilic drugs (following IV injection) postsurgery indicates that the BBB integrity is maintained overall [32,33]. However, other studies using Cr-51-EDTA transport have shown a significant effect of probe implantation on BBB permeability [34].

1.2.1 "Offline" vs. "online" sampling

The analysis of microdialysis samples can be divided into two general categories, i.e., offline and online analyses, the former of which is the most common. For offline analysis, a specified volume of sample (usually 1–20 μL) is collected in vials or tubes for later analysis. The temporal resolution that can be obtained in these

experiments is usually determined by the microdialysis flow rate, analyte recovery, and the sensitivity of the analytical method. At the typical microdialysis flow rate of 1 μL/min, most offline experiments have temporal resolutions from 5–10 min. An exception to this is if the analyte concentration is very high. Then smaller volume samples can be collected and diluted prior to analysis. However, in general, sample volumes of less than one microliter become very difficult to manipulate offline due to problems with surface tension and evaporation.

Online sample analysis offers several potential advantages over offline analysis. In an online system, the sample collection, manipulation, injection, and analysis steps are all integrated on a planar device in a continuous, streamlined fashion. Therefore, problems related to handling submicroliter volumes of sample (sample loss, mislabeling, evaporation, and surface tension) as well as sample degradation that can occur with sample exposure to air (e.g., ascorbic acid and catecholamines) can be avoided [35,36]. Also, such systems are usually capable of manipulating and analyzing submicroliter sample volumes, which allow high temporal resolution analysis to be performed. Such studies can yield continuous near real-time data, which provide immediate feedback on the biological process under investigation. When microdialysis sampling is directly coupled to a separation and detection method, it yields a separation-based sensor that can be used to monitor several analytes simultaneously in near real time.

1.2.2 General considerations

When developing an online assay based on microdialysis sampling, several parameters must be taken into consideration. These parameters include:

i. Perfusion flow rate

ii. Volume of sample available for analysis

iii. Concentration range of analyte in the sample

iv. Sensitivity and limit of detection of the analytical method scheme

v. Speed of analytical method

vi. Frequency of analysis required to draw valid conclusions (temporal resolution desired)

1.2.2.1 Perfusion flow rate and sample volume for analysis

Analyte recovery in microdialysis is defined by the overall mass transport of the analyte across the probe. It can be expressed as the ratio of concentration of the analyte in the dialysate to that present in the extracellular space or sampling solution. The amount of analyte that diffuses into the probe is a function of the concentration gradient that is produced across the dialysis membrane. This concentration gradient is established and maintained by continuously infusing the probe with fresh perfusate (e.g., aCSF in brain probes). The analytes of interest passes through the probe and is transported to the outlet end for collection or analysis. Higher recoveries (more concentrated samples) are obtained when probes are perfused at lower flow rates because this allows more time for the analyte to diffuse from the extracellular fluid

across the membrane into the perfusate. Typical microdialysis perfusate flow rates are between 0.5 and 2.0 μL min–1. Recoveries for small molecules are usually between 10 and 40% at these flow rates. If a flow rate of 100 nL min–1 is employed, the relative recovery approaches 100% [37]. However, use of these very low flow rates places a substantial burden on the analytical method. The method must be able to analyze submicroliter samples if one minute temporal resolution is desired as well as provide the requisite sensitivity to detect the analyte at this concentration. As seen from **Fig. 1.4**, the absolute recovery (mass of substance recovered in a particular period of time) increases with higher flow rates. Therefore, with systems that require relatively large sample volumes (e.g., HPLC), it is better to sample at higher flow rates. This will lead to higher absolute recoveries as well as allow more frequent injections of sample.

The major applications of microdialysis are to measure the *in vivo* concentration of endogenous (neurotransmitters, peptides) or exogenous (therapeutic drugs) compounds. Therefore, analyte recovery is an important consideration in microdialysis experiments. Recovery can be described by the following equation:

$$R = 1 - \exp\left(-1/(Q_d(R_d + R_m + R_e)\right)$$

where Q_d is the volumetric flow rate of perfusate, R_d, R_m and R_e are the resistances of dialysate, membrane and extracellular space, respectively, to the mass transfer of analyte(s). From this equation, one can derive the factors that affect recovery. R_e

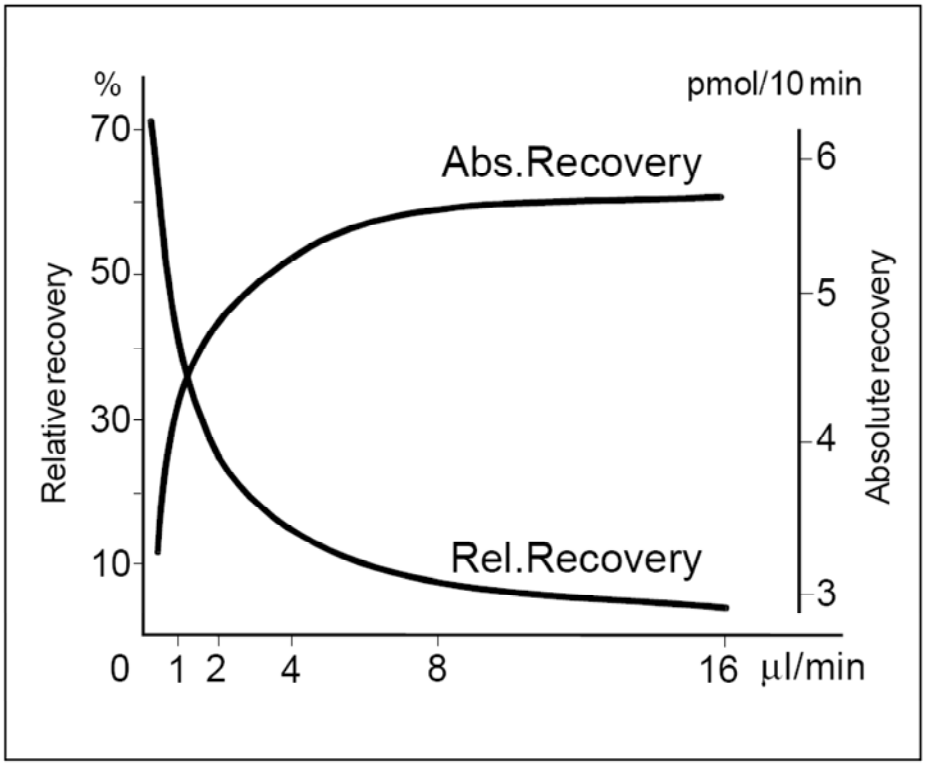


Fig. 1.4: Relative and absolute recoveries of dopamine as a function of flow rate (from "Microdialysis – principles of recovery" by Agneta Eliasson, 1991).

includes factors such as the rate of diffusion of the analyte of interest through the extracellular space as well as metabolism and other active reactions that the analyte may undergo. R_m incorporates the probe-related factors such as probe dimension, MW cutoff and the diffusion coefficient of the analyte across the probe membrane. Rd takes into consideration the diffusion of the analyte in the microdialysate [21,37].

It is generally assumed that $R_m >> R_e$, which implies that recovery information obtained for a probe *in vitro* can be applied to *in vivo* experiments. However, the caveat attached to such an assumption is the fact that the diffusion of the analyte from distant extracellular space to the area around the probe can be slow and is dependent on the diffusion coefficient of the analyte(s) in the extracellular matrix. For large peptide neuromodulators, such sluggish diffusion can affect the recovery. In particular, peptides can also undergo metabolism and other kinetic processes in the brain before reaching the probe membrane. Other considerations include determining the effects of loss of tissue integrity due to surgery and changes in blood flow and immunological reactions following insertion of the probe.

For many *in vivo* applications, enough information can be obtained by monitoring the percent change of analyte with respect to the basal experiment concentration. In these experiments, the untreated animal acts as its own control. These are usually performed to demonstrate proof-of-concept rapid changes in analytes after some intervention in which the outcome is known a priori (change due to some neurochemical pathway) [21]. During such experiments, it is generally assumed that that the probe is performing consistently throughout the duration of the

experiment. However, careful consideration needs to be given to this assumption during the course of the experiment. For example, a tissue reaction could be triggered during perfusion that could lead to altered analyte concentrations and erroneous conclusions. Monitoring changes in endogenous compounds is an excellent application for online experiments that can make measurements with high temporal resolution on a single, awake animal where the animal can serve as its own control.

Several additional factors need to be taken into consideration if more accurate *in vivo* concentration is warranted (such as PK studies). Some of those factors include the probe membrane type and its size, location of sampling probe, and flow rate. In addition, for an accurate assessment of *in vivo* concentration, the probe must be calibrated. There are several methods that have been reported for probe calibration with the "no-net flux" as the most popular method [38,39]. In this method, a known concentration of analyte is added to the perfusate. The concentration is usually varied over a range that is both higher and lower than the expected extracellular concentration of the exogenous or endogenous compound. The concentration of the analyte in the dialysate is measured and considered to be at the "no net flux" condition when there is no exchange of analyte between extracellular space and perfusate. This point is considered to be the extracellular fluid concentration. The major drawback of this method is the long time required to reach steady-state conditions, usually from several hours to a day. Hence, for online systems, this method of probe calibration may not be practical due to the tediousness of this process.

An easier method of probe calibration is called “retrodialysis” or “reverse dialysis” and is particularly applicable for *in vivo* experiments. The underlying assumption of this method is that diffusion across the probe membrane is quantitatively equal in both directions. This method uses an internal standard whose physical, biological, and pharmacokinetic properties closely resemble the compound under scrutiny. Such a compound is added at a known concentration to the perfusate, and its rate of disappearance is calculated from the equation,

$$\text{Recovery (\%)} = 100 - (100 \times C_{dialysate}/C_{perfusate})$$

Calibration should ideally be done before an experiment when there is no analyte present in the tissue; this is to maintain the membrane concentration gradient during the experiment. Also, a thorough perfusion of probe with physiological solution should be performed to remove the drug delivered to the tissue during retrodialysis [29,40]. Other *in vivo* recovery estimation methods include the “variation of perfusion flow rate method” and the “delivery method” [40].

1.2.2.2. Temporal resolution

Temporal resolution can be defined as the smallest increment of time over which the change in a dynamic process can be observed. For neurochemical experiments, microdialysis is usually coupled online to an instrument with the goal of analyzing samples with high temporal resolution. Many neurochemical events, such as neurotransmitter release, occur at a time scale of seconds or less. This results in very fast and transient changes in the concentration of substances in the extracellular

fluid. Therefore, the primary objective in such experiments is to analyze samples as frequently as possible in order to detect these fast changes; otherwise, the concentration change could be missed due to dilution and averaging of the signal in the sample.

In the choice of an analytical method, the first question that needs to be addressed is whether the device or the method is sensitive enough to actually detect the quantity of analyte(s) present in the small volume of sample generated by very fast sampling. If a probe is perfused at 1 μL/min and the perfusate contains an analyte at micromolar concentrations, then the analytical system must have sufficient mass sensitivity to detect 1 pmol of analyte if 1 min temporal resolution is desired. If the analytical method is capable of detecting only 10 pmol or higher, this means that a much larger sample (10 μL) must be collected in order to measure the analyte of interest. This limits the temporal resolution to 10 min at a flow rate of 1 μL/min.

In many cases with on-line systems, the temporal resolution of the technique is defined by the analysis time. If the analysis time is longer than the duration of the event being measured, then the change will appear digital and appear in the next injection. On the other hand, in those cases where the analysis step is much faster than the event being measured, it is possible to detect the change in concentration as a function of time. In this case, the rise time is defined as the time required during for the signal to increase from 10% to 90% of maximum intensity [41]. For very fast analyses, the rise time becomes dependent on the rate of diffusion of analyte across the probe membrane. However, in most cases, it is the dead volume in the system,

injection method, and the flow rate of the dialysate that determine how fast a concentration change can be measured with an on-line system.

As mentioned in the previous section, higher analyte recovery can be achieved by using very slow flow rates. At flow rates of 100 nL/min, analyte recoveries of 100% have been reported [37]. However, the increase in concentration of the analyte in the perfusate is offset by the extremely small sample volumes that are generated per unit time. In this case, an analytical method that is both sensitive and capable of analyzing submicroliter sample volumes is necessary if 1 min temporal resolution is to be obtained. This is one of the driving forces for the use of capillary and microchip electrophoresis for the analysis of microdialysis samples.

For most online separation-based microdialysis systems (described later in this review), analysis is performed on such as amino acid neurotransmitters whose *in vivo* concentrations are relatively high and well within the detection limits of laser induced fluorescence (LIF) detection. In such cases, the factor that dictates temporal resolution is the time that is required to separate the compounds so that serial analyses can be performed without overlapping the analysis peaks from two different runs. In other words, the difference in injection time between run 1 and run 2 should ideally be the separation time for run 1 so that as soon as run 1 ends, run 2 begins and this cycle can be repeated to get uninterrupted *in vivo* data. If the analysis time can be made very short (a few seconds), such a mechanism leads to high temporal resolution. As discussed in the previous section, there are three mass transfer processes occurring in series that are associated with recovery from a microdialysis probe. The probe

sample analytes that are present in the extracellular matrix surrounding the probe. However, many times the analyte needs to diffuse from the regions farther away from the probe surface to the surrounding matrix in order to be sampled. For slow diffusing analytes, the recovery will be low, indicating that one will have to wait longer to obtain enough mass to get detected. In addition, diffusion of analyte can occur within the microdialysate due to Taylor dispersion while it is being pumped to the analysis system, compromising the temporal integrity of the zones and thereby diffusing zones of analyte that have different concentrations. Such phenomena can be mitigated by increasing the flow rate or reducing the length and inner diameter of the tubing.

1.2.2.3. Low temporal resolution studies

Online microdialysis systems can also be useful for studies where high temporal resolution is not essential. A classic example is pharmacokinetic (PK) experiments in which analysis is required every few minutes over a period of hours. Most drugs exhibit pharmacokinetic profiles (absorption-distribution-metabolism excretion) that last for a few hours to several days. Therefore, measuring the average concentration over a period of time (10–20 min) is sufficient for PK modeling studies. Another application that does not require high temporal resolution but where continuous online monitoring is useful is the *in vitro* monitoring of products of bioreactors. Here, the time-course of the experiment is usually several hours or days. In this case, sample collection and analysis every 15 min can provide adequate information regarding the progress of the reaction system [42].

1.2.2.4. Other considerations

The development of a robust interface between microdialysis sampling and the analytical system can be challenging. Tubing and connectors associated with such an interface can produce an increase in dead volume in the system that leads to a delayed response in online monitoring experiments as well as Taylor dispersion causing band broadening. For experiments involving awake, freely moving animals, there is the additional dead volume associated with the swivel that connects the probe to the analytical system. In extreme cases, the dialysis membrane itself can be the limiting factor with regard to temporal resolution [24].

1.3. Microdialysis - liquid chromatography (MD-LC)

One of the most common separation methods used for online analysis of microdialysis samples is liquid chromatography (LC). Such a system usually consists of LC pumps, online injector with injection loop, LC column, detector, and data recorder (**Fig. 1.5**). The online injector is an integral part of online MD-LC systems as such an instrument can load and inject samples from an injection loop continuously without the requirement for autosamplers and fraction collectors. Online sampling is usually accomplished using automated valves that reduce sample loss and make it possible to perform continuous monitoring. An early report by Steele *et al*. employed multiple sample loops to continuously inject microdialysis samples without having to divert any sample to waste. In this setup, no temporal information was lost by discarding sample, and each sample represented an integrated time period depending

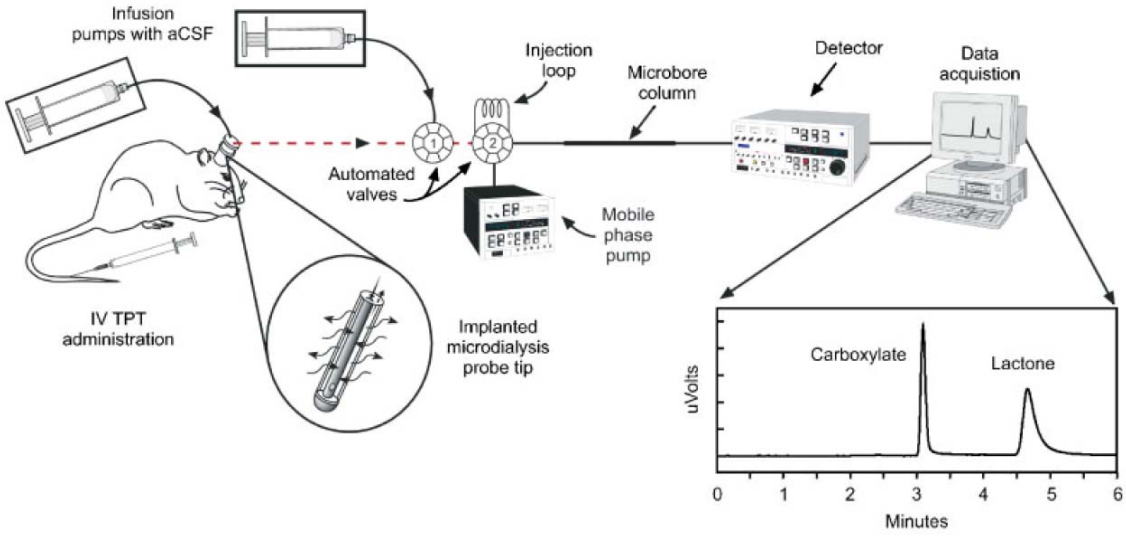


Fig. 1.5: Setup of an online microdialysis-LC system (from Leggas *et al*. [97])

on the time of sample collection. This system was well suited for pharmacokinetic studies [43]. Such a system can be compared to segmented flow systems discussed later where minimal or no sample is wasted.

The choice of columns for online instruments has mostly been limited to microbore and capillary columns with internal diameters ranging form 0.5 to 2 mm, 100–250 mm in length, and particle size 5 μm. The primary advantage of using microbore columns for online systems is their ability to perform small volume sample analysis at low perfusion rate through the microdialysis probe. Also, because of smaller diameter, there is less analyte dilution, resulting in improved detection limits [44].

One challenge of microbore and capillary LC separations is the need for low flow rate, pulse-free pumps as well as low dead volume and low dispersion requirements to minimize band broadening. Dead volume for microbore column systems can be reduced by minimizing the length and the internal diameter of all tubing; the flow cell for analysis also needs to have a low volume to avoid mixing and dispersion, all of which can increase band broadening. The use of microbore columns for analysis of tirazepam and its metabolites with an improved temporal resolution of 14 min compared to 23 min achieved with a conventional LC column was reported by McLaughlin *et al*. [19]. Online *in vivo* analysis of fluconazole in blood and dermis was performed by Mathy *et al*. These investigators reported an increase of temporal resolution from 40 min to 24 min by using a microbore column [45]. Capillary liquid chromatography has also been employed with online systems employing mass

spectrometric (MS) detection. Capillary columns are typically 50 to 500 μm in inner diameter and 5 to 200 cm in length. In addition to low sample volume requirements, these columns produced better ionization efficiencies resulting in improved limits of detection for MS [46].

The detection method employed for online MD-LC systems is dependent on the analyte of interest (Table 1). UV detection has been a popular detection scheme for online monitoring of drugs for pharmacokinetic studies. Electrochemical detection has been employed in a number of studies involving the detection of catecholamines and other redox active compounds of biological interest. Fluorescence detection (FL) has also been popular, but usually requires derivatization of analytes prior to analysis. One example of online MD-LC-FL was reported by Yoshitake *et al*. in which serotonin sampled from brain was derivatized post-column with benzylamine in the presence of potassium hexaferrocyanate [47].

Mass spectrometric detection has also been employed for on-line MD-LC. Shackman *et al*. used MD-LC-MS to monitor acetylcholine *in vivo* with limits of detection of 8 amol [46]. In another study, the pharmacokinetics of melatonin (administered i.v. in rats) was monitored over 15 hours using online microdialysis with LC-MS/MS [48].

There have been several applications of such online MD-LC systems over the past decade for *in vivo* monitoring of drugs, neurotransmitters, and other important biomolecules. Applications included primarily pharmacokinetic, metabolism, and

neurochemical studies. Further information regarding the region of sampling, analyte of interest, and method of detection used for these studies can be found in Table 1.1.

Table 1.1: Microdialysis-LC

Region of interest	Analyte(s)	Detection scheme
Rat common bile duct	Cefepime, moropenem, diclofenac	UV [85-87]
Rat jugular vein	Cefsulodin, ceftriaxone, cefmetazole, cephaloridine chloramphenicol, glucuronide	UV [88-92]
Rat liver	Glutathione	EC [93]
Rat brain	Serotonin, trazodone	EC [94,95]
Rat blood jugular vein and brain striatum	Cephalexin	UV [96]
Rat brain lateral ventricle	Topotecan lactone, carboxylate	FL [97]
Rat brain striatum	Monoamines and their metabolites	EC [44]
Rat urinary bladder	Arsenite (AsIII), arsenate (AsV), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)	Hydride generation atomic absorption spectrometry (HGAAG) [98]
Rat brain striatum and heart ventricle	Ascorbic acid	EC [36]
Rat brain striatum	Acetyl choline	EC [36]
Rat brain	Rhodamine 123	FL [99]
Rat bile duct	Camptothecin	FL [100]
Rat brain striatum	Serotonin	FL [47]
Rat brain and plasma	EAB 515	FL [101]
Rat brain	Malondialdehyde	FL [102]
Rat brain striatum	Acetyl choline	MS [46]
Rat jugular vein	Melatonin	MS [48]

1.3.1 Comments

The narrow diameter of capillary LC (cLC) columns offers the advantage of high mass sensitivity, low flow rate requirements, and low consumption of sample and reagents that are very suitable for online microdialysate analysis [49]. The separation times are relatively long, which can be detrimental for designing systems with very high temporal resolution. In addition, the robustness of cLC columns is less than that of conventional LC columns, and specialized equipment is required to load samples to such columns, which can limit its applicability [50].

1.4. Microdialysis - capillary electrophoresis (MD-CE)

Capillary electrophoresis (CE) is particularly attractive for the analysis of microdialysis samples because it has very low sample volume requirements (nL to pL) and can perform extremely fast separations. In CE, both the analysis speed and separation efficiency improve with field strength (in the absence of Joule heating). Therefore by using very short capillaries and high field strengths very fast, highly efficient separations can be accomplished. When microdialysis sampling is interfaced directly to CE, it is possible to make reproducible nanoliter injections of sample into the CE capillary. The electrophoretic separations range in duration from a few minutes to several seconds. This makes it possible to continuously monitor biological processes with excellent temporal resolution.

Laser-induced fluorescence (LIF) detection is the most popular method for online MD-CE, primarily because of its high sensitivity and the availability of

inexpensive lasers and laser-based detectors. There are also a large number of reagents commercially available for fluorescence derivatization of amines, thiols, carbohydrates, carboxylic acids, and other functional groups. NDA (naphthalene-2,3-dicarboxaldehyde) and OPA (o-phthalaldehyde) are the most common reagents employed for the detection of amino acid neurotransmitters and other primary amines *in vivo*. Both of these reagents are fluorogenic and exhibit fast reaction kinetics, making them well suited for precolumn derivatization of amino acids prior to analysis by CE-LIF (See **Fig 1.6** for reaction scheme). Online reaction times for OPA/β-ME and NDA/CN with primary amines have been reported to be 10–30 s and 120–240 s, respectively [51]. Table 2 summarizes experiments performed using online MD-CE. The relevant parameters listed include the tissue region of interest, analyte measured, and detection method.

A key component of online microdialysis-CE systems is the development of an interface that is capable of injecting discrete nanoliter-size sample plugs from continuous hydrodynamic flow from the microdialysis probe (μL). The first report of an online MD-CE system by Hogan *et al*. used a nanoliter injection valve as the interface between the MD system and the CE separation capillary with a continuously running CE separation. Perfusate from the dialysis experiment was collected in the nanoliter injection loop of the valve, and a special transfer line delivered the sample from the valve to the separation capillary inlet The system was used to monitor antineoplastic agents in blood with a temporal resolution of 90 s [52]. A similar system incorporating online derivatization with NDA/CN was used for monitoring

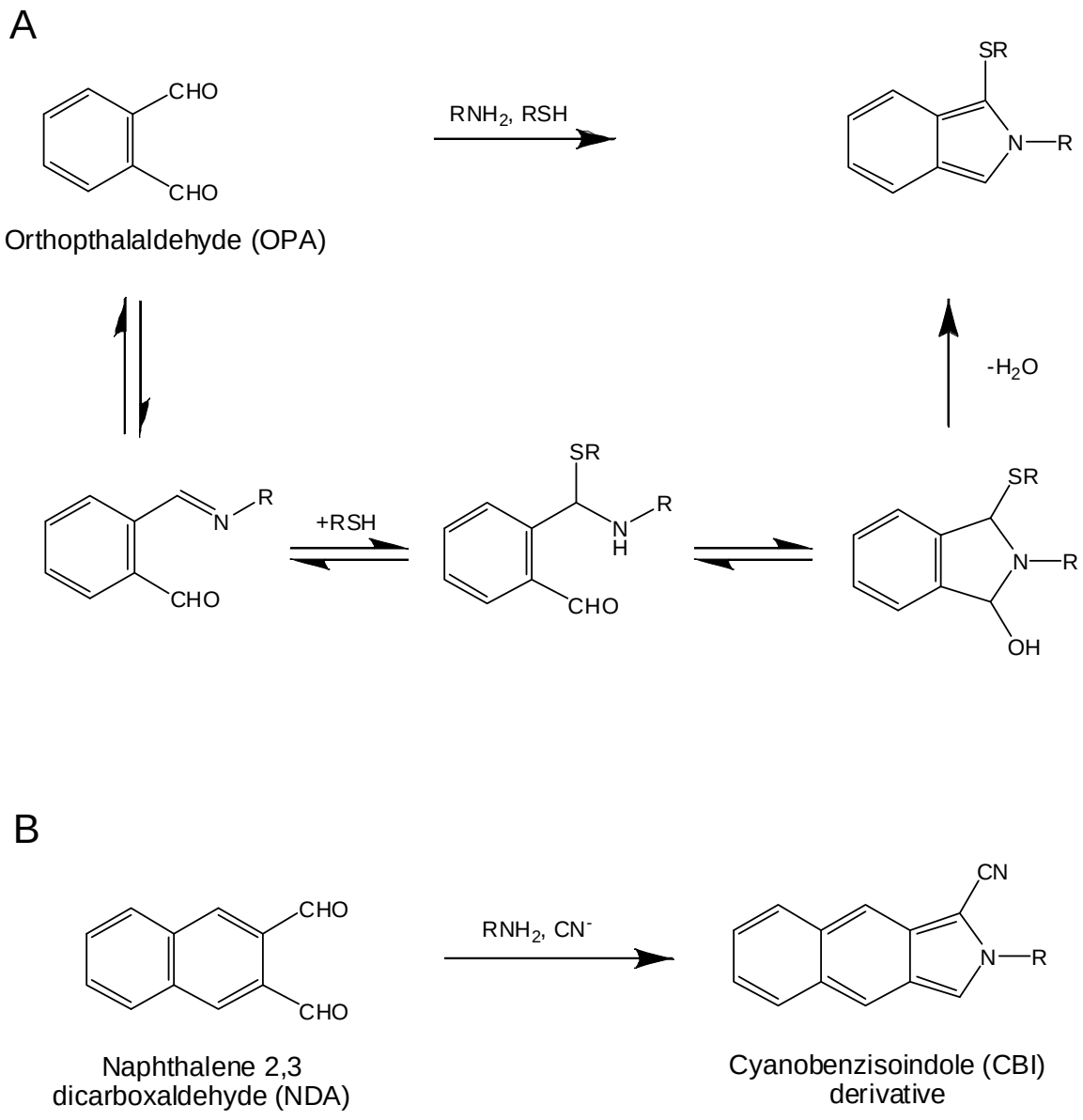


Fig. 1.6: Reaction schemes for OPA and NDA. A. Reaction of OPA with primary amine (RNH_2) and thiol (RSH) with the key reaction intermediates. B. Reaction of NDA with primary amine CN^- that produces fluorescent CBI derivative. (from De Montigny et. al. [108])

aspartate and glutamate release in the brain [53]. Later, the transdermal delivery of nicotine was monitored using on an online microdialysis-capillary electrophoresis system with electrochemical detection [54]. The system incorporated a carbon fiber working electrode and a cellulose acetate decoupler before the separation capillary to shield the animal from high voltage. The cutaneous nicotine concentration was monitored for 24 hours following application of a Nicotrol patch. The temporal resolution was 10 min.

Lada *et al*. used a flow-gated interface instead of a valve to inject microdialysis samples into the separation capillary. This interface made it possible to perfuse the microdialysis probe at submicroliter/min flow rates and still obtain temporal resolutions of 65–85 s [55]. Subsequently, the same group demonstrated extremely fast separations of glutamate and aspartate using a short capillary (6.5 cm) with a temporal resolution of 12 s [24]. A similar system was utilized to separate and perform quantitative analysis of multiple neurotransmitters in the brain (45 s temporal resolution) using micellar electrokinetic chromatography (MEKC) [56]. A system designed with a sheath-flow cuvette for improved sensitivity (15-fold) (by reducing background fluorescence and laser scattering) was reported by Bowser *et al*. [57]. The separation capillary (10 mm ID, 150 mm OD) outlet was connected to a quartz cuvette (outer dimension 2 mm x 2 mm sq.; inner dimension 2 μm x 2 μm sq) and sheath buffer flow was maintained outside the capillary by a siphoning effect that resulted in laminar flow profile of the analytes leaving the capillary. This system was

used to monitor amino acids in the brain of a freely moving animal. Seventeen amino acids were separated in 30 s following precolumn derivatization with NDA [51].

The most popular application of online MD-CE systems *in vivo* has been measuring amino acid neurotransmitters in the brain. In general, the change in concentration from basal is measured following an external stimulus. In most cases, a drug or other substance is added to the perfusate to evoke the response. A common experiment is that of high potassium stimulation, which causes the release of excitatory amino acids into the extracellular fluid [53,56-59]. An electropherogram produced from such an experiment is shown in **Fig. 1.7**. The glutamate reuptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) has also been administered via the microdialysis probe for monitoring glutamate and aspartate [24,60]. Other experiments include monitoring changes in ascorbic acid following subcutaneous injection of amphetamine [55], monitoring brain amino acids following intraperitoneal injections of saline (0.9% v/v) and ethanol (20% v/v) [51], and monitoring dopamine in the brain following injection of cocaine [61].

1.4.1. Comments

Compared to capillary LC, capillary electrophoresis can produce very fast analysis times due to application of high field strengths (30 kV). Therefore, high temporal resolution can be attained for on-line systems. The major drawback of on-line systems using fused silica capillaries is the need for connecting tubing between the microdialysis system and the capillary electrophoresis system that can lead to

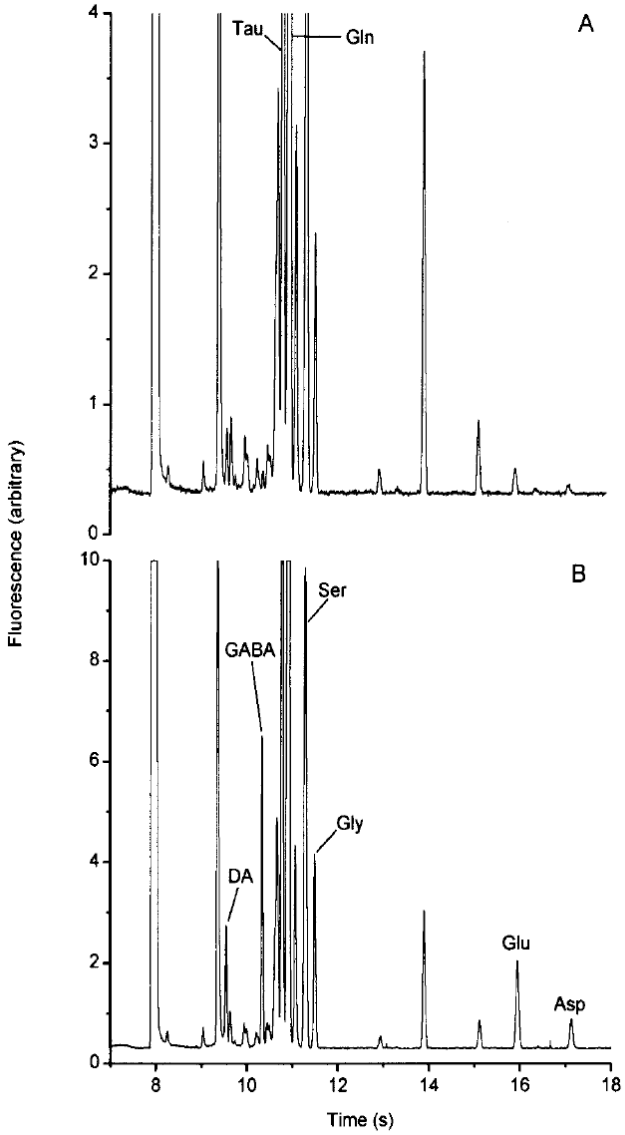


Fig. 1.7: Change in concentration of amino acids following potassium ion stimulation. A. Basal. B. After K+ stimulation. (Bowser *et al*. [57])

band broadening and loss of temporal resolution. This is particularly the case with awake animal systems that use liquid swivels. For extremely fast separations, very short capillaries are employed at high field strengths. In humid environments, arcing can be a problem. Lastly, the on-line systems using conventional capillary electrophoresis are relatively large and do not lend themselves to the development of on-animal analysis systems. In contrast, the microchip systems (next section) have the potential to be completely miniaturized for use in on-animal sensing.

Table 1.2: Microdialysis – CE

Region of interest	Analyte(s)	Detection scheme
Rat brain striatum	Glucose, lactate	EC [84]
Rat brain striatum	Dopamine	FL [61]
Rat brain coronal slice	Serine, taurine, glutamate GABA	FL [103]
Rat brain striatum	D-serine	FL [104]
Larval tiger salamander retina	Amino acid neurotransmitters	FL [105]
Larval tiger salamander retina	D,L-serine	FL [106]
Rat Brain striatum	Amino acid neurotransmitters	FL [57]
Rat dermis	Nicotine	EC [54]
Rat brain striatum	O-phosphoethanolamine, excitatory amino acid	FL [60]
Rat brain striatum	Glutamate and aspartate	FL [24]
Rat caudate nucleus	Glutathione and cysteine	FL [59]
Rat caudate nucleus	Primary amines	FL [56]
Rat caudate nucleus	Ascorbate	UV [58]
Rat brain hippocampus	Glutamate and aspartate	FL [53]
Rat vein (opposite to jugular vein)	Antineoplastic agent	FL [52]

1.5. Microdialysis - microchip electrophoresis (MD-MC)

More recently, microchip electrophoresis has become an attractive analytical platform for the online analysis of microdialysis samples. Microchips have several advantages for online analysis of microdialysis samples. These include the ability to manipulate samples on-chip using a combination of electroosmotic and hydrodynamic flow, integrated sample preparation or derivatization, nL to pL sample volume requirements, fast analysis times, and the ability to integrate the detector directly into the chip. Most of the research in this area has exploited one or both of the two major advantages of microchip-based devices. The first is the ability to perform very fast separations on-chip for monitoring fast biochemical processes. The second is miniaturization of the system for on-animal sensing.

Most of the work over the past several years has been focused on the development of interfaces between the microdialysis sampling system and the microchip electrophoresis system. The major challenge has been to develop an interface that can inject nL to pL volume samples continuously and reproducibly into the electrophoresis channel while still achieving adequate temporal resolution (frequency of injecting and analyzing samples) for the experiment of interest. Other challenges include interfacing the external tubing used for microdialysis sampling with analysis chip. In addition, fast and efficient derivatization strategies must be incorporated online for detection of amino acid neurotransmitters and peptides.

Microdialysis sampling coupled to microchip electrophoresis for online analysis was first reported by Huynh *et al*. [62]. The microchip device was fabricated

from soda-lime glass and consisted of a twin T channel (**Fig. 1.8A**). A continuous stream of perfusate was delivered to the microchip using a syringe pump that continuously perfused the cylindrical 4 mm CMA/12 microdialysis probe with 20 mM boric acid buffer. A commercially available microtight union (Upchurch Scientific) was used to connect the PEEK tubing from the microdialysis sampling system to the electrophoresis chip. Injection was accomplished using a gated voltage that cut off the hydrodynamic flow at the injection cross, allowing introduction of a plug of sample into the separation channel for analysis. Such an injection scheme made it possible to continuously inject discrete plugs of microdialysis sample into the separation channel for fast electrophoretic separation of analyte. The system was used to successfully monitor the activity of the enzyme β-galactosidase. This enzyme catalyzes the hydrolysis of fluorescein mono-β-galactoside (FMG) to produce fluorescein. Both the substrate and the product were monitored using the online system with LIF detection (**Fig. 1.8B**). The lag time (time needed for the device to respond to a concentration change) on this device was 5–7 min.

A similar setup was employed for online sampling, derivatization, and detection of an *in vitro* mixture of amino acids and peptides [63]. On-chip labeling of analytes was accomplished using NDA and 2-mercaptoethanol (2ME). The NDA and 2ME were dissolved in 20 mM borate buffer and were added to the run buffer reservoir. Following a gated injection scheme, the plug of sample reacted with the derivatization reagent at the beginning of the electrophoretic separation. The analytes were separated and detected with a temporal resolution of 30–40 s.

Kennedy's group employed a gated injection scheme to continuously monitor *in vivo* amino acid neurotransmitters using a microdialysis microchip setup with an effective temporal resolution of 2–4 min [64]. The chip device was fabricated from borosilicate glass (**Fig. 1.9A**). Reaction channels were incorporated for the derivatization of the sample collected via a side-by-side microdialysis probe (fabricated in-house) implanted in the brain of an anesthetized rat. The precolumn reaction was achieved by mixing OPA and 2ME with the stream of microdialysate (**Fig. 1.9B**). Following the reaction, a gated injection scheme introduced plugs of sample into the separation channel for analysis by LIF. The interface in this device consisted of capillary tubing from the probe outlet connected to the chip via an Upchurch fitting.

A similar approach was employed by Cellar *et al*. for low-flow, push-pull-based microdialysis sampling. This system also employed online derivatization followed by injection into a short fused silica CE capillary for separation and LIF detection [65]. The chip incorporated a number of valves fabricated using a multilayer soft lithography technique and actuated by nitrogen gas pressure controlled by solenoid valves. In this and the previous study, changes in concentration of the endogenous amino acid neurotransmitter (glutamate) were monitored following perfusion with high potassium. The effect of administration of the glutamate reuptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) through the microdialysis probe on extracellular glutamate was also investigated.

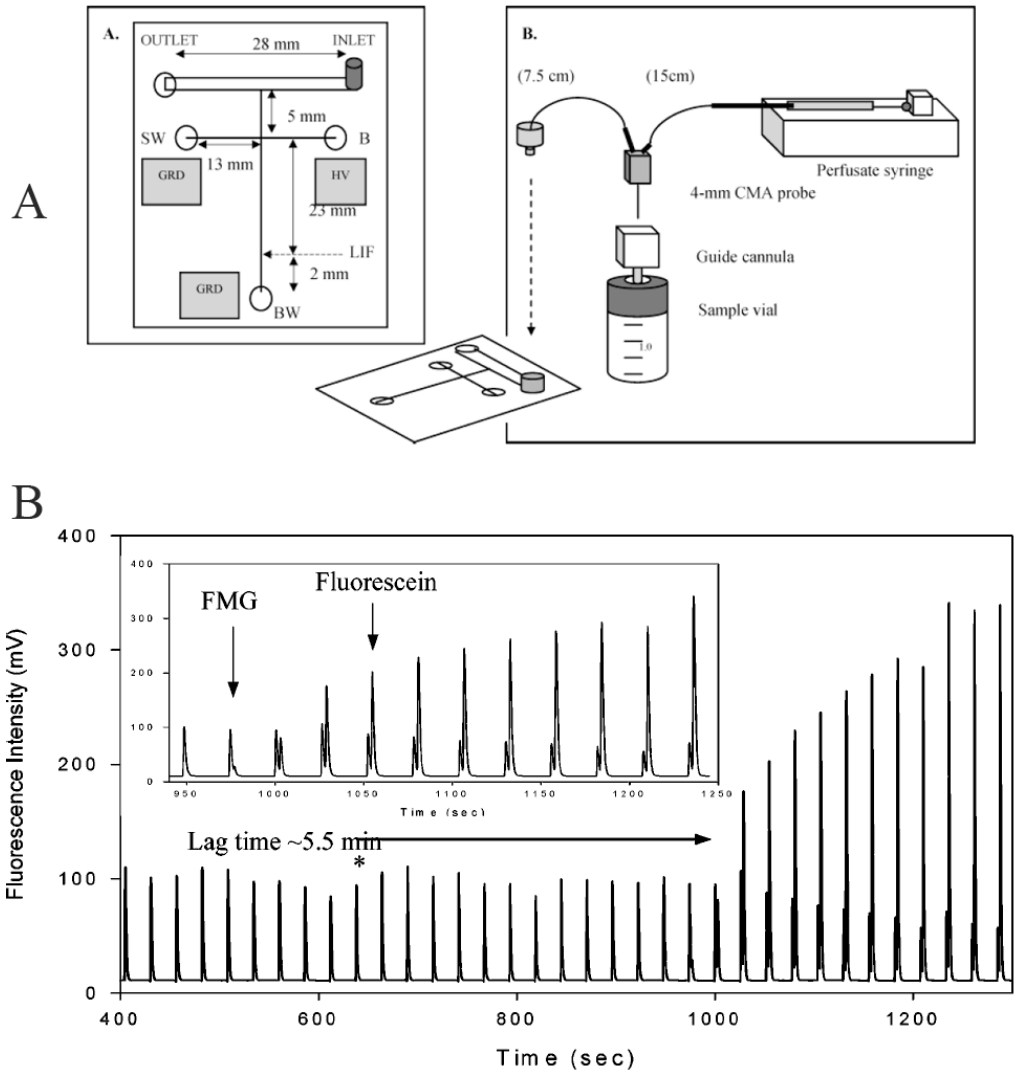


Fig. 1.8: Online *in vitro* microdialysis microchip. A. Chip design and setup. B. *In vitro* enzyme assay of FMG (lag time 5.5 min). (From Huynh *et al*. [62])

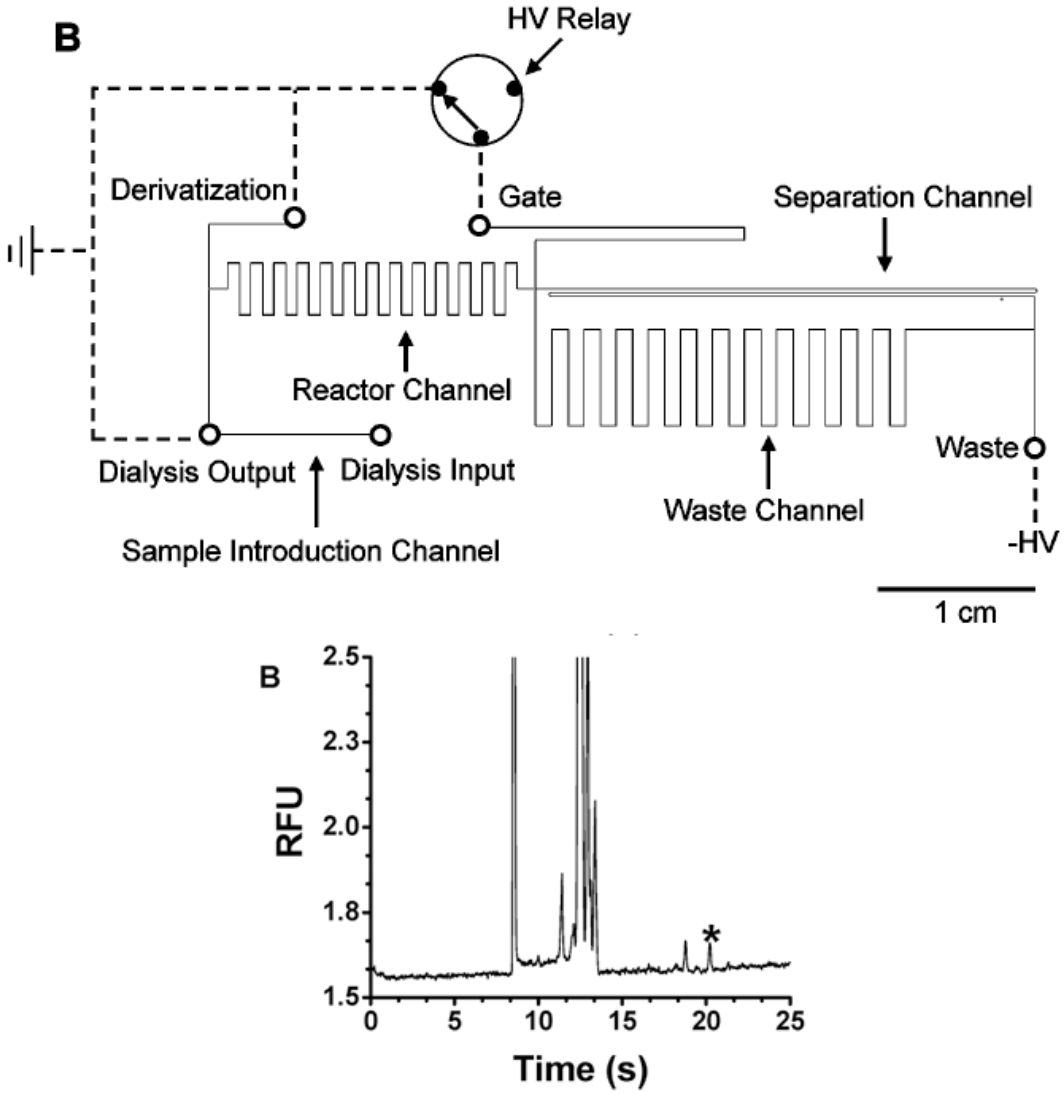


Fig. 1.9: Online *in vivo* microdialysis microchip. A. Chip design. B. Microdialysis sample analysis (marked peak is glutamate). (From Sandlin *et al*. [64])

The use of a solenoid-controlled pneumatic valving method for sample injection in an online microdialysis microchip system has been described by the Martin group [41, 66]. The general design (**Fig. 1.10**) consists of one or two nitrogen-actuated PDMS microvalves that are used to inject or manipulate the flow of fluid within the chip. The CE separation channel is situated at a right angle to the flow channels. Sample injection was achieved by actuation of the valve at the flow channel/separation channel junction and was followed by electrophoresis with LIF or electrochemical detection.

In the first report Li *et al*. used this approach for continuous injection and separation of fluorescein and dichlorofluorescein with temporal resolution of 20 s [66]. All fluids were delivered from the syringe pump or from the probe using a capillary. The lag and rise times (time needed for the response to change from 10% to 90% of the total change) were reported to be approximately 6 min and 2 min, respectively. More recently, amperometric detection has been incorporated into the system by Mecker *et al*. In this study, the stimulated release of dopamine from PC12 cells cultured in a Petri dish was monitored using a 5 mm cylindrical microdialysis probe placed over the cells. In this case, the lag time was approximately 5 min and the rise time was 2 min [41].

The use of segmented flow to create reaction chambers for derivatization has also been investigated for online microdialysis-microchip electrophoresis. Wang *et al*. developed a system in which aqueous sample plugs were created in a stream of oil (perfluorodecalin) [67]. The frequency and size of the droplets were dependent on

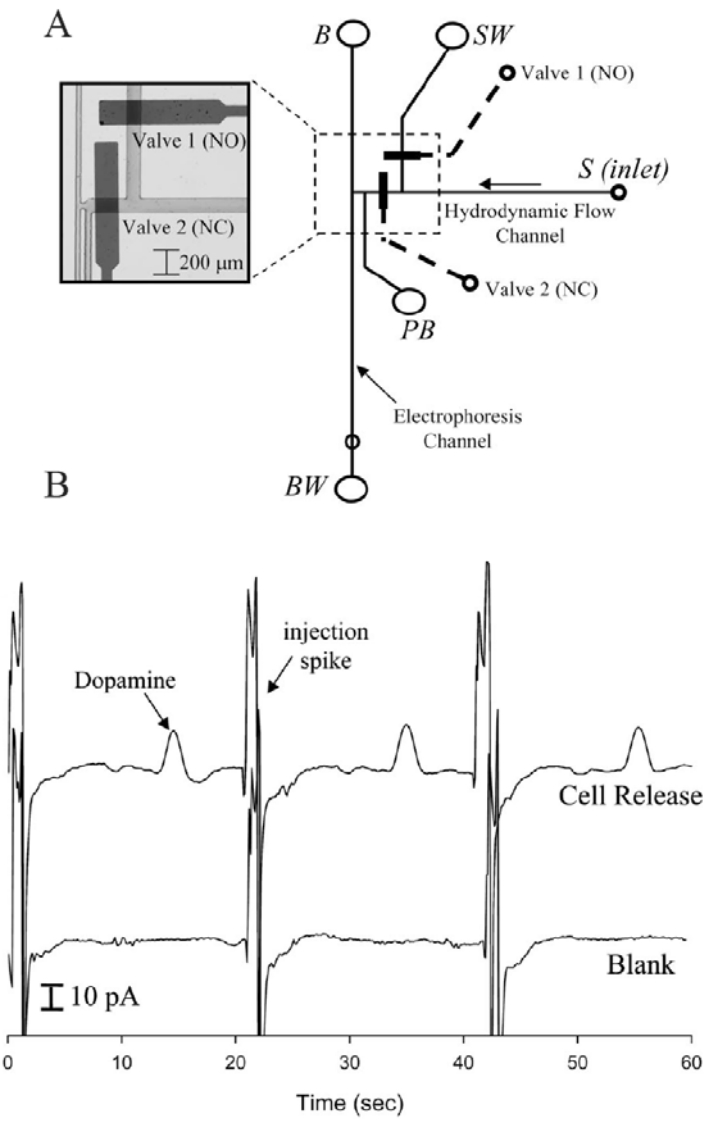


Fig. 1.10: PDMS microchips with pneumatic valving. A. Chip with 2 valves (1 and 2). B, BW and SW are buffer, buffer waste and sample waste, respectively. NO and NC refer to valves that are "normally open" and "normally closed," respectively. B. Online detection of dopamine from PC 12 cells. (From Mecker *et al*. [41])

the relative perfusion rates of oil and water from the respective syringe pumps (**Fig. 1.11A**). The plug of aqueous sample was interrogated at the end of a 7-cm-long capillary connected to the chip. With this system, glucose was measured online using glucose oxidase and Amplex Red (**Fig. 1.11B**). The same reaction scheme was employed for monitoring extracellular glucose in rat brain by microdialysis sampling; a change in glucose concentration in the brain was brought about by perfusion of 100 mM potassium through the probe.

This interface has also been employed to perform electrophoretic separations from a continuous segmented flow, which can potentially be used for CE separation of microdialysis samples [68]. In this case, the chip consisted of a flow channel (75 μm deep and 250 μm wide) and an electrophoresis channel (7.5 μm deep and 20 μm wide) that were thermally bonded with a K-shaped interface overlap for sample injection **(Fig. 1.12A)**. Aqueous plugs were generated between the oil phase (perfluorodecalin) segments based on optimization studies reported previously by the Ismagilov group [69].

For injection of discrete plugs for CE separation, two modes of injection were described. For the "discrete injector," a series of sample plugs were injected; the injection volume was dependent on the electrophoresis field strength and temporal width of the plug. For the "desegmenting injector," the sample plugs coalesce at the K junction from which electrokinetic injections can be made repeatedly for analysis. Primary amines were derivatized on-chip with NDA/CN and then analyzed using the injection schemes described above. A concentration change experiment was also

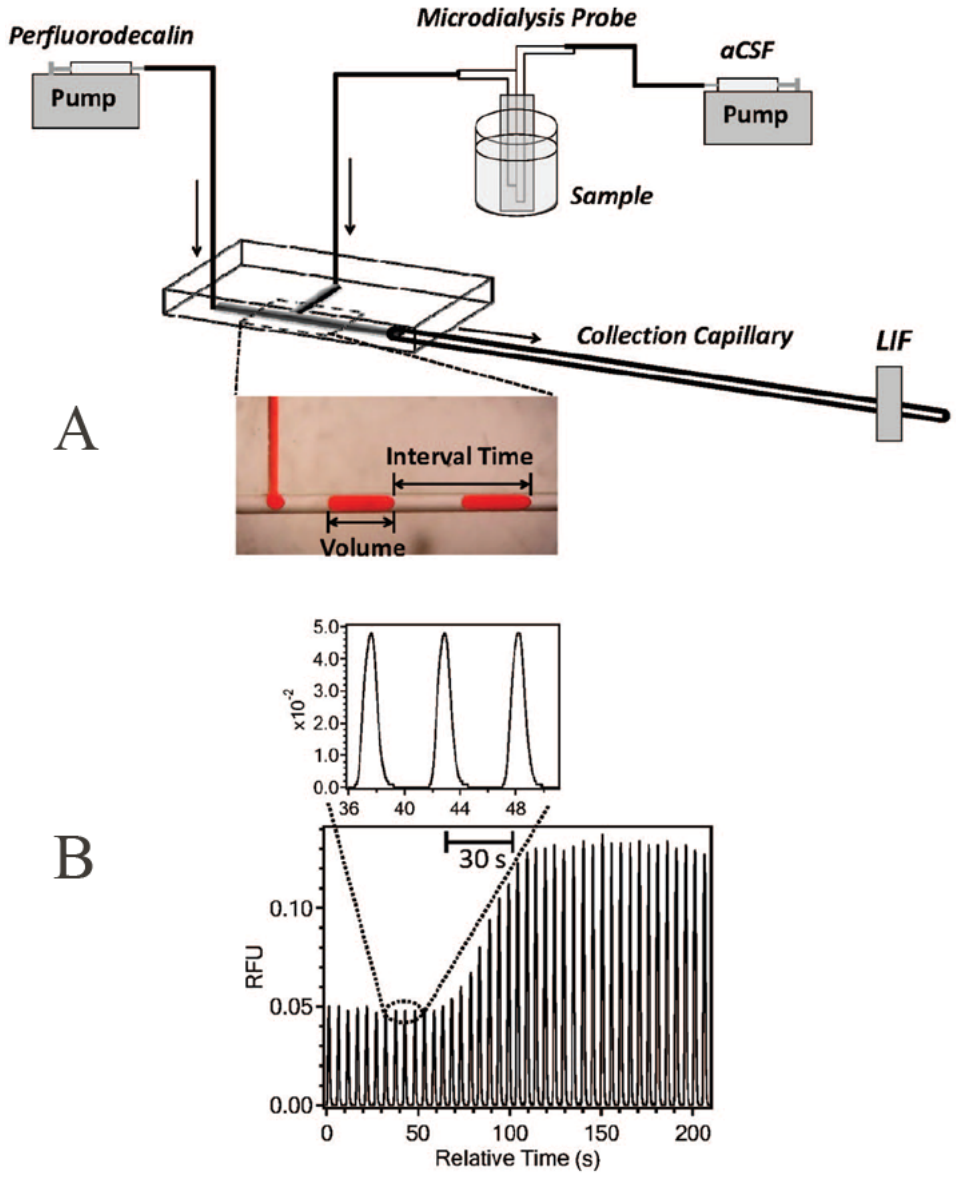


Fig. 1.11: Segmented flow for flow-through analysis. A. Setup B. LIF response for glucose after reaction with glucose oxadase (GOX)/horseradish peroxidase (HRP) and Amplex Red to produce fluorescent Resorufin. The concentration of glucose was changed at the probe from 0.2 mm to 1 mm. (From Wang *et al*. [67])

performed with the amino acids in which the change in concentration from 1 μM to blank solution was registered on chip in 20 s (**Fig. 1.12B**). These methods offer a more reliable way to analyze sample plugs by electrophoresis while still preserving temporal information.

1.5.1 Comments

The development of online microchip-based systems is ideal for studies requiring high temporal resolution. However, there can be several challenges associated with building such devices. First, the separation must be optimized and careful consideration needs to be given to the separation and identification of the analyte(s) of interest in the matrix being sampled. Interfacing the electrophoretic separation with the hydrodynamic flow can be a long, drawn-out process in which extensive investigation is required to determine the optimal microchannel design and dimensions for the specific application. For example, setting up repeated injections from a continuous flow of microdialysate can be challenging in a gated injection scheme. Second, fabricating glass-based chips can be challenging in many lab facilities as it involves a high temperature thermal bonding step that can yield poor success rates. Glass microchips are commercially available for such applications but, for many labs, are cost prohibitive. Although PDMS microchip devices are easy to fabricate they suffer from analyte adsorption and changing EOF. Several complex modification approaches have been suggested in the literature to reduce adsorption

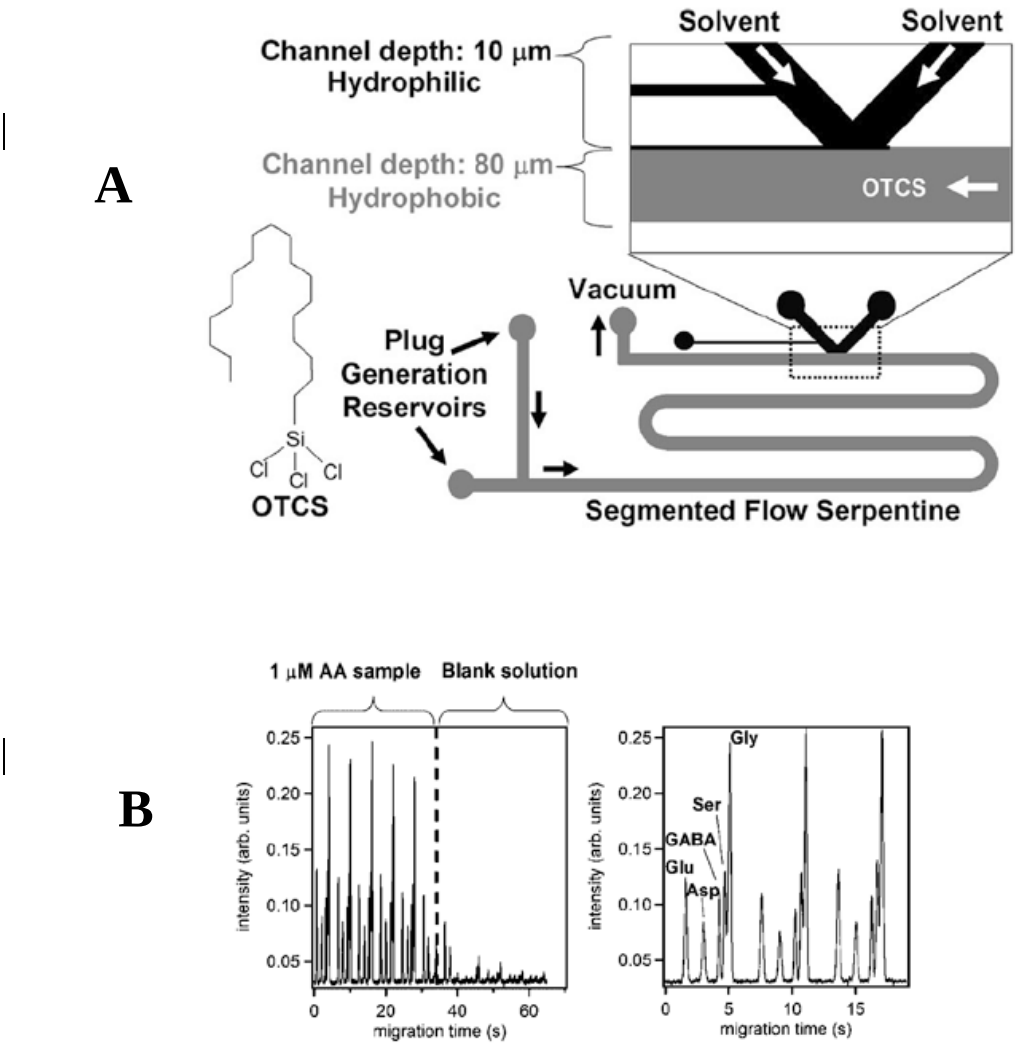


Fig. 1.12: Glass microchip for segmented flow and electrophoretic separation. A. Design B. Amino acid analysis after derivatization with NDA/CN. (From Roman *et al*. [68])

and maintain the EOF for a longer period of time on such devices [70]. Third, the performance of the chip is dependent on a number of factors such as removal of waste (reduce hydrostatic pressure) and frequent replenishment of buffer; integrating such steps on chip may require a fair degree of automation. Lastly, much of the equipment used for such systems must be custom built or is lab specific (e.g., software, power supplies). Therefore, a wide variety of technical resources and expertise needs to be available for developing these systems.

Table 1.3: Microdialysis – microchip electrophoresis

Region of interest	Analyte(s)	Detection scheme
In-vitro enzyme assay	Conversion of FMG to fluorescein by β-gal	LIF [62]
Rat brain striatum	Glutamate	LIF [64]
In vitro analysis	Gly-Pro, Leu-Enk, glutamate, aspartate, fluorescein, dichlorofluorescein, GABA, serine, glycine	LIF [63,66] [68]
In vitro analysis (PC12 cell)	Dopamine	EC [41]

1.6. Microdialysis coupled to biosensors

Biosensors are analytical devices with a biological recognition element that produce an electrical signal in response to a biological change. An ideal biosensor should be able to perform continuous and reliable monitoring of analyte from complex body fluids of patients over a significant period of time. There are many types of biosensors. Many are enzyme-based and employ either electrochemical or optical detection. Online microdialysis-biosensor systems should be able to handle low sample volumes (microliter) if high temporal resolution is required. Also, such systems should ideally exhibit high sensitivity and specificity for the analyte(s) of interest in the presence of other endogenous electroactive analytes [71]. A flow-through biosensor has been reported for direct coupling to continuous low-flow microdialysis. Analyte selectivity for glucose and lactate was achieved in this device using immobilized oxidoreductase enzymes followed by amperometric detection of hydrogen peroxide [72].

Online microdialysis sampling coupled to biosensors has been reported for analytes such as ascorbate [73], glucose, lactate [74,75] and glutamate [74,76,77]. The simultaneous monitoring of glucose and lactate in rats under hypoxic conditions was reported by Jones *et al*. [78]. Yao *et al*. also reported an online system for multianalyte *in vivo* monitoring. The biosensor consisted of a triple enzyme electrode that selectively detected glucose, l-lactate, and pyruvate without significant cross-reactivity [79]. Similar flow injection-based online systems were described by the

same group for L-glutamate, acetylcholine and dopamine [80] and D-/L-lactic acid [81].

Gramsbergen *et al*. developed an online system for glucose and lactate to monitor ischemic events in freely moving rats. The analytes were monitored by flow injection analysis with enzyme-based amperometric detection [82]. The development of a flow-through sensor with chemiluminescence detection for glucose monitoring in an awake rabbit has also been described [83]. Recently, online monitoring of glucose and lactate from rat brain was performed following ischemia and reperfusion. The sensor employed methylene green adsorbed on single-walled carbon nanotubes for detection [84].

1.6.1 Comments

Biosensors can yield good temporal resolution since no separation step is required. However, most biosensors are developed for a single analyte, so it is not possible to analyze several compounds of a similar class (catecholamines, amino acids) simultaneously as it is with separation-based sensors. Microdialysis does have an advantage over placing the sensor directly into the tissue of interest in that the probe membrane will eliminate fouling and an immune response that could be generated by sensor implantation. In addition, sensor arrays have been found to be very useful for many types of analytes and are especially valuable in the clinical setting.

Table 1.4: Microdialysis – biosensor

Region of interest	Analyte(s)	Detection scheme
Rat brain striatum	Glucose, glutamate, lactate	EC [74,77,82,84]
Rat subcutaneous tissue	Glucose, lactate	EC [75]
Rat cortex	Glucose, lactate	EC [78]
Frontal lobe of rat brain	Glucose, l-lactate, pyruvate, acetyl choline, dopamine	EC [79,80]
Rat jugular	Glucose	Chemiluminescence [83]

1.7. Concluding remarks

Microdialysis is a well established sampling technique that has found application in many areas, most notably in monitoring biological compounds *in vivo*. Advances in fabrication technologies have led to the development of innovative microchip technologies suitable for real-world monitoring applications. Microdialysis/microchip systems offer several advantages, including lower cost, faster analysis for improved temporal resolution, and simplified connections and fittings for device fabrication and operation. While the performance of conventional bench-top systems is currently superior to that of the separation-based devices, initial reports clearly demonstrate the potential of coupling microdialysis to microchip platforms.

Future developments will see the optimization of device design and the investigation of alternative materials for microchip, electrode, and membrane fabrication. It is envisioned that further advances in fabrication and integration procedures will allow the development of implantable/wearable microdialysis/microchip systems for personal or on-animal monitoring. Integration of the separation-based systems with powerful detection techniques such as mass spectrometry will further improve the detection capability of these systems for biological, pharmaceutical, and environmental monitoring.

1.8 References

[1] J.A. Bourne, Intracerebral microdialysis: 30 years as a tool for the neuroscientist, Clin. Exp. Pharmacol. Physiol. 30 (2003) 16-24.

[2] H.J. Holovics, C.R. Anderson, B.S. Levine, H.-W. Hui, C.E. Lunte, Investigation of drug delivery by iontophoresis in a surgical wound utilizing microdialysis, Pharm. Res. 25 (2008) 1762-1770.

[3] A. Kim, L.A. Suecof, C.A. Sutherland, L. Gao, J.L. Kuti, D.P. Nicolau, *In vivo* microdialysis study of the penetration of daptomycin into soft tissues in diabetic versus healthy volunteers, Antimicrob. Agents Chemother. 52 (2008) 3941-3946.

[4] S. Bielecka-Grzela, A. Klimowicz, Application of cutaneous microdialysis to evaluate metronidazole and its main metabolite concentrations in the skin after a single oral dose, J. Clin. Pharm. Ther. 28 (2003) 465-469.

[5] K. Lanckmans, R. Clinckers, A. Van Eeckhaut, S. Sarre, I. Smolders, Y. Michotte, Use of microbore LC-MS/MS for the quantification of oxcarbazepine and its active metabolite in rat brain microdialysis samples, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 831 (2006) 205-212.

[6] D.A. Richards, M.A. Silva, N. Murphy, S.J. Wigmore, D.F. Mirza, Extracellular amino acid levels in the human liver during transplantation: a microdialysis study from donor to recipient, Amino Acids 33 (2007) 429-437.

[7] M.I. Davies, C.E. Lunte, Simultaneous microdialysis sampling from multiple sites in the liver for the study of phenol metabolism, Life Sci. 59 (1996) 1001-1013.

[8] M.A. Gilinsky, A.A. Faibushevish, C.E. Lunte, Determination of myocardial norepinephrine in freely moving rats using *in vivo* microdialysis sampling and liquid chromatography with dual-electrode amperometric detection, J. Pharm. Biomed. Anal. 24 (2001) 929-935.

[9] K.E. Price, S.S. Vandaveer, C.E. Lunte, C.K. Larive, Tissue-targeted metabonomics: Metabolic profiling by microdialysis sampling and microcoil NMR, J. Pharm. Biomed. Anal. 38 (2005) 904-909.

[10] J.M. Ault, C.M. Riley, N.M. Meltzer, C.E. Lunte, Dermal microdialysis sampling *in vivo*, Pharm. Res. 11 (1994) 1631-9.

[11] H. Huang, Y. Zhang, R. Yang, X. Tang, Determination of baicalin in rat cerebrospinal fluid and blood using microdialysis coupled with ultra-performance liquid chromatography-tandem mass spectrometry, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 874 (2008) 77-83.

[12] L.-C. Lin, L.-C. Hung, T.-H. Tsai, Determination of (-)-epigallocatechin gallate in rat blood by microdialysis coupled with liquid chromatography, J. Chromatogr., A 1032 (2004) 125-128.

[13] K.W. Ward, G.M. Pollack, Use of intrauterine microdialysis to investigate methanol-induced alterations in uteroplacental blood flow, Toxicol. Appl. Pharmacol. 140 (1996) 203-210.

[14] K.L. Woo, C.E. Lunte, The direct comparison of health and ulcerated stomach tissue: A multiple probe microdialysis sampling approach, J. Pharm. Biomed. Anal. 48 (2008) 85-91.

[15] K.L. Woo, C.E. Lunte, The development of multiple probe microdialysis sampling in the stomach, J. Pharm. Biomed. Anal. 48 (2008) 20-26.

[16] T. Zhu, B.W.Y. Cheung, L.L. Cartier, G.S. Giebink, R.J. Sawchuk, Simultaneous intravenous and intramiddle-ear dosing to determine cefditoren influx and efflux clearances in middle ear fluid in freely moving chinchillas, J. Pharm. Sci. 92 (2003) 1947-1956.

[17] A.L. Freed, J.D. Cooper, M.I. Davies, S.M. Lunte, Investigation of the metabolism of substance P in rat striatum by microdialysis sampling and capillary electrophoresis with laser-induced fluorescence detection, J. Neurosci. Methods 109 (2001) 23-29.

[18] K.L. Kostel, S.M. Lunte, Evaluation of capillary electrophoresis with post-column derivatization and laser-induced fluorescence detection for the determination of substance P and its metabolites, J. Chromatogr., B: Biomed. Sci. Appl. 695 (1997) 27-38.

[19] K.J. McLaughlin, A.A. Faibushevich, C.E. Lunte, Microdialysis sampling with on-line microbore HPLC for the determination of tirapazamine and its reduced metabolites in rats, Analyst 125 (2000) 105-110.

[20] M. Telting-Diaz, D.O. Scott, C.E. Lunte, Intravenous microdialysis sampling in awake, freely-moving rats, Anal. Chem. 64 (1992) 806-10.

[21] J.A. Stenken, Methods and issues in microdialysis calibration, Anal. Chim. Acta 379 (1999) 337-357.

[22] J.K. Huff, K.E. Heppert, M.I. Davies, The microdialysis shunt probe: profile of analytes in rats with erratic bile flow or rapid changes in analyte concentration in the bile, Curr. Sep. 18 (1999) 85-90.

[23] Q. Wu, C. Liu, R.D. Smith, Online microdialysis desalting for electrospray ionization-mass spectrometry of proteins and peptides, Rapid Commun. Mass Spectrom. 10 (1996) 835-838.

[24] M.W. Lada, T.W. Vickroy, R.T. Kennedy, High temporal resolution monitoring of glutamate and aspartate *in vivo* using microdialysis online with capillary electrophoresis with laser-induced fluorescence detection, Anal. Chem. 69 (1997) 4560-4565.

[25] S. Kottegoda, I. Shaik, A. Shippy Scott, Demonstration of low flow push-pull perfusion, J. Neurosci. Methods 121 (2002) 93-101.

[26] M. Orlowska-Majdak, W.Z. Traczyk, D. Szymanski, Hippocampal vasopressin release evoked by N-methyl D-aspartate (NMDA) microdialysis, Physiol. Res. 52 (2003) 373-382.

[27] J.M.R. Delgado, J. Lerma, R. Martin del Rio, J.M. Solis, Dialytrode technology and local profiles of amino acids in the awake cat brain, J. Neurochem. 42 (1984) 1218-28.

[28] J.K. Huff, M.I. Davies, Microdialysis monitoring of methylphenidate in blood and brain correlated with changes in dopamine and rat activity, J. Pharm. Biomed. Anal. 29 (2002) 767-777.

[29] M. Brunner, H. Derendorf, Clinical microdialysis: Current applications and potential use in drug development, TrAC, Trends Anal. Chem. 25 (2006) 674-680.

[30] M.C. Grabb, V.M. Sciotti, J.M. Gidday, S.A. Cohen, D.G. van Wylen, Neurochemical and morphological responses to acutely and chronically implanted brain microdialysis probes, J. Neurosci. Methods 82 (1998) 25-34.

[31] H. Benveniste, J. Drejer, A. Schousboe, N.H. Diemer, Regional cerebral glucose phosphorylation and blood flow after insertion of a microdialysis fiber through the dorsal hippocampus in the rat, J. Neurochem. 49 (1987) 729-34.

[32] H. Benveniste, J. Drejer, A. Schousboe, N.H. Diemer, Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis, J. Neurochem. 43 (1984) 1369-74.

[33] E.C. de Lange, M. Danhof, A.G. de Boer, D.D. Breimer, Critical factors of intracerebral microdialysis as a technique to determine the pharmacokinetics of drugs in rat brain, Brain Res. 666 (1994) 1-8.

[34] O. Major, T. Shdanova, L. Duffek, Z. Nagy, Continuous monitoring of blood-brain barrier opening to Cr51-EDTA by microdialysis following probe injury, Acta. Neurochir. Suppl. 51 (1990) 46-8.

[35] G. Jin, Q. Cheng, J. Feng, F. Li, On-line microdialysis coupled to analytical systems, J. Chrom. Sci. 46 (2008) 276-287.

[36] P.-J. Tsai, J.-P. Wu, N.-N. Lin, J.-S. Kuo, C.-S. Yang, *In vivo*, continuous and automatic monitoring of extracellular ascorbic acid by microdialysis and online liquid chromatography, J. Chromatogr., B: Biomed. Appl. 686 (1996) 151-156.

[37] S. Menacherry, W. Hubert, J.B. Justice, Jr., *In vivo* calibration of microdialysis probes for exogenous compounds, Anal. Chem. 64 (1992) 577-83.

[38] J.B. Justice, Jr., Quantitative microdialysis of neurotransmitters, J. Neurosci. Methods 48 (1993) 263-76.

[39] P. Lonnroth, P.A. Jansson, U. Smith, A microdialysis method allowing characterization of intercellular water space in humans, Am. J. Physiol. 253 (1987) E228-31.

[40] M.J. Cano-Cebrian, T. Zornoza, A. Polache, L. Granero, Quantitative *in vivo* microdialysis in pharmacokinetic studies: some reminders, Curr. Drug Metab. 6 (2005) 83-90.

[41] L.C. Mecker, R.S. Martin, Integration of Microdialysis Sampling and microchip electrophoresis with electrochemical detection, Anal. Chem. 80 (2008) 9257-9264.

[42] N. Torto, T. Laurell, L. Gorton, G. Marko-Varga, Recent trends in the application of microdialysis in bioprocesses, Anal. Chim. Acta 379 (1999) 281-305.

[43] K.M. Steele, C.E. Lunte, Microdialysis sampling coupled to on-line microbore liquid chromatography for pharmacokinetic studies, J. Pharm. Biomed. Anal. 13 (1995) 149-54.

[44] C.S. Chaurasia, C.-E. Chen, C.R. Ashby, *In vivo* on-line HPLC-microdialysis: simultaneous detection of monoamines and their metabolites in awake freely-moving rats, J. Pharm. Biomed. Anal. 19 (1999) 413-422.

[45] F.-X. Mathy, B. Vroman, D. Ntivunwa, A.J. De Winne, R.K. Verbeeck, V. Preat, On-line determination of fluconazole in blood and dermal rat microdialysates by microbore high-performance liquid chromatography, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 787 (2003) 323-331.

[46] H.M. Shackman, M. Shou, N.A. Cellar, C.J. Watson, R.T. Kennedy, Microdialysis coupled on-line to capillary liquid chromatography with tandem mass spectrometry for monitoring acetylcholine *in vivo*, J. Neurosci. Methods 159 (2007) 86-92.

[47] T. Yoshitake, R. Iizuka, J. Kehr, H. Nohta, J. Ishida, M. Yamaguchi, Determination of serotonin in microdialysis samples from rat brain by microbore column liquid chromatography with post-column derivatization and fluorescence detection, J. Neurosci. Methods 109 (2001) 91-96.

[48] P.S.H. Wong, K. Yoshioka, F. Xie, P.T. Kissinger, *In vivo* microdialysis/liquid chromatography/tandem mass spectrometry for the online monitoring of melatonin in rat, Rapid Commun. Mass Spectrom. 13 (1999) 407-411.

[49] T. Takeuchi, Capillary columns in liquid chromatography, Anal. Bioanal. Chem. 375 (2003) 26-7.

[50] K.N. Schultz, R.T. Kennedy, Time-resolved microdialysis for *in vivo* neurochemical measurements and other applications, Annu. Rev. Anal. Chem. 1 (2008) 627-661.

[51] M. Shou, A.D. Smith, J.G. Shackman, J. Peris, R.T. Kennedy, *In vivo* monitoring of amino acids by microdialysis sampling with on-line derivatization by naphthalene-2,3-dicarboxyaldehyde and rapid micellar electrokinetic capillary chromatography, J. Neurosci. Methods 138 (2004) 189-197.

[52] B.L. Hogan, S.M. Lunte, J.F. Stobaugh, C.E. Lunte, Online coupling of *in vivo* microdialysis sampling with capillary electrophoresis, Anal. Chem. 66 (1994) 596-602.

[53] S.Y. Zhou, H. Zuo, J.F. Stobaugh, C.E. Lunte, S.M. Lunte, Continuous *in Vivo* Monitoring of Amino Acid Neurotransmitters by Microdialysis Sampling with Online Derivatization and Capillary Electrophoresis Separation, Anal. Chem. 67 (1995) 594-9.

[54] J. Zhou, D.M. Heckert, H. Zuo, C.E. Lunte, S.M. Lunte, Online coupling of *in vivo* microdialysis with capillary electrophoresis/electrochemistry, Anal. Chim. Acta 379 (1999) 307-317.

[55] M.W. Lada, G. Schaller, M.H. Carriger, T.W. Vickroy, R.T. Kennedy, On-line interface between microdialysis and capillary zone electrophoresis, Anal. Chim. Acta 307 (1995) 217-225.

[56] M.W. Lada, R.T. Kennedy, Quantitative *in vivo* monitoring of primary amines in rat caudate nucleus using microdialysis coupled by a flow-gated interface to capillary electrophoresis with laser-induced fluorescence detection, Anal. Chem. 68 (1996) 2790-2797.

[57] M.T. Bowser, R.T. Kennedy, *In vivo* monitoring of amine neurotransmitters using microdialysis with on-line capillary electrophoresis, Electrophoresis 22 (2001) 3668-3676.

[58] M.W. Lada, R.T. Kennedy, Quantitative *in vivo* measurements using microdialysis on-line with capillary zone electrophoresis, J. Neurosci. Methods 63 (1995) 147-52.

[59] M.W. Lada, R.T. Kennedy, *In vivo* monitoring of glutathione and cysteine in rat caudate nucleus using microdialysis online with capillary zone electrophoresis-laser induced fluorescence detection, J. Neurosci. Methods 72 (1997) 153-159.

[60] F. Robert, L. Bert, S. Parrot, L. Denoroy, L. Stoppini, B. Renaud, Coupling on-line brain microdialysis, precolumn derivatization and capillary

electrophoresis for routine minute sampling of O-phosphoethanolamine and excitatory amino acids, J. Chromatogr. A 817 (1998) 195-203.

[61] M. Shou, C.R. Ferrario, K.N. Schultz, T.E. Robinson, R.T. Kennedy, Monitoring dopamine *in vivo* by microdialysis sampling and on-line CE-laser-induced fluorescence, Anal. Chem. 78 (2006) 6717-6725.

[62] B.H. Huynh, B.A. Fogarty, R.S. Martin, S.M. Lunte, On-line coupling of microdialysis sampling with microchip-based capillary electrophoresis, Anal. Chem. 76 (2004) 6440-6447.

[63] B.H. Huynh, B.A. Fogarty, P. Nandi, S.M. Lunte, 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, J. Pharm. Biomed. Anal. 42 (2006) 529-34.

[64] Z.D. Sandlin, M. Shou, J.G. Shackman, R.T. Kennedy, Microfluidic electrophoresis chip coupled to microdialysis for *in vivo* monitoring of amino acid neurotransmitters, Anal. Chem. 77 (2005) 7702-7708.

[65] N.A. Cellar, S.T. Burns, J.-C. Meiners, H. Chen, R.T. Kennedy, Microfluidic chip for low-flow push-pull perfusion sampling *in vivo* with on-line analysis of amino acids, Anal. Chem. 77 (2005) 7067-7073.

[66] M.W. Li, B.H. Huynh, M.K. Hulvey, S.M. Lunte, R.S. Martin, Design and characterization of poly(dimethylsiloxane)-based valves for interfacing continuous-flow sampling to microchip electrophoresis, Anal. Chem. 78 (2006) 1042-1051.

[67] M. Wang, G.T. Roman, K. Schultz, C. Jennings, R.T. Kennedy, Improved temporal resolution for *in vivo* microdialysis by using segmented flow, Anal. Chem. 80 (2008) 5607-5615.

[68] G.T. Roman, M. Wang, K.N. Shultz, C. Jennings, R.T. Kennedy, Sampling and electrophoretic analysis of segmented flow streams using virtual walls in a microfluidic device, Anal. Chem. 80 (2008) 8231-8238.

[69] H. Song, D.L. Chen, R.F. Ismagilov, Reactions in droplets in microfluidic channels, Angew. Chem., Int. Ed. 45 (2006) 7336-7356.

[70] K. Miyaki, H.L. Zeng, T. Nakagama, K. Uchiyama, Steady surface modification of polydimethylsiloxane microchannel and its application in simultaneous analysis of homocysteine and glutathione in human serum, J. Chromatogr. A. 1166 (2007) 201-6.

[71] M. Zhang, L. Mao, Enzyme-based amperometric biosensors for continuous and on-line monitoring of cerebral extracellular microdialysate, Front. Biosci. 10 (2005) 345-352.

[72] M.M. Rhemrev-Boom, M.A. Jonker, K. Venema, R. Tiessen, J. Korf, G. Jobst, On-line continuous monitoring of glucose or lactate by ultraslow microdialysis combined with a flow-through nanoliter biosensor based on poly(m-phenylenediamine) ultra-thin polymer membrane as enzyme electrode, Analyst 126 (2001) 1073-1079.

[73] M. Miele, M. Fillenz, *In vivo* determination of extracellular brain ascorbate, J. Neurosci. Methods 70 (1996) 15-19.

[74] M.G. Boutelle, L.K. Fellows, C. Cook, Enzyme packed bed system for the on-line measurement of glucose, glutamate, and lactate in brain microdialysate, Anal. Chem. 64 (1992) 1790-4.

[75] W.A. Kaptein, J.J. Zwaagstra, K. Venema, J. Korf, Continuous ultraslow microdialysis and ultrafiltration for subcutaneous sampling as demonstrated by glucose and lactate measurements in rats, Anal. Chem. 70 (1998) 4696-700.

[76] M.O.M. Berners, M.G. Boutelle, M. Fillenz, Online Measurement of brain glutamate with an enzyme/polymer-coated tubular electrode, Anal. Chem. 66 (1994) 2017-21.

[77] M. Miele, M. Berners, M.G. Boutelle, H. Kusakabe, M. Fillenz, The determination of the extracellular concentration of brain glutamate using quantitative microdialysis, Brain Res. 707 (1996) 131-3.

[78] D.A. Jones, J. Ros, H. Landolt, M. Fillenz, M.G. Boutelle, Dynamic changes in glucose and lactate in the cortex of the freely moving rat monitored using microdialysis, J. Neurochem. 75 (2000) 1703-1708.

[79] T. Yao, T. Yano, H. Nishino, Simultaneous *in vivo* monitoring of glucose, L-lactate, and pyruvate concentrations in rat brain by a flow-injection biosensor system with an on-line microdialysis sampling, Anal. Chim. Acta 510 (2004) 53-59.

[80] T. Yao, G. Okano, Simultaneous determination of L-glutamate, acetylcholine and dopamine in rat brain by a flow-injection biosensor system with microdialysis sampling, Anal. Sci. 24 (2008) 1469-1473.

[81] Y. Nanjo, T. Yano, R. Hayashi, T. Yao, Optically specific detection of D- and L-lactic acids by a flow-injection dual biosensor system with on-line microdialysis sampling, Anal. Sci. 22 (2006) 1135-1138.

[82] J.B. Gramsbergen, J. Skjoth-Rasmussen, C. Rasmussen, K.L. Lambertsen, On-line monitoring of striatum glucose and lactate in the endothelin-1 rat model of transient focal cerebral ischemia using microdialysis and flow-injection analysis with biosensors, J. Neurosci. Methods 140 (2004) 93-101.

[83] B. Li, Z. Zhang, Y. Jin, Chemiluminescence flow sensor for *in vivo* on-line monitoring of glucose in awake rabbit by microdialysis sampling, Anal. Chim. Acta 432 (2001) 95-100.

[84] Y. Lin, N. Zhu, P. Yu, L. Su, L. Mao, Physiologically relevant online electrochemical method for continuous and simultaneous monitoring of striatum glucose and lactate following global cerebral ischemia/reperfusion, Anal. Chem. 81 (2009) 2067-2074.

[85] Y.L. Chang, M.H. Chou, M.F. Lin, C.F. Chen, T.H. Tsai, Determination and pharmacokinetic study of unbound cefepime in rat bile by liquid chromatography with on-line microdialysis, J. Chromatogr. A. 914 (2001) 77-82.

[86] Y.L. Chang, M.H. Chou, M.F. Lin, C.F. Chen, T.H. Tsai, Determination and pharmacokinetic study of meropenem in rat bile using on-line microdialysis and liquid chromatography, J. Chromatogr., A 961 (2002) 119-124.

[87] S.C. Liu, T.H. Tsai, Determination of diclofenac in rat bile and its interaction with cyclosporin A using on-line microdialysis coupled to liquid chromatography, J. Chromatogr., B: Biomed. Appl. 769 (2002) 351-356.

[88] T.H. Tsai, F.C. Cheng, Y.F. Chen, C.F. Chen, On-line microdialysis coupled with microbore liquid chromatography with ultraviolet detection for continuous monitoring of free cefsulodin in rat blood, J. Chromatogr., A 914 (2001) 83-87.

[89] T.H. Tsai, F.C. Cheng, L.C. Hung, C.F. Chen, Determination of unbound ceftriaxone in rat blood by on-line microdialysis and microbore liquid chromatography, Int. J. Pharm. 193 (1999) 21-26.

[90] T.R. Tsai, F.C. Cheng, L.C. Hung, C.F. Chen, T.H. Tsai, Determination of unbound cefmetazole in rat blood by on-line microdialysis and microbore liquid chromatography: a pharmacokinetic study, J. Chromatogr., B: Biomed. Sci. Appl. 736 (1999) 129-134.

[91] T.-H. Tsai, F.-C. Cheng, L.-C. Hung, C.-F. Chen, Online microdialysis coupled with microbore liquid chromatography for the determination of unbound chloramphenicol and its glucuronide in rat blood, J. Chromatogr., B: Biomed. Sci. Appl. 720 (1998) 165-169.

[92] T.H. Tsai, H.Y. Kao, C.F. Chen, Measurement and pharmacokinetic analysis of unbound cephaloridine in rat blood by on-line microdialysis and microbore liquid chromatography, Biomed. Chromatogr. 15 (2001) 79-82.

[93] C.-S. Yang, P.-J. Tsai, W.-Y. Chen, L. Liu, J.-S. Kuo, Determination of extracellular glutathione in livers of anesthetized rats by microdialysis with online high-performance liquid chromatography, J. Chromatogr., B: Biomed. Appl. 667 (1995) 41-8.

[94] C.M. Portas, B. Bjorvatn, S. Fagerland, J. Gronli, V. Mundal, E. Sorensen, R. Ursin, Online detection of extracellular levels of serotonin in dorsal raphe nucleus and frontal cortex over the sleep/wake cycle in the freely moving rat, Neuroscience 83 (1998) 807-814.

[95] F.-C. Cheng, T.-H. Tsai, Y.-S. Wu, J.-S. Kuo, C.-F. Chen, Pharmacokinetic and pharmacodynamic analyses of trazodone in rat striatum by *in vivo* microdialysis, J. Pharm. Biomed. Anal. 19 (1999) 293-300.

[96] T.H. Tsai, L.C. Hung, Y.L. Chang, A.Y.C. Shum, C.F. Chen, Simultaneous blood and brain sampling of cephalexin in the rat by microdialysis and microbore liquid chromatography: application to pharmacokinetics studies, J. Chromatogr., B: Biomed. Sci. Appl. 740 (2000) 203-209.

[97] M. Leggas, Y. Zhuang, J. Welden, Z. Self, C.M. Waters, C.F. Stewart, Microbore HPLC method with online microdialysis for measurement of topotecan lactone and carboxylate in murine CSF, J. Pharm. Sci. 93 (2004) 2284-2295.

[98] W.-C. Tseng, M.-H. Yang, T.-P. Chen, Y.-L. Huang, Automated, continuous, and dynamic speciation of urinary arsenic in the bladder of living organisms using microdialysis sampling coupled on-line with high performance liquid chromatography and hydride generation atomic absorption spectrometry, Analyst 127 (2002) 560-564.

[99] Q. Wang, H. Yang, D.W. Miller, W.F. Elmquist, Effect of the P-glycoprotein inhibitor, cyclosporin A, on the distribution of rhodamine-123 to the brain: an *in vivo* microdialysis study in freely moving rats, Biochem. Biophys. Res. Commun. 211 (1995) 719-26.

[100] T.H. Tsai, T.R. Tsai, Y.F. Chen, C.J. Chou, C.F. Chen, Determination of unbound 20(S)-camptothecin in rat bile by on-line microdialysis coupled to microbore liquid chromatography with fluorescence detection, J. Chromatogr., B: Biomed. Sci. Appl. 732 (1999) 221-225.

[101] B.K. Malhotra, M. Lemaire, J.F. Brouillard, R.J. Sawchuk, High-performance liquid chromatographic analysis of (S)-[alpha]-amino-5-phosphonomethyl[1,1'-biphenyl]-3-propanoic acid (EAB 515) in brain and blood microdialysate (on-line) and in plasma ultrafiltrate of freely moving rats, J. Chromatogr., B: Biomed. Appl. 679 (1996) 167-176.

[102] C.S. Yang, P.J. Tsai, W.Y. Chen, J.S. Kuo, On-line, continuous and automatic monitoring of extracellular malondialdehyde concentration in anesthetized rat brain cortex, J. Chromatogr., B: Biomed. Sci. Appl. 752 (2001) 33-38.

[103] K.B. O'Brien, M.T. Bowser, Measuring D-serine efflux from mouse cortical brain slices using online microdialysis-capillary electrophoresis, Electrophoresis 27 (2006) 1949-1956.

[104] C.M. Ciriacks, M.T. Bowser, Measuring the effect of glutamate receptor agonists on extracellular D-serine concentrations in the rat striatum using online microdialysis-capillary electrophoresis, Neurosci. Lett. 393 (2006) 200-205.

[105] K.B. O'Brien, M. Esguerra, R.F. Miller, M.T. Bowser, Monitoring Neurotransmitter Release from Isolated Retinas Using Online Microdialysis-Capillary Electrophoresis, Anal. Chem. 76 (2004) 5069-5074.

[106] K.B. O'Brien, M. Esguerra, C.T. Klug, R.F. Miller, M.T. Bowser, A high-throughput on-line microdialysis-capillary assay for D-serine, Electrophoresis 24 (2003) 1227-1235.

[107] N. Plock, C. Kloft, Microdialysis - theoretical background and recent implementation in applied life-sciences, Eur. J. Pharm. Sci. 25 (2005) 1-24.

[108] P. De Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Naphthalene-2,3-dicarboxyaldehyde/cyanide ion: a rationally designed fluorogenic reagent for primary amines, Anal. Chem. 59 (1987) 1096-101.

Chapter 2

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

("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", B. Huynh, B. Fogarty, P. Nandi, S. Lunte, J Pharm Biomed Anal. 2006 Nov 16;42(5):529-34.)

2.1. Introduction

Microdialysis is a continuous sampling technique widely implemented for the measurement of neuroactive compounds in the central nervous system (CNS) extracellular space. This technique has been applied to a variety of research areas including pharmacokinetics [1-3], neuroscience [4,5] and biotechnology [6]. Microdialysis has been used to sample a variety of neurochemically important compounds including amino acids (aspartate, glutamate) [7-9], 5-hydroxytryptamine (5-HT) [10], catecholamines (dopamine, norepinephrine, epinephrine) [11-13], and their metabolites (3,4-dihydroxyphenylacetic acid, homovanillic acid, dopamine glucuronide) [14-17] and neuropeptides (angiotension 1, methionine enkephalin, and neurotensin) [18-23].

For online microdialysis systems, CE is a very attractive mode for separation-based analysis; this is attributed to the fact that small volumes (nL) of sample can be quickly injected in CE systems from the uninterrupted microdialysate flow stream along with the easy integration of highly sensitive laser induced fluorescence (LIF) detection. For similar reasons, microchip electrophoresis can be an even better alternative to CE as the channel dimensions in microchips are typically shorter and narrower than CE channels and therefore sample volume requirements are smaller (pL) and the high field strengths lead to extremely fast analysis times (less than 2 minutes). Also, microchip electrophoresis has a small footprint and can be custom built based on the application with the incorporation of commercially available interfacing products for hydrodynamic flow. In addition, fluorescence detection can

be easily employed for such devices (glass and PDMS). PDMS is very popular material for such devices because of their low cost and their optical transparency at wavelengths as low as 280 nm with a refractive index of 1.43 [24]. Also, PDMS microchips are easier to fabricate compared to glass.

The first coupling of online microdialysis with microchip electrophoresis was reported by our group in 2004 [25]. The online system facilitated delivery of microdialysis sample to a twin-t chip where sample was continuously injected by gated injection and analyzed. The conversion of fluorescein mono-β-d galactopyranoside (FMG) to fluorescein by the enzyme β-galactosidase was monitored using this device. The reaction was carried out *in vitro* in an off-chip vial and sampled by microdialysis. However, in order to use such systems for the analysis of real world samples, incorporation of derivatization and alternative detection methods are absolutely necessary. For many *in vivo* monitoring applications, derivatization of analytes with a fluorophore must be accomplished either off-chip or on-chip for high sensitivity fluorescence detection.

Derivatization strategies have been reported in microchip devices using precolumn [26-31] and postcolumn reactors [32-34]. The microchip platform is particularly attractive for incorporating derivatization because of integration of reaction channels with virtually zero dead volumes and short mass transport times [35]. The sample can be derivatized prior to injection and separation using a "precolumn" reactor; alternatively, the analyte can be derivatized in a "postcolumn" reactor after separation. In postcolumn reactors, the derivatization reagents should be

introduced into the separation channel prior to the electrophoresis ground; therefore in the true sense it is actually an "on-column" reaction. However, this is referred to as "postcolumn" derivatization as the reaction occurs close to the detector [35].

For the rapid derivatization of analytes containing primary amines (e.g. amino acids and peptides), ortho-phthalaldehyde/2-mercaptoethanol (OPA/2-ME) has been a popular choice for on-chip derivatization [26,32]. However, OPA derivatives are quite unstable as the isoindole derivatives exhibit a time-dependent degradation. It can therefore sometimes be tricky to optimize precolumn OPA/RSH because the product can degrade before injection leading to irreproducible results. This could be particularly be a problem if multiple analytes with different optimum derivatization times need to be analyzed.

An analog of OPA that exhibits very similar reaction chemistry with primary amines 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 with CN^- as the nucleophile [36]. It has 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 [37,38] and capillary electrophoresis [39]. NDA was also employed for on-column derivatization of reduced glutathione on a microchip electrophoresis device [40]. Strategies with LIF detection has also been reported with other derivatizing agents [30,41].

The present study aims to evaluate the application of NDA/2ME for the on-column derivatization of amino acids and peptides by microchip electrophoresis. The reagents (NDA/2-ME) were incorporated into the background electrolyte. Theoretically, such an approach should lead to simultaneous separation and derivatization of analytes of analytes (if the reaction occurs very fast) before being detected. The devices used for these studies described in this paper consist of a glass layer containing etched microfluidic channels that was plasma sealed with a layer of poly(dimethylsiloxane) (PDMS) [42]. This fabrication approach was a quick and easy alternative to the production of similar devices reported by our laboratory that were composed exclusively of glass [25]. Specifically, the hybrid device precludes 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 were constructed for continuous on-line sampling from a syringe pump 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 into an already highly-integrated device. This separation-based sensor can ultimately be employed for high-temporal resolution monitoring of primary amines of biological significance.

2.2. Materials and Methods

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 hydroxide, acetone, and isopropyl alcohol were from Fisher Scientific (Fair Lawn, NJ). Naphthalene 2,3-dicarboxaldehyde (NDA) was purchased from Bioanalytical Systems (BAS, West Lafayette, IN). 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 were 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).

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 [25], the negative mask transparency was placed on top of a piece of soda lime glass with predeposited layers of chrome and AZ1500 photoresist. The glass was exposed

to UV light for 15 s and 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. 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 the particulate buildup 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 measured 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 Figure 2.1A): 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 (Sulfur Springs, TX). The fluidic connector was modified from a low dead-volume union (nanoport, Upchurch Scientific, Oak Harbor, WA) (Figure 2.1B). To produce the PDMS layer, a 7:1 (w/w)

mixture of Sylgard 184 and curing agent mixture was poured onto a silicon wafer and allowed to bake in an oven at 70°C 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.

Device coupling to syringe and microdialysis sampling: 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. The general setup for these experiments has been described previously [25] and a schematic representation is shown in Figure 2.1C. Briefly, the modified nanoports (Figure 2.1B) 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 (BR-4, 4mm cylindrical probe, BAS, West Lafayette, IN) were packaged with a plastic protective cap; this cap was cut using a razor blade to expose the probe tip (Figure 2.1B). 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 buffer, was connected to the inlet of the 4-mm microdialysis brain probe (BAS).

Device operation conditions: A solution of 25 mM boric acid buffer adjusted to pH 9.2 with 1 N NaOH was used as the electrophoresis buffer. The device was conditioned by flushing the microchip with 0.1 N 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 (Figure 2.1A). Voltages were applied using a four-channel bipolar high voltage system (Jenway, Essex, England, UK) that was controlled by a Toshiba laptop computer. During device operation, the voltage was applied continuously to electroosmotically prevent sample leakage into 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 [25,43].

For 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

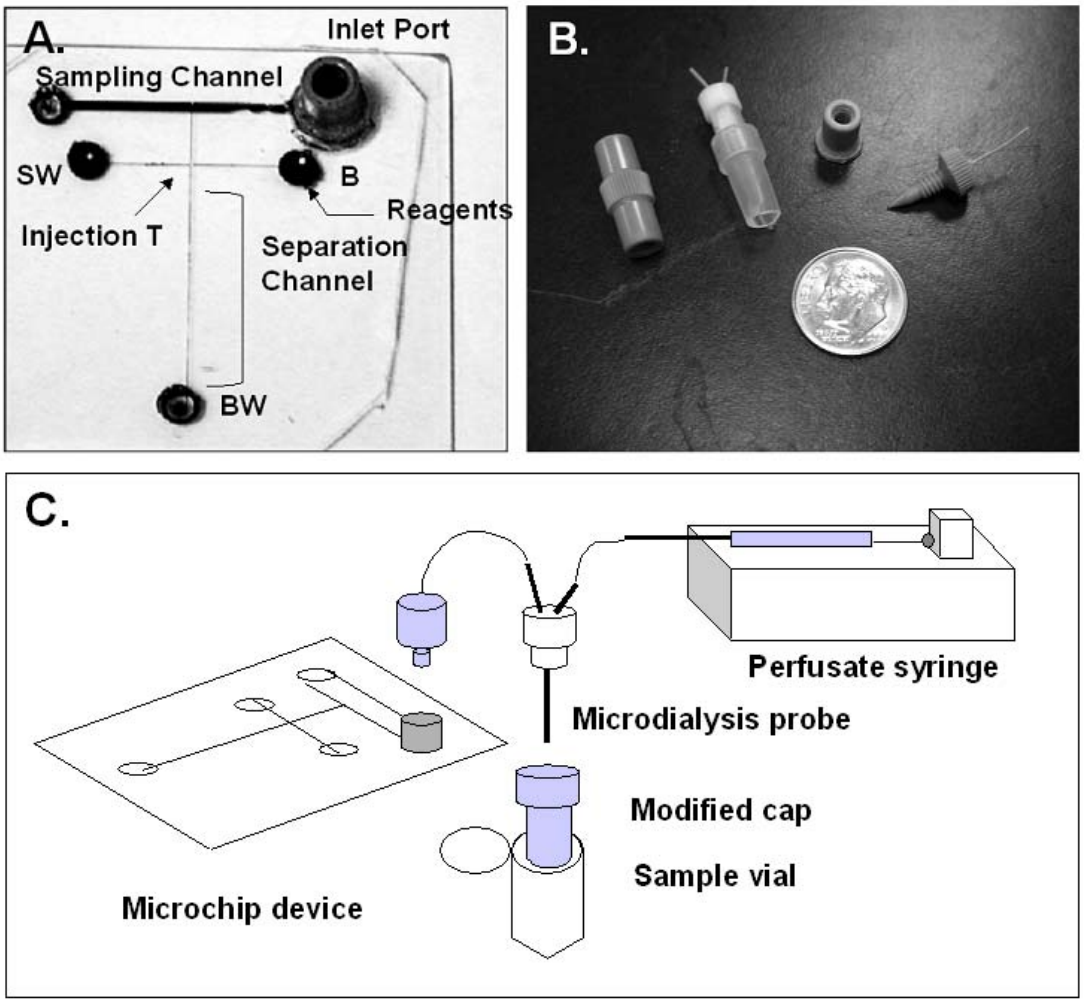


Fig. 2.1: A) Picture of PDMS-glass hybrid device used in initial studies. Device is constructed by etching channels into the glass layer and irreversibly sealing with a layer of PDMS. Reservoirs: B = buffer, SW = sample waste, BW = buffer waste. Derivatization reagents were added into the buffer reservoir. 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.

leucine enkephalin, 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 still 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). The experiments were carried out under device operating conditions of 2000 V applied voltage and an injection time of 1 s.

LIF detection: A home-built LIF detection setup similar to that previously described was employed [25]. 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 degrees into a long working distance objective (N.A. = 0.6, 40X 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 a X-Y-Z translatable stage. Fluorescence was collected with the same microscope objective, passed through the dichroic mirror, and reflected 90 degrees 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. Analogue 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.

2.3. Results and Discussion

2.3.1. 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 containing etched fluidic channels is much easier for device fabrication compared to high-temperature glass bonding of two glass plates. By using the PDMS sealing method, devices were quickly and easily assembled for use. In cases where channel blockages were observed, the PDMS layer of the device could be removed with a razor blade and the glass layer 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, in our application, we did not observe any problems associated with hydrophobic interactions such as peak tailing

or band broadening. 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 these studies.

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

A microdialysis-microchip device based on a similar design in glass was characterized previously for optimal microdialysis sample flow, voltage application scheme and injection time [25]. To increase the utility of this system for the 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 the target for the 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 sec 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 employed. Next, a simple mixture of two amino acids, 5 mM Phe and Glu, was pumped into the device under the same conditions as described above, and sequential injections were carried out. Figure 2.2 shows repeated injections (30 secs interval) of mutiple amino

acids that were baseline-resolved with good reproducibility (RSD = 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 to be 12 and 18 s for Phe and Glu, respectively. Separations of simple mixtures of peptides and amino acids introduced from a syringe were also evaluated using this system. These mixtures, which consisted of A) L-enkephalin and Asp and B) Gly-Pro and Glu, were analyzed and the results are shown in Figure 2.3. The concentrations of analytes in their respective mixtures were 5 mM. In these experiments, 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 [44,45]. 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 but at the cost of longer analysis times (300 s).

Estimated injection plug lengths of 20 μm correspond to approximately 12 fmol injected 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 and glycine, respectively

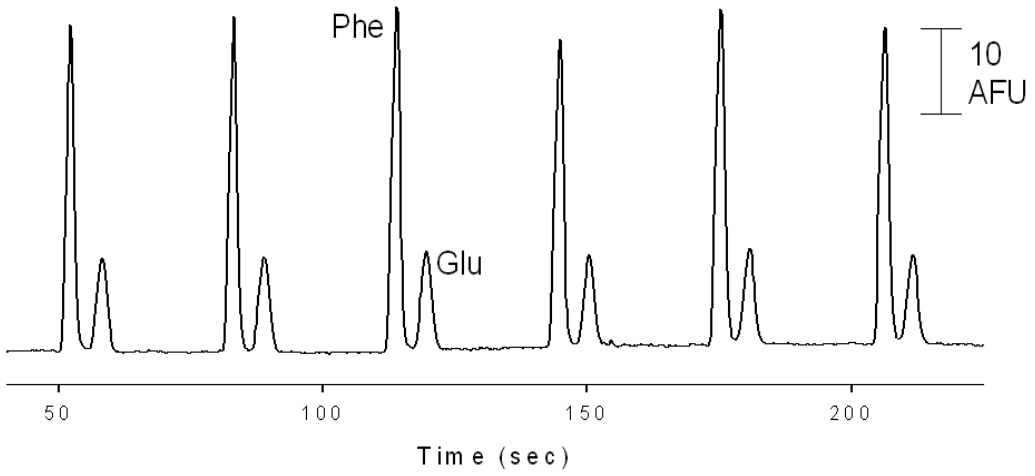


Fig. 2.2: Sequential injections and separations of 5 mM concentrations Phe and Glu, derivatized on-chip with a mixture of 0.6 mM NDA and 1.2 mM 2ME. Conditions were as follows: applied voltage of 2300 V was used with a 1 s sample fill time and a flow rate of 1.0 ml/min. LIF detection was conducted 20 mm from the injector t.

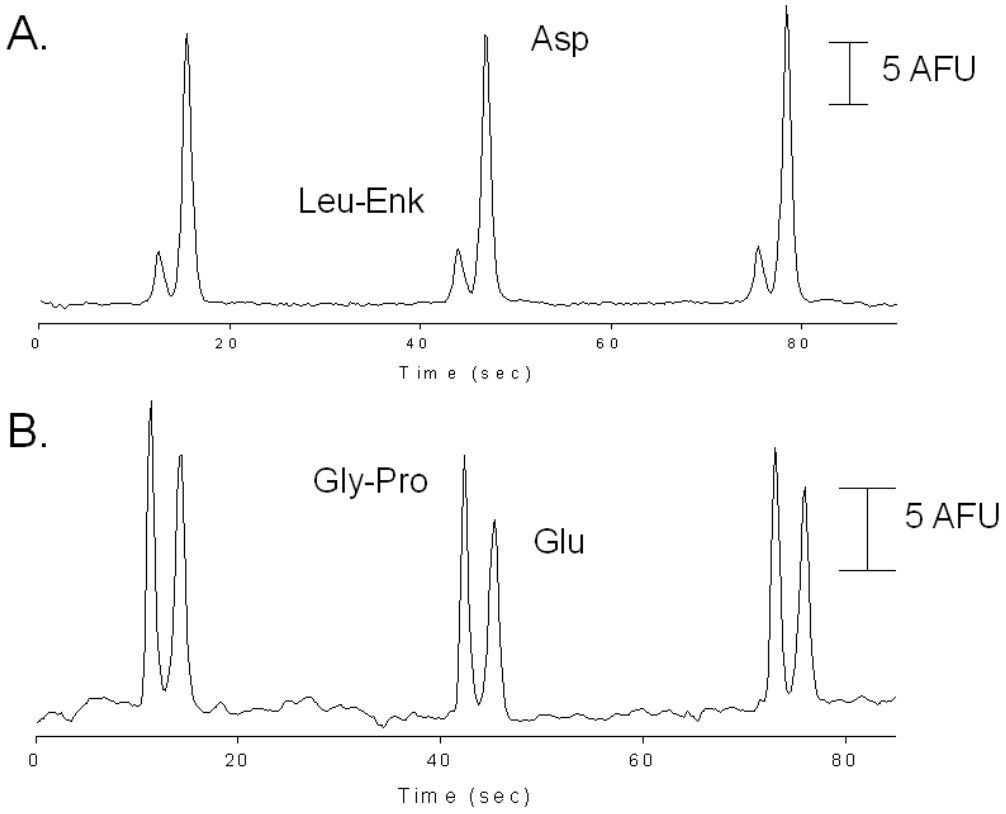


Fig. 2.3: Amino acid and peptide separations. Amino acids and peptides were derivatized on-chip using NDA/2ME. Consecutive injections of A) Leu-enkephalin and Asp and B) Gly-Pro and Glu. Conditions were similar to those used in Figure 2.

were reported by Jacobson *et al*. [26]. More recently, Kennedy's group 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 [46]. For postcolumn derivatization, detection limits were 200, 130, and 120 fmol for Arg, Gly, and Thr, respectively [32]. The limits of detection (LOD) for this work employing on-column derivatization were better than those reported for a postcolumn scheme; however, precolumn derivatization limits of detection were 2–3 orders of magnitude lower than the LOD demonstrated 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 could also 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 [47].

2.3.3. 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) evaluate on-column NDA/2ME derivatization for amino acids and peptides on-chip; and 3) incorporate

the additional feature of on-chip sample derivatization to our on-line system. The latter 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. Figure 2.4 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 (RSD = 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. Analysis could be run around 15 times without replenishing reservoir B or reconditioning the chip with NaOH to regenerate the EOF.

2.4. Conclusion

The incorporation of on-chip sample derivatization was carried out using an easily- assembled microchip CE device coupled online to microdialysis sampling. This miniaturized analysis system exhibits a high degree of process integration, including sample preparation, delivery, sample gating, dynamic derivatization, 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, 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 to develop a micro-total analysis system for high-temporal resolution biological monitoring of primary amines.

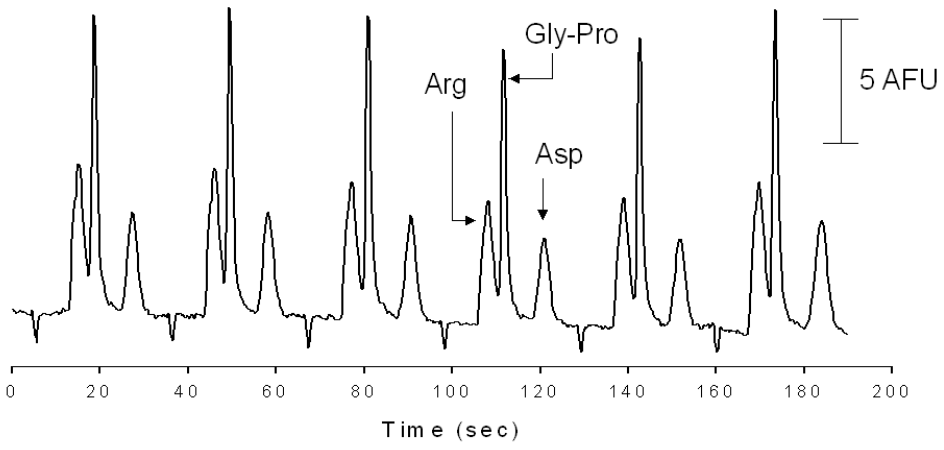


Fig. 2.4: Continuous separations of a 3-component mixture sampled continuously through a 4-mm microdialysis probe at a flow rate of 1.0 ml/min. Analyte concentrations are as follows: 7.95 mM Arg, 10 mM Gly-Pro, and 10 mM Asp. Derivatization conditions were similar to those used in Figure 2.2.

2.5. References

[1] D.K. Hansen, M.I. Davies, S.M. Lunte, C.E. Lunte, Pharmacokinetic and metabolism studies using microdialysis sampling, J. Pharm. Sci. 88 (1999) 14-27.

[2] M.I. Davies, A review of microdialysis sampling for pharmacokinetic applications, Anal. Chim. Acta 379 (1999) 227-249.

[3] W.F. Elmquist, R.J. Sawchuk, Application of Microdialysis in Pharmacokinetic Studies, Pharm. Res. 14 (1997) 267-288.

[4] U. Ungerstedt, E. Rostami, Microdialysis in neurointensive care, Curr. Pharm. Des. 10 (2004) 2145-2152.

[5] Y. Deguchi, Application of *in vivo* brain microdialysis to the study of blood-brain barrier transport of drugs, Drug Metab. Pharmacokinet. 17 (2002) 395-407.

[6] N. Torto, T. Laurell, L. Gorton, G. Marko-Varga, Recent trends in the application of microdialysis in bioprocesses, Anal. Chim. Act. 379 (1999) 281-305.

[7] T. Bruhn, T. Christensen, N.H. Diemer, *In vivo* cellular uptake of glutamate is impaired in the rat hippocampus during and after transient cerebral ischemia: a microdialysis extraction study, J. Neurosci. Res. 66 (2001) 1118-26.

[8] E. Silva, L. Hernandez, Q. Contreras, F. Guerrero, G. Alba, Noxious stimulation increases glutamate and arginine in the periaqueductal gray matter in rats: a microdialysis study, Pain 87 (2000) 131-5.

[9] C.S. Yang, P.J. Tsai, W.Y. Chen, W.J. Tsai, J.S. Kuo, On-line derivatization for continuous and automatic monitoring of brain extracellular glutamate levels in anesthetized rats: a microdialysis study, J. Chromatogr. B 734 (1999) 1-6.

[10] B. Langen, C. Rundfeldt, ELB139 an agonist at the benzodiazepine binding site increases 5-HT in the striatum and prefrontal cortex of rats: a microdialysis study, Pharmacology Biochemistry and Behavior 86 (2007) 79-85.

[11] J. Bergquist, A. Sciubisz, A. Kaczor, J. Silberring, Catecholamines and methods for their identification and quantitation in biological tissues and fluids, J. Neurosci. Meth. 113 (2002) 1-13.

[12] D.-C. Chen, D.-Z. Zhan, C.-W. Cheng, A.-C. Liu, C.-H. Chen, Determination of urine catecholamines by capillary electrophoresis with dual-electrode amperometric detection, J. Chromatogr. B 750 (2001) 33-39.

[13] F.-C. Cheng, J.-S. Kuo, H.-M. Huang, D.-Y. Yang, T.-F. Wu, T.-H. Tsai, Determination of catecholamines in pheochromrocytoma cell (PC-12) culture medium by microdialysis-microbore liquid chromatography, J. Chromatogr. A 870 (2000) 405-411.

[14] L. Bezin, C. Ortemann, M. Ogier, B. Astier, J.F. Pujol, B. Renaud, L. Lambas-Senas, Enhanced tail pinch-induced activation of catecholamine metabolism in the pericerulean area of RU 24722-treated rats, Brain Res. 1030 (2004) 1-10.

[15] S. Boulet, S. Mounayar, A. Poupard, A. Bertrand, C. Jan, M. Pessiglione, E.C. Hirsch, C. Feuerstein, C. Francois, J. Feger, M. Savasta, L. Tremblay, Behavioral recovery in MPTP-treated monkeys: neurochemical mechanisms studied by intrastriatal microdialysis, J. Neurosci. 28 (2008) 9575-84.

[16] H. Chen, F.C. Jing, C.L. Li, P.F. Tu, Q.S. Zheng, Z.H. Wang, Echinacoside prevents the striatal extracellular levels of monoamine neurotransmitters from diminution in 6-hydroxydopamine lesion rats, J. Ethnopharmacol. 114 (2007) 285-9.

[17] P. Uutela, L. Karhu, P. Piepponen, M. KaÌˆenmaÌˆki, R.A. Ketola, R. Kostiainen, Discovery of dopamine glucuronide in rat and mouse brain microdialysis samples using liquid chromatography tandem mass spectrometry, Anal. Chem. 81 (2008) 427-434.

[18] Q. Li, J.K. Zubieta, R.T. Kennedy, Practical Aspects of *in Vivo* Detection of Neuropeptides by Microdialysis Coupled Off-Line to Capillary LC with Multistage MS, Anal. Chem. (2009) 2242-2250.

[19] B. Bernay, M.-C. Gaillard, V. Guryca, A. Emadali, L. Kuhn, A. Bertrand, I. Detraz, C. Carcenac, M. Savasta, E. Brouillet, J. Garin, J.-M. Elalouf, Discovering new bioactive neuropeptides in the striatum secretome using *in vivo* microdialysis and versatile proteomics, Mol. Cell. Proteomics 8 (2009) 946-958.

[20] H.L. Behrens, R. Chen, L. Li, Combining microdialysis, NanoLC-MS, and MALDI-TOF/TOF to detect neuropeptides secreted in the crab, Cancer borealis, Anal. Chem. 80 (2008) 6949-58.

[21] K.T. Myasein, J.S. Pulido, R.M. Hatfield, C.A. McCannel, R.F. Dundervill, 3rd, S.A. Shippy, Sub-microlitre dialysis system to enable trace level peptide detection from volume-limited biological samples using MALDI-TOF-MS, Analyst 132 (2007) 1046-52.

[22] M. Emmett, P. Andren, R. Caprioli, Specific molecular mass detection of endogenously released neuropeptides using *in vivo* microdialysis/mass spectroscopy, J. Neurosci. Meth. 62 (1995) 141-147.

[23] P.E. Andren, R.M. Caprioli, Determination of extracellular release of neurotensin in discrete rat brain regions utilizing *in vivo* microdialysis/electrospray mass spectrometry, Brain Res. 845 (1999) 123-129.

[24] K.W. Ro, K. Lim, H. Kim, J.H. Hahn, Poly(dimethylsiloxane) microchip for precolumn reaction and micellar electrokinetic chromatography of biogenic amines, Electrophoresis 23 (2002) 1129-37.

[25] B.H. Huynh, B.A. Fogarty, R.S. Martin, S.M. Lunte, On-Line Coupling of Microdialysis Sampling with Microchip-Based Capillary Electrophoresis, Anal. Chem. 76 (2004) 6440-6447.

[26] S.C. Jacobson, R. Hergenroder, A.W. Moore, J.M. Ramsey, Precolumn reactions with electrophoretic analysis integrated on a microchip, Anal. Chem. 66 (1994) 4127-4132.

[27] J. Wang, M.P. Chatrathi, B. Tian, Micromachined separation chips with a precolumn reactor and end-column electrochemical detector, Anal. Chem. 72 (2000) 5774-5778.

[28] J. Wang, G. Chen, M. Pumera, Microchip separation and electrochemical detection of amino acids and peptides following precolumn derivatization with naphthalene-2,3-dicarboxyaldehyde, Electroanalysis 15 (2003) 862-865.

[29] K.W. Ro, J.H. Hahn, Precolumn diastereomerization and micellar electrokinetic chromatography on a plastic microchip: rapid chiral analysis of amino acids, Electrophoresis 26 (2005) 4767-73.

[30] N.P. Beard, J.B. Edel, A.J. deMello, Integrated on-chip derivatization and electrophoresis for the rapid analysis of biogenic amines, Electrophoresis 25 (2004) 2363-2373.

[31] O. Yassine, P. Morin, O. Dispagne, L. Renaud, L. Denoroy, P. Kleimann, K. Faure, J.L. Rocca, N. Ouaini, R. Ferrigno, Electrophoresis PDMS/glass chips with continuous on-chip derivatization and analysis of amino acids using naphthalene-2,3-dicarboxaldehyde as fluorogenic agent, Anal. Chim. Acta. 609 (2008) 215-22.

[32] S.C. Jacobson, L.B. Koutny, R. Hergenroder, A.W. Moore, J.M. Ramsey, Microchip capillary electrophoresis with an integrated postcolumn reactor, Anal. Chem. 66 (1994) 3472-3476.

[33] K. Fluri, G. Fitzpatrick, N. Chiem, D.J. Harrison, Integrated capillary electrophoresis devices with an efficient postcolumn reactor in planar quartz and glass chips, Anal. Chem. 68 (1996) 4285-4290.

[34] J. Wang, M.P. Chatrathi, A. Ibanez, A. Escarpa, Micromachined separation chips with post-column enzymatic reactions of "class" enzymes and end-column electrochemical detection: Assays of amino acids, Electroanalysis 14 (2002) 400-404.

[35] M. Pumera, Microfluidics in amino acid analysis, Electrophoresis 28 (2007) 2113-24.

[36] P. De Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Naphthalene-2,3-dicarboxyaldehyde/cyanide ion: a rationally designed fluorogenic reagent for primary amines Anal. Chem. 59 (1987) 1096-1101.

[37] K. Dave, J.F. Stobaugh, C.M. Riley, Reversed-phase liquid chromatography of the opioid peptides-2. Quantitative structure-retention relationships and isocratic retention prediction, J Pharm. Biomed. Anal. 10 (1992) 49-60.

[38] K. Dave, J.F. Stobaugh, T.M. Rossi, C.M. Riley, Reversed-phase liquid chromatography of the opioid peptides. 3. Development of a microanalytical system for opioid peptides involving microbore liquid chromatography, post-column derivatization and laser-induced fluorescence detection, J. Pharm. Biomed. Anal. 10 (1992) 965-977.

[39] S.D. Gilman, A.G. Ewing, Post-column derivatization for capillary electrophoresis using naphthalene-2,3-dicarboxaldehyde and 2-mercaptoethanol, Anal. Meth. Instrum. 2 (1995) 133-41.

[40] J. Qin, N. Ye, L. Yu, D. Liu, Y. Fung, W. Wang, X. Ma, B. Lin, Simultaneous and ultrarapid determination of reactive oxygen species and reduced glutathione in apoptotic leukemia cells by microchip electrophoresis, Electrophoresis 26 (2005) 1155-1162.

[41] M. Kato, Y. Gyoten, K. Sakai-Kato, T. Toyo'oka, Rapid analysis of amino acids in Japanese green tea by microchip electrophoresis using plastic microchip and fluorescence detection, J. Chrom. A. 1013 (2003) 183-189.

[42] J. Cooper McDonald, D.C. Duffy, J.R. Anderson, D.T. Chiu, H. Wu, O.J.A. Schueller, G.M. Whitesides, Fabrication of microfluidic systems in poly(dimethylsiloxane), Electrophoresis 21 (2000) 27-40.

[43] Y. Lin, G. Lee, C. Li, G. Huang, S. Chen, Flow-through sampling for electrophoresis-based microfluidic chips using hydrodynamic pumping, J. Chromatogr. A 937 (2001) 115-125.

[44] L.J. Jin, B.C. Giordano, J.P. Landers, Dynamic labeling during capillary or microchip electrophoresis for laser-induced fluorescence detection of protein-SDS complexes without pre- or postcolumn labeling, Anal. Chem. 73 (2001) 4994-4999.

[45] B.C. Giordano, L. Jin, A.J. Couch, J.P. Ferrance, J.P. Landers, Microchip laser-induced fluorescence detection of proteins at submicrogram per milliliter

levels mediated by dynamic labeling under pseudonative conditions, Anal. Chem. 76 (2004) 4705-4714.

[46] Z.D. Sandlin, M. Shou, J.G. Shackman, R.T. Kennedy, Microfluidic electrophoresis chip coupled to microdialysis for *in vivo* monitoring of amino acid neurotransmitters, Anal. Chem. 77 (2005) 7702-7708.

[47] A.L. Freed, J.D. Cooper, M.I. Davies, S.M. Lunte, Investigation of the metabolism of substance P in rat striatum by microdialysis sampling and capillary electrophoresis with laser-induced fluorescence detection, J. Neurosci. Methods 109 (2001) 23-9.

Chapter 3

Development of a poly(dimethylsiloxane) (PDMS) based microchip device for continuous online *in vivo* microdialysis sampling

3.1 Introduction

The brain extracellular space is a complex jelly-like matrix comprised of numerous chemical entities such as ions, neurotransmitters, peptides, metabolites and extracellular matrix molecules [1]. The analysis of amino acid neurotransmitters in the brain extracellular fluid is an important aspect of many *in vivo* neurochemical investigations such as learning and behavior, oxidative stress, seizure, epilespsy and other neurological disorders [2-5]. Microdialysis is a popular *in vivo* sampling method that can be used to continuously retrieve analytes from the brain extracellular space so that they can subsequently be interrogated by appropriate analytical methods [6]. Also, microdialysis sampling studies can provide information on the concentrations of exogenous compounds in the extracellular fluid of tissues following administration (i.e drug substances) [7]. This sampling technique has been used to investigate drug transport and metabolism at the blood brain barrier (BBB) [8]. Pharmacokinetic and pharmacodynamic studies performed with microdialysis samples can be used to draw conclusions regarding the delivery characteristics of the drug as well as its therapeutic efficacy [9,10].

Brain microdialysis sampling is accomplished by surgically implanting a concentric cannula probe into the specific brain region of interest. The probes are continuously perfused with a solution of ionic strength similar to the extracellular fluid of the region of implantation. Small molecular weight compounds present in the extracellular space diffuse across the probe's semipermeable membrane into the continuous flowing perfusate based on the concentration gradient. Microdialysis

creates a continuous stream of sample and hence is an ideal technique to be coupled to an analytical system that is capable of analyzing discrete sample plugs from a continuous stream. The value of developing such analysis systems lies in the ability to perform "continuous" and "online" analysis that ultimately results in high throughput and temporal resolution. Various online instrumental setups have been reported in the literature that involve microdialysis coupled to liquid chromatography (LC) or capillary electrophoresis (CE) with laser-induced fluorescence (LIF), mass spectrometry (MS), UV or electrochemical (EC) detection scheme [11,12].

Microchip electrophoresis is a technique that is well suited to perform flow-through sampling and separation on one integrated device. In addition to advantages associated with miniaturization, such systems offer the ability to be custom designed to handle, manipulate and inject discrete plugs from pressure driven flows. For *in vivo* continuous monitoring applications, the outlet from the microdialysis probe can be coupled directly to the microchip device creating a continuous flow of sample through the chip. Discrete plugs can then be injected by hydrodynamic or electrokinetic injection into an electrophoresis channel for separation [13,14]. Ultimately, such a system is capable of continuous online near real time analysis of microdialysis samples yielding useful information regarding temporal changes in the concentration of analyte(s) in the brain or other tissues.

One of the primary advantages of microchip based devices for the analysis of microdialysis sample is the ability to sample from submicroliter flow rates. Low flow rates (low microliter to nanoliter per minute) are ideal for microdialysis as this leads

to higher relative recovery of the analyte and ultimately improves the limits of detection for the integrated device [15]. Such low flow rates are also very compatible with microchip devices because they result in lower back pressures and less fluid buildup within the device. This provides the opportunity to build microchip devices out of less robust substrates (e.g. PDMS) without requiring complicated surface modification [16].

During the development of a microdialysis-microchip electrophoresis system with LIF detection for *in vivo* application, incorporation of various key components into the chip device needs to be considered. These components include a microdialysis-chip interface capable of handling the hydrodynamic flow, mixing channels for analyte derivatization, a separation channel to accomplish the electrophoretic separation and a detector module. Our group reported the first system that coupled microdialysis with microchip electrophoresis [17]. Since then, there have been a few additional reports of online microdialysis-microchip separation systems for *in vitro* and *in vivo* applications. Most notable among those is the device developed by Kennedy group using glass chips for continuous *in vivo* monitoring of amino acids in rat brain using side-by-side microdialysis probes. This method employed microdialysis sampling, precolumn derivatization with OPA and β-ME and gated injection followed by amino acid analysis on the chip device [18]. The Martin group has also described an innovative method for coupling microdialysis to microchip that employs soft lithographic valves that are actuated to inject plugs of

sample from microdialysis sample flow. This device has been used to detect dopamine release from PC-12 cells *in vitro* with electrochemical detection [19].

The present study concerns the development of a PDMS microchip-based analysis system that integrates *in vivo* microdialysis sampling along with reagent delivery and mixing by means of hydrodynamic flow for on-chip derivatization followed by injection and analysis of the derivatized sample by LIF detection. This device utilizes a simple design in PDMS; it is substantially easier to construct compared to other devices that employs glass bonding or soft lithographic valves. Also, the chip is designed to make it possible to optimize and manipulate flows with minimal effort. The system was evaluated for the analysis of amino acid standards and rat brain striatum microdialysis samples using naphthalene-2, 3-dicarboxaldehyde (NDA) and cyanide (CN) as the derivatization reagents. To the best of our knowledge, this is the first report of a PDMS-based microchip device for online *in vivo* analysis.

3.2 Materials and Methods

Chemicals and materials: The following chemicals and materials were used: boric acid, SDS, acetonitrile, disodium fluorescein, amino acid standards, sodium cyanide, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bocarbonate and disodium phosphate were purchased from Sigma (St. Louis, MO). Naphthalene-2,3-dicarboxaldehyde was received from Molecular Probes (Eugene, OR). All aqueous buffers and solutions were prepared using Milli-Q water

and filtered using 0.22 μm Cameo Teflon syringe cartridges from Osmonics (Minnetonka, MN). Brain probes (4mm length, CMA 12) were obtained from CMA (North Chelmsford, MA).

Chip Fabrication: All microchip devices were fabricated from poly (dimethylsiloxane) (PDMS) obtained from Ellsworth Adhesive (Minneapolis, MN). Channel molds were prepared on a silicon wafer (Telic Co.) using SU8-10 photoresist (Newton, MA) and a high resolution negative transparency (IGI, Minneapolis, MN) containing the chip design using standard photolithographic techniques.

The chip was fabricated by pouring an approximately 5 mm thick layer of a mixture of PDMS and curing agent (10.5:1.5) on the channel mold followed by curing for 25-30 minutes at 90^{o}C in a convection oven. Reservoirs were then punched with biopsy punches (4 mm diameter) and inlets for hydrodynamic flow were produced with 20 gauge luer stub. A second thin PDMS layer (approximately 1 mm in thickness) was semi cured in the oven for 15-18 minutes at 90^{o}C until this PDMS layer was tacky to touch. The channel layer was then conformally contacted with the semi cured layer and again cured in the oven at 85^{o}C overnight for a semi-permanent seal. To interface the chip with hydrodynamic flow, stainless steel pins were embedded in the flow inlets. The other end of each pin was connected to the outlet of a syringe pump or that of a microdialysis probe via FEP tubing.

In vitro experiments: For these studies, the microdialysis probe was placed in a vial containing a solution of the amino acids of interest in aCSF. The inlet of the

probe was connected to a 1 mL syringe containing water via FEP tubing, and outlet of the probe was connected to microfluidic device.

In vivo experiments: Sprague-Dawley (SD) rats weighing 225g-250g were anesthetized by inhalation of isoflurane followed by a subcutaneous (s.c.) injection of a mixture of acepromazine (0.37 mg/mL), ketamine (37.5 mg/mL) and xylazine (1.9 mg/mL). Additional doses of ketamine were injected as needed to maintain the level of anesthesia during the experiment. The body temperature of the rat was maintained by placing the animal on a heating pad. The area from the base of the skull to approximately the front of the eyes, the hair on the lower abdomen and inner thigh were shaved as closely as possible.

The animal was placed on its back for insertion of femoral cannula. A small nick was made in the femoral vein and micro-renethane cannula was inserted with aid of dental pick. The incision was closed properly with staples. The animal was placed on its front for implantation of the microdialysis probe. The guide cannula followed by microdialysis probe was implanted in the striatum region using stereotaxic coordinates: A/P (+0.2), M/L (+3.2), D/V (7.5). Dental acrylic was applied in the area that was shaved to anchor the guide cannula in place. Post surgery, the animal was allowed to recover for 2 hours with simultaneous perfusion of the implanted probe with aCSF.

Microdialysis sampling: For *in vivo* microdialysis sampling, the brain microdialysis probe was implanted in the rat striatum. The input of the probe was connected to 1 mL syringe containing artificial cerebrospinal fluid (aCSF was

composed of 120mM of NaCl, 3mM KCl, 20mM $NaHCO_3$, 1.2mM $CaCl_2$, 1.0mM $MgCl_2$, 0.25mM Na_2HPO_4) via FEP tubing. The syringe was placed in a syringe pump (CMA) and all experiments were performed with a flow rate of 1.0 μL/min for each inlet.

High potassium aCSF studies: For these studies, a liquid switch (CMA) was placed between the syringe pump and microdialysis probe that enabled switching from aCSF to high potassium aCSF (composed of 20mM of NaCl, 103mM of KCl, 20mM $NaHCO_3$, 1.2mM $CaCl_2$, 1.0mM $MgCl_2$, 0.25mM Na_2HPO_4) without interruption. Post recovery, a set of 5 basal microdialysis samples was collected from the anesthetized rat and then perfusate was switched to high K^+ aCSF followed by sample collection.

Derivatization reactions: For offline analysis, amino acid standards or microdialysate were derivatized with equal parts by volume of 7 mM NDA (dissolved in acetonitrile) and 10 mM NaCN (dissolved in 50 mM boric acid buffer, pH 9.2). For online derivatization, 7 mM NDA (dissolved in 1:1 acetonitrile:H_2O) and 10mM NaCN (50 mM boric acid buffer, pH 9.2 and 5% acetonitrile) were used.

Microchip operation: During offline experiments, an injection voltage was applied across the sampling channel (50V/cm) and a separation voltage (400V/cm) was applied across the serpentine channel. For online experiments, the same injection and separation method was followed except that the sample pool reservoir was manually cleared of the previous sample and allowed to fill up for approximately 30s for the next sample. This procedure was followed for all online experiments.

LIF detection system: The detection system consisted of an epi-fluorescent microscope (Nikon Ti series). The chip device was placed on a microscope glass slide (Gold Seal cover glass #3334) and positioned on the microscope stage with clamps. Fluorescent light (mercury lamp source connected to the microscope by fiber optic cable) was focused at a point of the separation channel usually at a distance of 0.2cm from the waste reservoir through a 40X objective. Filter cubes housing the appropriate excitation/emission filters and dichroic mirrors were installed inside the carousel. Fluorescein studies were performed using FITC filter cube (B1-E) obtained from Nikon. For the detection of CBI derivatives, a custom built cube was purchased from Chroma (25 mm diameter z442/10X clean-up filter, 25 mm diameter 510hq/50m bandpass emission filter and 25.5x36mm laser dichroic filter and transmit hq510/50m). A photomultiplier tube (Hamamatsu) was aligned to the side port of the microscope to collect the emission from the sample. Data was collected in a computer using Chromgraph software that was coupled to the PMT by means of a preamplifier (Stanford Research Systems) and data acquisition module (Chromgraph Interface). Electropherograms were analyzed using Origin software.

3.3 Results and discussions

3.3.1 Offline studies and characterization

Although glass is an ideal substrate for electrophoresis, the fabrication of glass microchips is challenging and time consuming. The major challenge is associated with the reproducibility of the bonding process used to seal the glass plates. Several

groups have proposed techniques to improve the reproducibility of glass bonding [20-22]; however, due to the inherent dependence of bonding success on the glass surface and microscopic structural defects, there are a number of problems associated with the transfer of this technique from one lab group to the next. Also, we have found that bonding of complex channel designs in glass can be problematic because of turns and angles. Hence, this device was fabricated out of PDMS to make the fabrication facile and reproducible.

Previous research regarding microdialysis-micochip electrophoresis utilized microchips with 3 cm separation channel [23]. However, microdialysis samples collected from brain extracellular space are quite complex and the separation is more complicated requiring greater peak capacity. Therefore, a serpentine channel with a separation channel length of 20cm was fabricated and characterized for these studies (**Fig 3.1A**). This design also incorporated a sampling channel (15 μm wide) that was narrower than the separation channel (40 μm wide) to facilitate more efficient pushback of sample and minimize sample leakage from the sampling to the separation channel during application of the separation voltage [24] .

The procedure for sample injection and separation on this device is illustrated in **Fig 3.1B**. An injection voltage of 0.3-0.5KV was applied across the sampling channel followed by a separation voltage of 400V/cm across the separation channel. The optimal injection times were found to be between 0.5-1sec. This injection scheme could be adapted quickly for optimization of the serpentine-channel chip for online analysis. Also, considering the fact that this design will be employed subsequently as

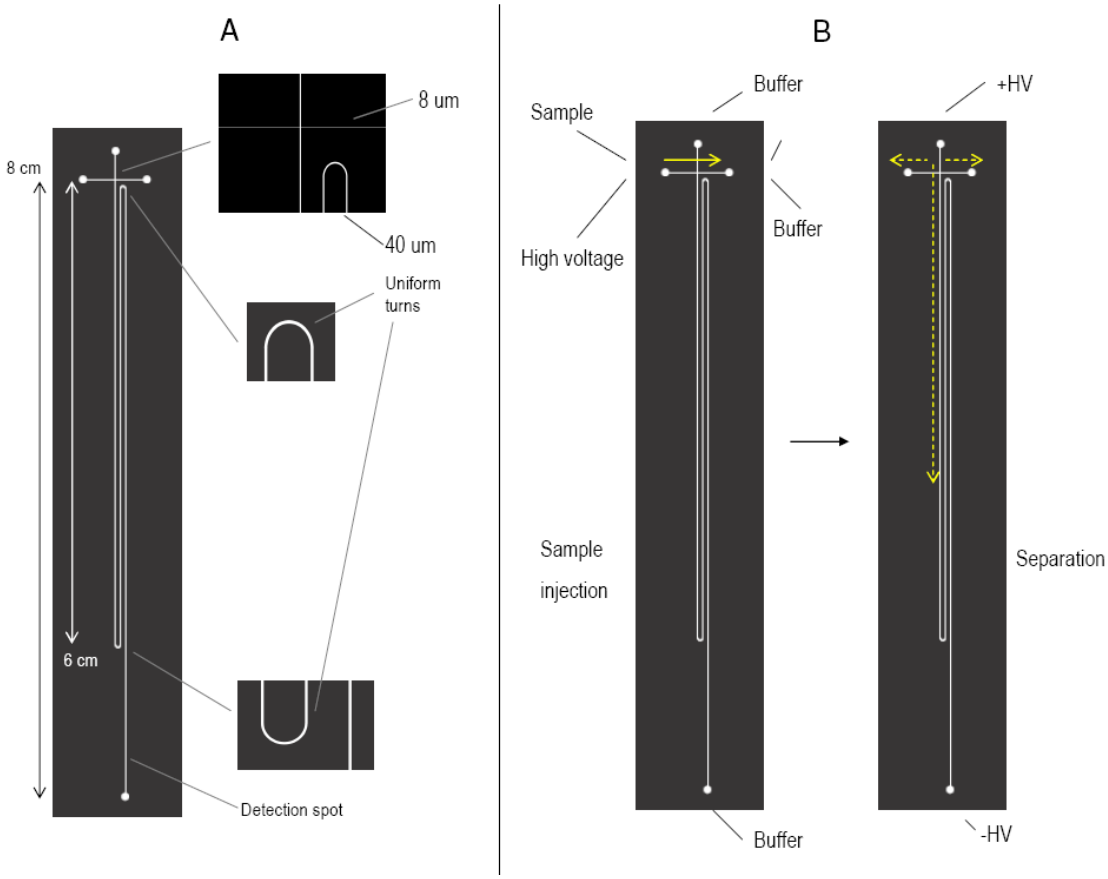


Figure 3.1: A. Microchip device with serpentine channel specifications. B. Injection and separation mechanism on such a device.

the separation component of *in vivo* microdialysis-microchip electrophoresis device, this injection method was found to be most easily integratable with the flow through sampling that is described later in this chapter. In addition, the short and narrow sample injection arms precluded possibilities to carry out gated injection on this design.

Roman *et al.* reported the development of a PDMS based serpentine microchip electrophoresis for high resolution analysis of amino acids and proteins. They also reported the use of SDS in the sample buffer to reduce analyte adsorption and generate a faster EOF on the chip device [25]. In our case, the separation was optimized for neuroactive amino acids by evaluating various buffer conditions containing SDS (**Fig 3.2**). It was empirically observed that a high salt buffer containing a high concentration of SDS offered better wetting properties and facilitated filling the hydrophobic PDMS channel compared to a low salt, low concentration SDS buffer. High currents and consequent Joule heating are considered major drawbacks for such buffers. However, in this case, it did not seem to affect the performance or the resolution of the devices (N=10 chips). This was likely due to fact that PDMS is gas permeable which provided some degree of heat exchange with the surrounding minimizing Joule heating.

Once the separation conditions were optimized, a set of basal microdialysis samples were collected from the rat brain striatum, derivatized offline with NDA and CN^- and analyzed on the chip (**Figs 3.3 and 3.4**). The microdialysis samples were

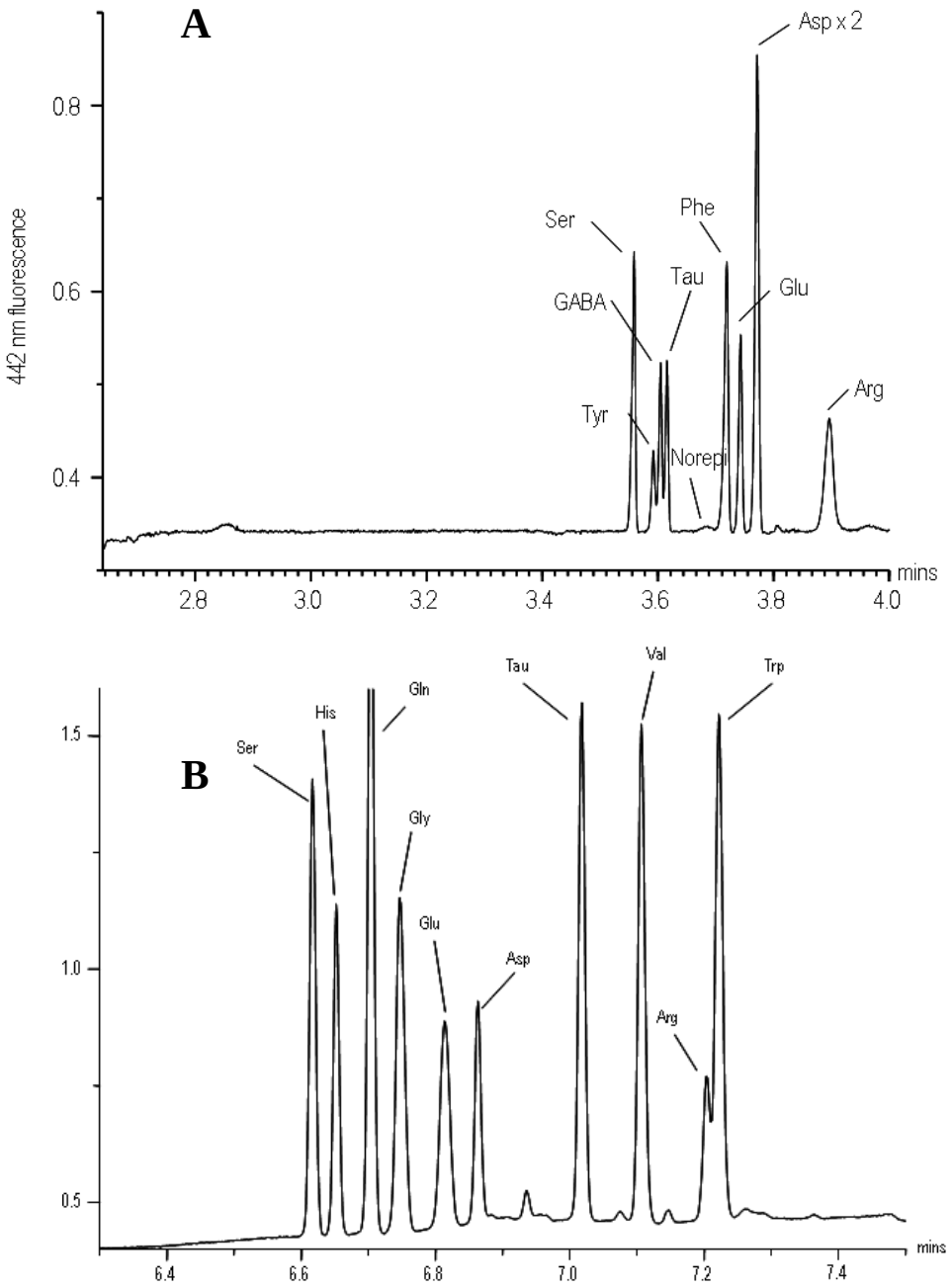


Figure 3.2: Optimization of separation of neuroactive amines under various buffer conditions on serpentine microchip. A. 20 mM borate, 10 mM SDS, 10% ACN at pH = 9.2; V = approx. 500 V/cm; approx. 3.3 uM amino acids; B. 200 mM boric acid, 80 mM SDS, 5 % acetonitrile (NDA/CN derivatization); ca. 3.3 uM of amino acids, 400 V/cm.

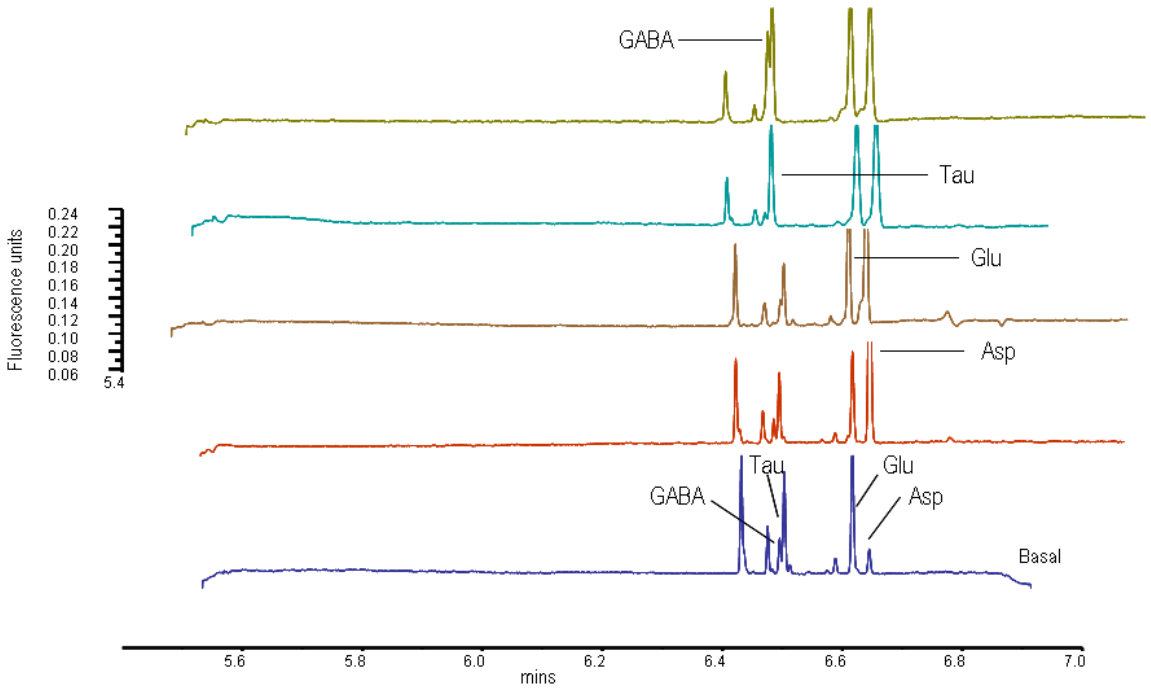


Figure 3.3: Peak identification in rat brain microdialysis sample (offline analysis). Each peak was identified by spiking the basal sample with 10μM of each analyte. Separation conditions are as follows: separation buffer composition is 20mM borate, 15mM SDS, 5% acetonitrile, pH 9.2, separation potential of 400V/cm, injection time 2 sec.

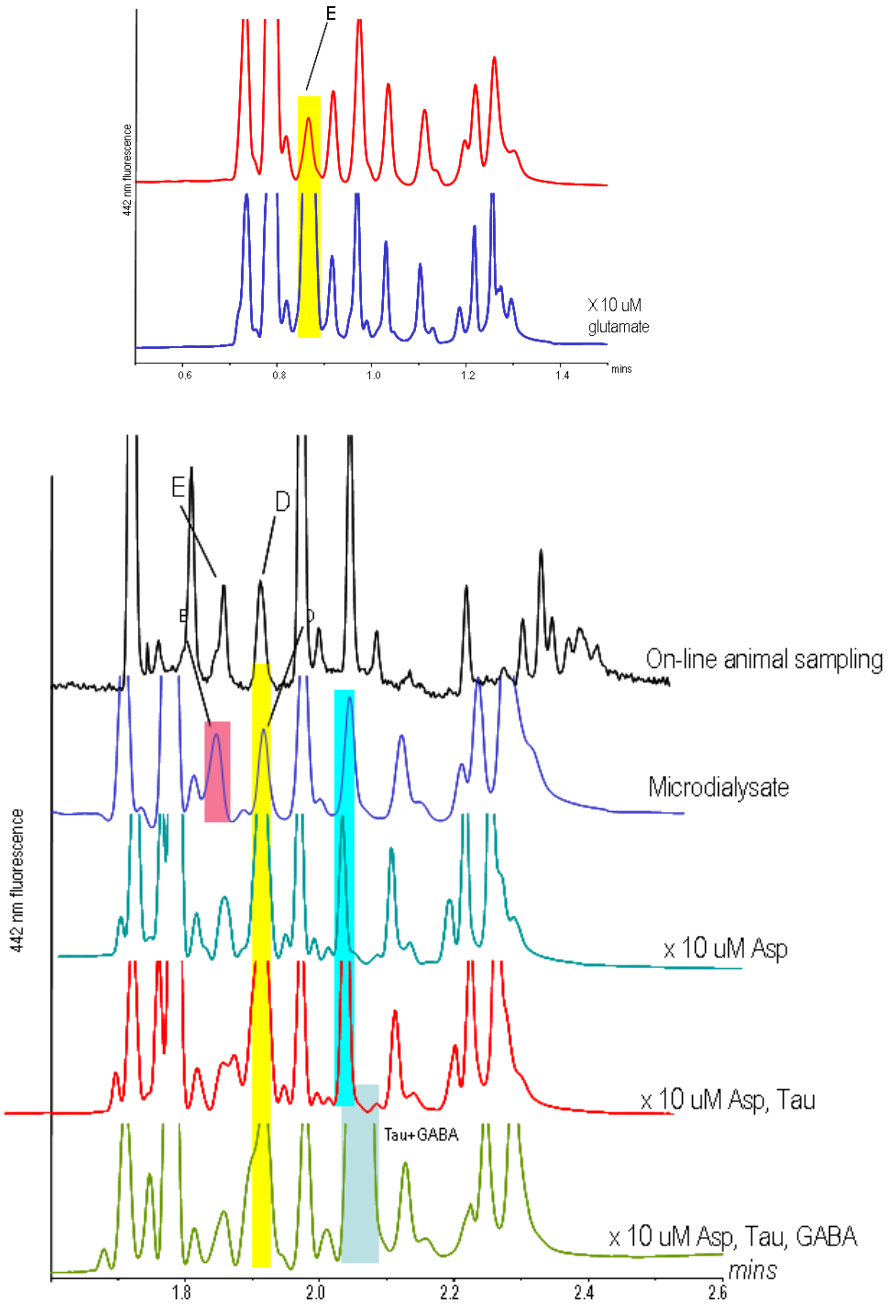


Figure 3.4: Peak identification in rat brain microdialysis sample (offline analysis). Each peak was identified by spiking the basal sample with 10µM of each analyte. Separation conditions are as follows: separation buffer composition is 200mM borate, 80mM SDS, 5% acetonitrile, pH 9.2, separation potential of 400V/cm, injection time 2 sec.

then spiked with glutamate (Glu), aspartate (Asp), GABA and taurine (Tau) for confirmation of peak identification.

3.3.2 High K^+ infusion

Infusion with high K^+ aCSF has been reported to depolarize neurons thereby increasing the concentration of excitatory amino acids in the extracellular space [26]. This effect has also been found to be immediate in previous studies [27,28]. Offline sample analysis was performed to show the application of this device to monitor *in vivo* concentration changes (**Fig 3.5**). Peak for Glu and Asp were identified based on spiking with standards and comparing their migration times. The data shows an increase in Glu after switching to high potassium aCSF.

3.3.3 Online microdialysis sampling and derivatization

The microchip was then modified for online monitoring. To integrate NDA/CN derivatization prior to the electrophoresis separation, additional channels for hydrodynamic flow were incorporated into the chip as shown in **Figure 3.6**. Briefly, NDA, NaCN and the microdialysis sample were introduced via hydrodynamic flow into the three inlets followed by mixing in the serpentine reaction channel. The channels in the mixer were optimized with wider dimensions (120μm) in order to avoid pressure buildup which could result in delamination or failure of the PDMS bilayer bond (see microchip fabrication).

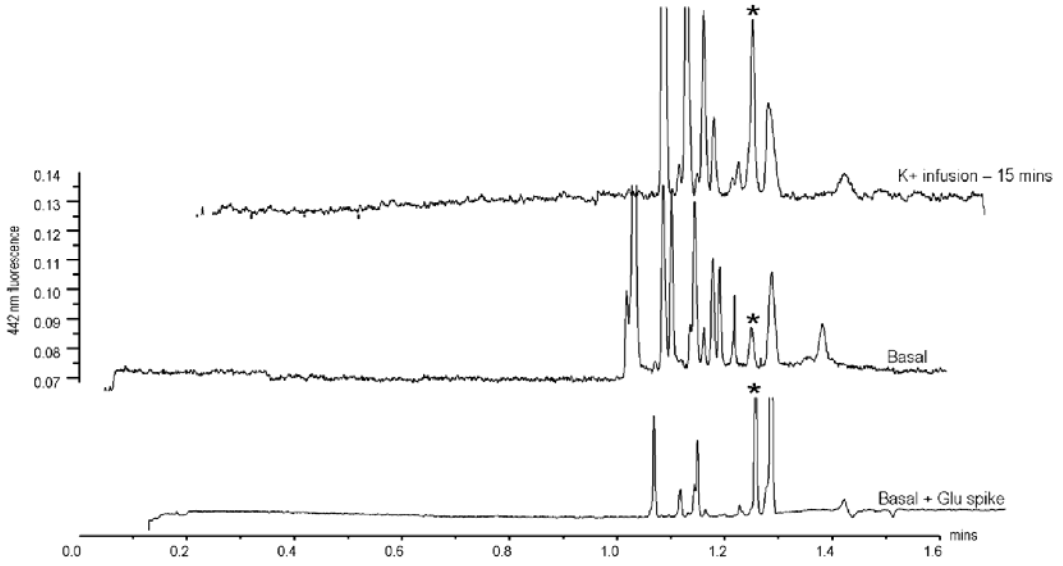


Figure 3.5: Offline analysis of rat brain microdialysis sample following perfusion of high K+ aCSF. High K+ leads to depolarization of the cell membrane resulting in the increase of glutamate in the brain extracellular space demonstrated here by the increase in height of the glutamate peak. Separation conditions are as follows: separation buffer composition is 20mM borate, 15mM SDS, 5% acetonitrile, pH 9.2, separation potential of 400V/cm, injection time 2 sec.

A 2mm inch diameter reservoir was constructed between the derivatization and the sampling channel that served as sample pool following derivatization. The reservoir also acted as a barrier between the pressure-driven flow from the derivatization channel and electrophoretic flow in the separation channel. This configuration made it possible to combine these two, which were optimized independently of each other without affecting injection or analysis. In addition, we were able to bypass challenging optimization experiments as generation of plugs from continuous stream requires a delicate balance between the pressure-driven flow and the electrophoretic flow in order to achieve reproducible gating and injection plugs.

The time scale of sample injection and separation with this approach was longer compared to gated injection because of manual intervention to remove sample which decreased temporal resolution. However, it also offered us the advantage of replenishing the separation buffer reservoirs periodically. For complex samples in gated injection scheme, closely eluting peaks can exhibit a loss in resolution as the buffer waste reservoir is filled up over time with sample. Eventually this can change the run buffer composition including buffer capacity, pH and ionic strength [29].

Studies were performed on derivatization channels to optimize the flow rate and mixing. To investigate the flow characteristics of the channel, three food dyes were used to represent microdialysis, NDA and CN^- flow from syringe pump (**Fig 3.6**). Micrographs were taken at various positions within the reaction channels. Three separate and uniform flow streams were observed at 1μL/min each (total 3μL/min)

for individual inlet at the Y-intersection. It was critical to ensure equal delivery of all the three components on chip for subsequent mixing and efficient reaction.

After establishing consistent flows from all three inlets, mixing was evaluated by visual inspection of color homogeneity within reaction channels. Previous reports have described alternative approaches to perform mixing in microchannels using various channel designs and geometries reviewed in a recent article by Mansur *et al.* [30]. In our design, a few turns were included in the mixer for simplicity. In the beginning of the derivatization channel, three separate streams of dye were observed. This fluidic behavior is common for microchannels due to the low Reynolds number generated from the small dimensions. However, it was observed that the turns induced some degree of turbulence in the channels which resulted in uniform mixing after the fluids passed through the first few turns (**Fig 3.6**). Further turbulence and mixing can be induced by introducing more turns but in our setup adequate mixing was observed to proceed with *in vivo* experiments. Compared to most previous reports, we were able to develop a uncomplicated system that exhibited mixing at low flow rates 0.5-1uL/min (for each inlet) from the syringe pump; however, quantitative analysis on mixing efficiency was not performed.

3.3.4 Online derivatization and sampling with change in concentration

In the first report of an online microdialysis microchip electrophoresis systems, Huynh *et al.* demonstrated *in vitro* change in concentration of fluorescein from 50mM to 100mM with lag time (time taken by the analyte to travel from the

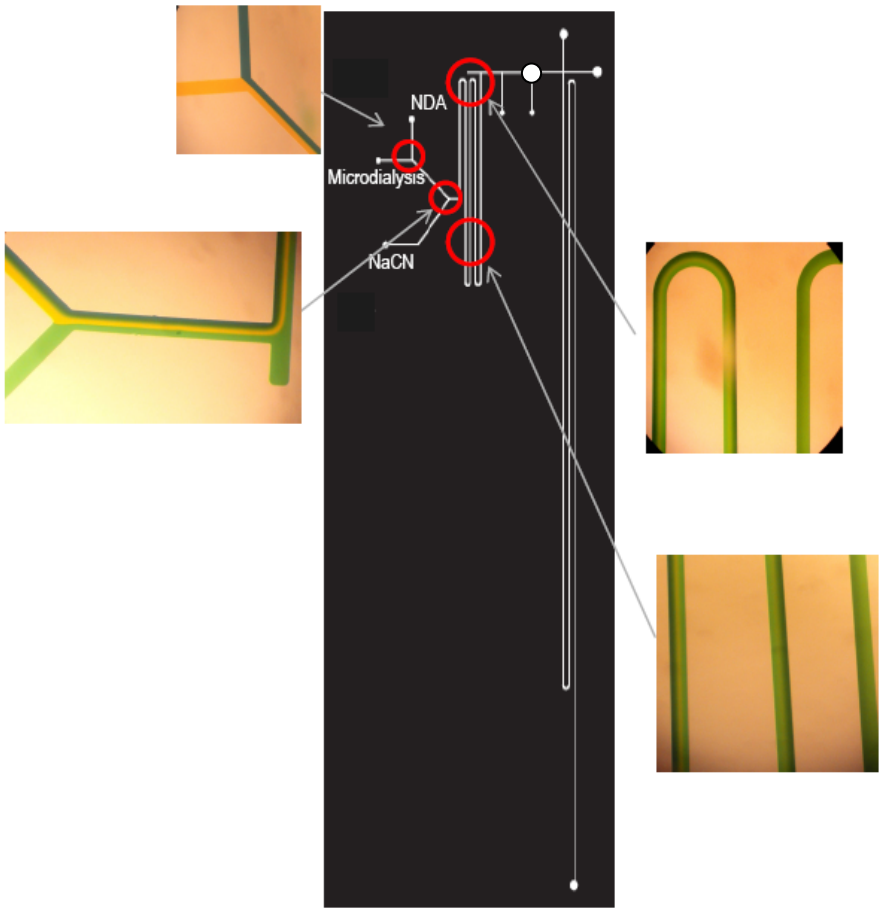


Figure 3.6: Demonstration of mixing in the serpentine derivatization channels. The micrographs in the panels reveal the flow pattern at various points of the derivatization channel. Compared to the left micrographs (segregated flow pattern due to low Reynolds number in microchannel), the right micrographs reveal a uniform color without any visual evidence of separate flow patterns.

probe to injection) of approximately 6.5mins. Sandlin *et al*. reported being able to monitor a step change in Glu concentrations from 0 to 100uM at various flow rates and their effect on the response time of 2 - 4mins.

In our chip the reaction and separation components were optimized separately. As with the previous online systems, we were interested in determining the response time of the system to a change in amino acid concentration via a microdialysis probe. Such an experiment was thought be more relevant than demonstrating concentration change with fluorescein or a single analyte in terms of *in vivo* applications with complex biological samples. Online sampling and derivatization was investigated *in vitro* for six amino acids (Glu, Asp, Gly, Tau, Arg, Trp) with 10X concentration change from 500nM to 5mM. The microdialysis sample was injected continuously and the change in response versus time is shown in **Figure 3.7**. The lag time (time between when the sample was introduced into the probe and when the change was detected) was approximately 25 minutes. There are few factors that contributed to this relatively long lag time, including dead volume from tubing length, flow rate and manual intervention during injections that increased analysis time for each run. The response time was not measured due to the time gap between the sample injections. Further studies will consider modifications needed to perform high throughput studies on this chip system through incorporation of a gated injection scheme.

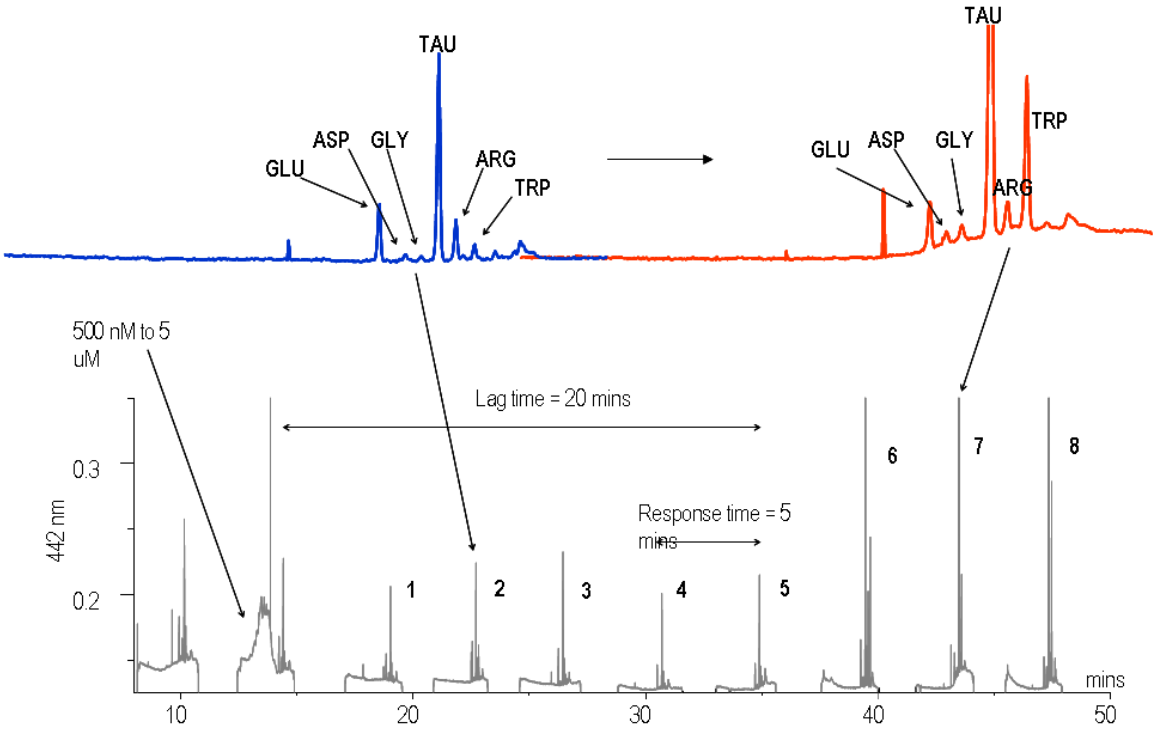


Figure 3.7: *In vitro* concentration change study with online derivatization of amino acids with NDA/CN^-. The concentration of amino acids was increased from 500nM to 5 µM at t = 15 mins. The lag time and the response times are indicated in the figure. Separation conditions are as follows: separation buffer composition is 20mM borate, 15mM SDS, 5% acetonitrile, pH 9.2, separation potential of 400V/cm, injection time 2 sec.

3.3.5 *In vivo* monitoring

Fluorescein has been used extensively as a marker for passive permeability at the blood brain barrier. To evaluate the capability of the device for *in vivo* experiments with fluorescein were performed. A rat was injected with a bolus dose of 0.5mg of fluorescein via femoral vein; followed by microdialysis sampling from the striatum and online analysis on chip. Fluorescein appeared in the microdialysate within five minutes after injection (**Fig 3.8**). Over a period of 90 mins, there was a decrease in fluorescein peak height suggesting evidence of clearance. It has been reported in the literature that fluorescein is cleared from the body via renal excretion, metabolism in liver and excretion via feces. Although no rigorous pharmacokinetic treatment was performed on the data, it was qualitatively demonstrated that this device can theoretically be used for quantitative assessment of changing analyte concentration in an *in vivo* setup. The time scale of clearance obtained in these studies is in agreement with previous publications [31,32].

To demonstrate an online *in vivo* application of microdialysis coupled to a microchip device, online microdialysis sampling was performed in the rat brain striatum followed by derivatization and analysis on the chip device. A method for separation of Glu and Asp in the microdialysis sample was developed earlier using offline sampling. Fluorescein was also incorporated into the separation as an exogenous compound as a marker for blood brain barrier (BBB) permeability.

A representative electropherogram from such a study is shown in **Figure 3.9**. After an initial equilibration period in the first two runs that was demonstrated by low

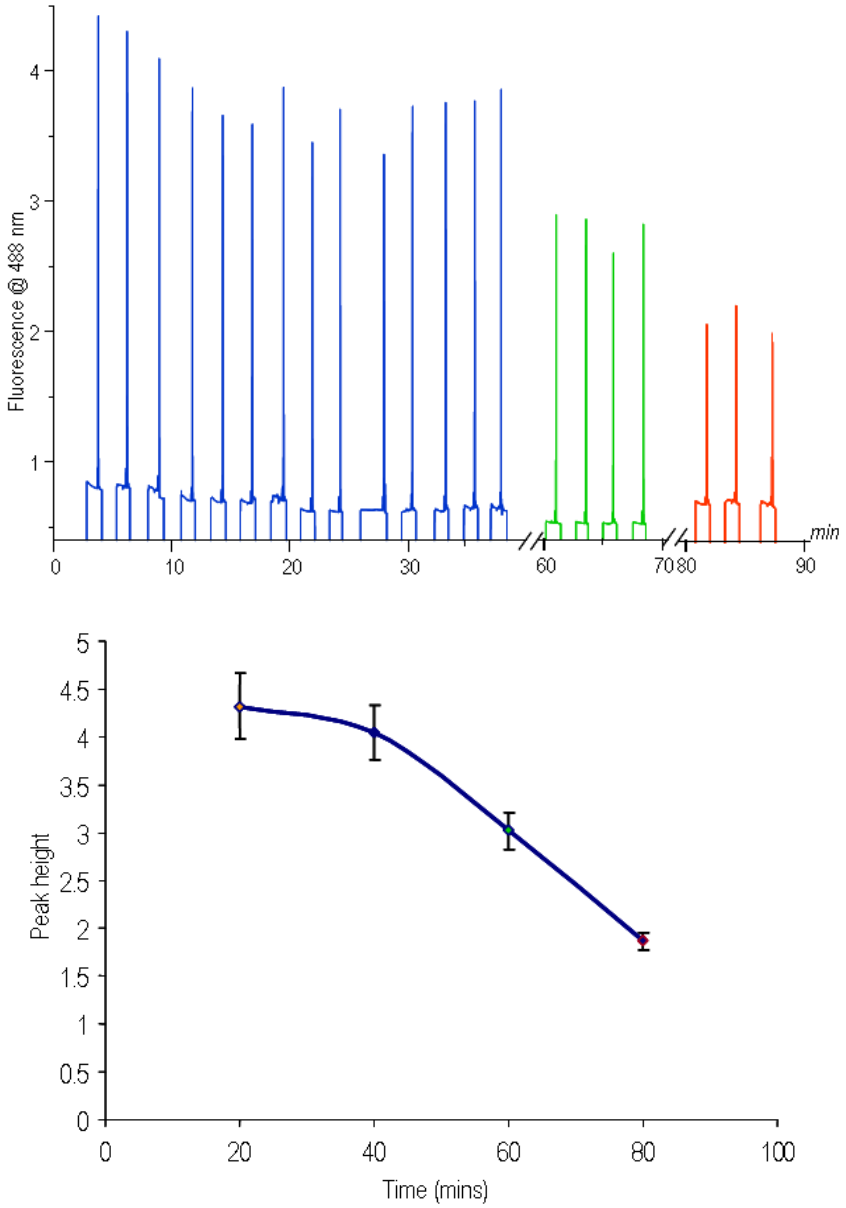


Figure 3.8: Online *in vivo* analysis of fluorescein from rat brain microdialysate. The fluorescein was administered via IV bolus dose in the femoral vein of SD rat. The bottom figure shows the graphical representation of the continuous set of injections shown at the top panel. Electrophoresis conditions are same as Figure 3.7.

peak height in Figure 3.9, , we were able to obtain reproducible runs (RSD 8.5% for Glu, 4.6% for Asp, N = 4 injections). The migration order is well in agreement with offline analysis and standard runs based on their migration time. A major concern for PDMS devices is adsorption of analytes and buffer components to the surface of the chip resulting in gradually deteriorating separation characteristics. However, we were able to run these chips for approximately 2 hours (N = 5 chips) without appreciable loss of resolution or signal. Such observations were probably a consequence of the ability to replenish buffer on this chip device at regular intervals without stopping the flow from the syringe pump and the microdialysis probe. In a gated injection approach, changing the buffer requires stopping the voltage which can result in flooding of separation channel with sample creating carry-over problems during subsequent analysis. These series of injections successfully demonstrated that the system is capable of monitoring the appearance of fluorescein in the brain as well as the amino acids of interest.

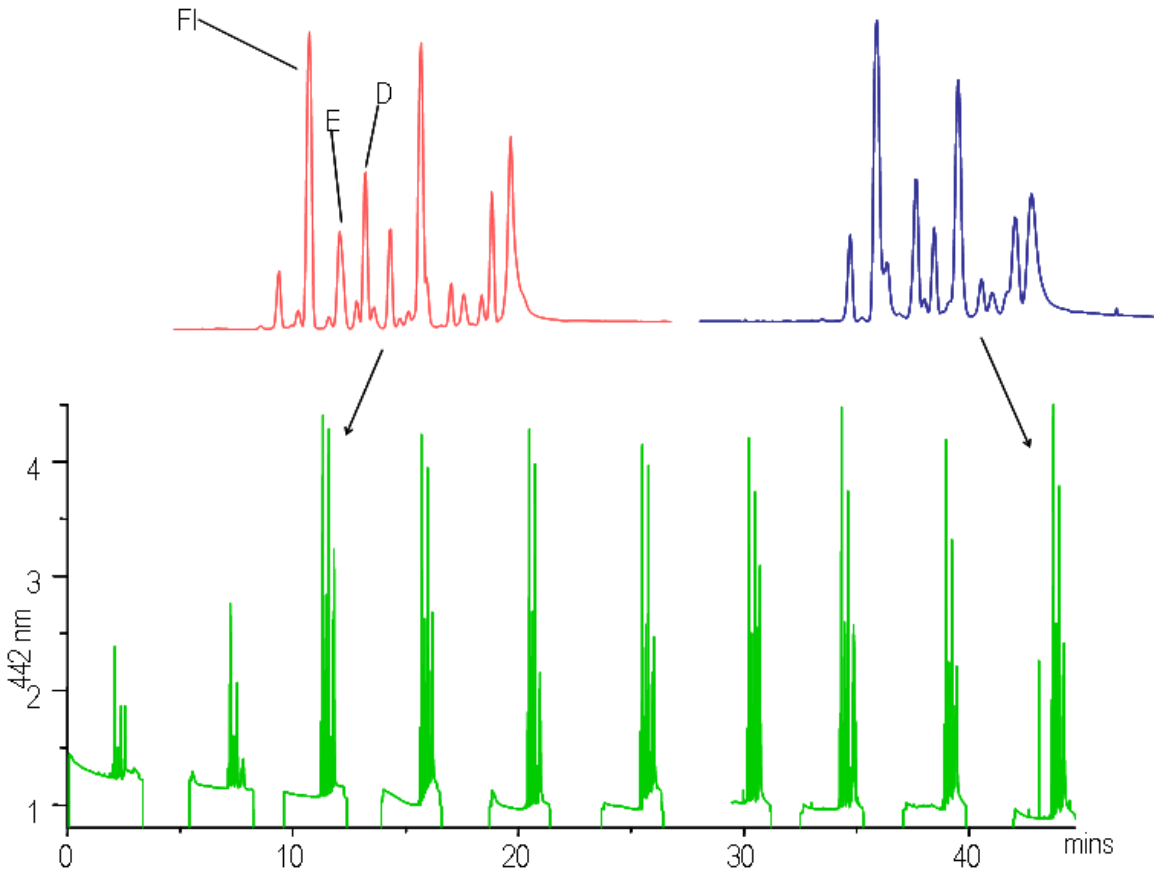


Figure 3.9: *In vivo* continuous monitoring with online derivatization of amino acid neurotransmitters (glu and asp) with fluorescein as the exogenous compound. Separation conditions same as Figure 3.7.

3.4 Conclusion

This work demonstrates the applicability of PDMS-based microchip devices for online *in vivo* studies. Previous reports of such devices included glass microchips and PDMS valving system, both of which can be challenging to implement. Also, microchips containing PDMS based valving suffer from the lack of ability to be scaled down to true miniaturized devices as multiple gas tanks are required to activate the valves. This study aimed to perform online *in vivo* sampling utilizing a simple design and fabrication technique that were easily adaptable for different kinds of experiments and labs. Optimization studies can be carried out separately and then combined in the same integrated device. With precise integration and automation for sample and buffer fill/removal, such a device can potentially be scaled down to a true miniaturized device. All of the fluidics inside this device are dictated by the application of voltage and not by hydrodynamic to electrophoretic flow balance as is the case with a gated injection. Also, this chip can be modified to perform high temporal resolution studies which will be the scope of a future publication. The ultimate goal is to apply this device to investigate changes in concentrations of amino acid neurotransmitters and blood brain permeability in neurological disorders and stroke.

3.5 References

[1] D.K. Binder, M.C. Papadopoulos, P.M. Haggie, A.S. Verkman, In vivo measurement of brain extracellular space diffusion by cortical surface photobleaching, J. Neurosci. 24 (2004) 8049-8056.

[2] R.M. Barbosa, C.F. Lourenco, R.M. Santos, F. Pomerleau, P. Huettl, G.A. Gerhardt, J. Laranjinha, In vivo real-time measurement of nitric oxide in anesthetized rat brain, Methods Enzymol. 441 (2008) 351-367.

[3] K. Hyland, Clinical utility of monoamine neurotransmitter metabolite analysis in cerebrospinal fluid, Clin. Chem. (Washington, DC, U. S.) 54 (2008) 633-641.

[4] A. Morales-Villagran, C. Sandoval-Salazar, L. Medina-Ceja, An analytical flow injection system to measure glutamate in microdialysis samples based on an enzymatic reaction and electrochemical detection, Neurochem. Res. 33 (2008) 1592-1598.

[5] T. Nishimura, A. Kubosaki, Y. Ito, A.L. Notkins, Disturbances in the secretion of neurotransmitters in IA-2/IA-2beta null mice: Changes in behavior, learning and lifespan, Neuroscience (Amsterdam, Neth.) 159 (2009) 427-437.

[6] S. Parrot, L. Bert, L. Mouly-Badina, V. Sauvinet, J. Colussi-Mas, L. Lambas-Senas, F. Robert, J.-P. Bouilloux, M.-F. Suaud-Chagny, L. Denoroy, B. Renaud, Microdialysis monitoring of catecholamines and excitatory amino acids in the rat and mouse brain: Recent developments based on capillary

electrophoresis with laser-induced fluorescence detection-A mini-review, Cell. Mol. Neurobiol. 23 (2003) 793-804.

[7] C. Hoecht, J.A.W. Opezzo, C.A. Taira, Microdialysis in drug discovery, Curr. Drug Discovery Technol. 1 (2004) 269-285.

[8] E.C.M. de Lange, M.B. Hesselink, M. Danhof, A.G. Boer, D.D. Breimer, The use of intracerebral microdialysis to determine changes in blood-brain barrier transport characteristics, Pharm. Res. 12 (1995) 129-33.

[9] W.F. Elmquist, R.J. Sawchuk, Application of microdialysis in pharmacokinetic studies, Pharm. Res. 14 (1997) 267-288.

[10] E.C.M. de Lange, B.A.G. de Boer, D.D. Breimer, Microdialysis for pharmacokinetic analysis of drug transport to the brain, Adv. Drug Delivery Rev. 36 (1999) 211-227.

[11] G. Jin, Q. Cheng, J. Feng, F. Li, On-line microdialysis coupled to analytical systems, J. Chromatogr. Sci. 46 (2008) 276-287.

[12] K.N. Schultz, R.T. Kennedy, Time-resolved microdialysis for in vivo neurochemical measurements and other applications, Annu. Rev. Anal. Chem. 1 (2008) 627-661.

[13] S. Attiya, A.B. Jemere, T. Tang, G. Fitzpatrick, K. Seiler, N. Chiem, D.J. Harrison, Design of an interface to allow microfluidic electrophoresis chips to drink from the fire hose of the external environment, Electrophoresis 22 (2001) 318-27.

[14] Y.-H. Lin, G.-B. Lee, C.-W. Li, G.-R. Huang, S.-H. Chen, Flow-through sampling for electrophoresis-based microfluidic chips using hydrodynamic pumping, J. Chromatogr., A 937 (2001) 115-125.

[15] J.A. Stenken, Methods and issues in microdialysis calibration, Anal. Chim. Acta 379 (1999) 337-357.

[16] N. Patrito, J.M. McLachlan, S.N. Faria, J. Chan, P.R. Norton, A novel metal-protected plasma treatment for the robust bonding of polydimethylsiloxane, Lab Chip 7 (2007) 1813-1818.

[17] B.H. Huynh, B.A. Fogarty, R.S. Martin, S.M. Lunte, On-Line Coupling of Microdialysis Sampling with Microchip-Based Capillary Electrophoresis, Anal. Chem. 76 (2004) 6440-6447.

[18] Z.D. Sandlin, M. Shou, J.G. Shackman, R.T. Kennedy, Microfluidic electrophoresis chip coupled to microdialysis for in vivo monitoring of amino acid neurotransmitters, Anal. Chem. 77 (2005) 7702-7708.

[19] L.C. Mecker, R.S. Martin, Integration of Microdialysis Sampling and Microchip Electrophoresis with Electrochemical Detection, Anal. Chem. (Washington, DC, U. S.) 80 (2008) 9257-9264.

[20] A. Iles, A. Oki, N. Pamme, Bonding of soda-lime glass microchips at low temperature, Microfluid. Nanofluid. 3 (2007) 119-122.

[21] Y. Akiyama, K. Morishima, A. Kogi, Y. Kikutani, M. Tokeshi, T. Kitamori, Rapid bonding of Pyrex glass microchips, Electrophoresis 28 (2007) 994-1001.

[22] P.B. Allen, D.T. Chiu, Calcium-assisted glass-to-glass bonding for fabrication of glass microfluidic devices, Anal. Chem. 80 (2008) 7153-7157.

[23] B.H. Huynh, B.A. Fogarty, R.S. Martin, S.M. Lunte, On-line coupling of microdialysis sampling with microchip-based capillary electrophoresis, Anal Chem 76 (2004) 6440-7.

[24] C.-X. Zhang, A. Manz, Narrow sample channel injectors for capillary electrophoresis on microchips, Anal. Chem. 73 (2001) 2656-2662.

[25] G.T. Roman, K. McDaniel, C.T. Culbertson, High efficiency micellar electrokinetic chromatography of hydrophobic analytes on poly(dimethylsiloxane) microchips, Analyst 131 (2006) 194-201.

[26] L. Zheng, D.A. Godfrey, H.J. Waller, T.G. Godfrey, K. Chen, Y. Sun, Effects of high-potassium-induced depolarization on amino acid chemistry of the dorsal cochlear nucleus in rat brain slices, Neurochem. Res. 25 (2000) 823-835.

[27] S.Y. Zhou, H. Zuo, J.F. Stobaugh, C.E. Lunte, S.M. Lunte, Continuous *in vivo* monitoring of amino acid neurotransmitters by microdialysis sampling with online derivatization and capillary electrophoresis separation, Anal. Chem. 67 (1995) 594-9.

[28] M.T. Bowser, R.T. Kennedy, In vivo monitoring of amine neurotransmitters using microdialysis with on-line capillary electrophoresis, Electrophoresis 22 (2001) 3668-3676.

[29] J.P. Landers, Introduction to capillary electrophoresis, Handb. Capillary Microchip Electrophor. Assoc. Microtech. (3rd Ed.) (2008) 3-74.

[30] E.A. Mansur, M. Ye, Y. Wang, Y. Dai, A state-of-the-art review of mixing in microfluidic mixers, Chin. J. Chem. Eng. 16 (2008) 503-516.

[31] A.G. Palestine, R.F. Brubaker, Pharmacokinetics of fluorescein in the vitreous, Invest. Ophthalmol. Visual Sci. 21 (1981) 542-9.

[32] H. Sun, D.R. Johnson, R.A. Finch, A.C. Sartorelli, D.W. Miller, W.F. Elmquist, Transport of fluorescein in MDCKII-MRP1 transfected cells and mrp1-knockout mice, Biochem. Biophys. Res. Commun. 284 (2001) 863-869.

Chapter 4

Development of a PDMS based microdialysis microchip device for continuous online monitoring with high temporal resolution.

4.1 Introduction

Microdialysis is a well characterized sampling technique that can be utilized to continuously recover analytes *in vivo*. Cerebral microdialysis is an important tool to study neurochemistry of the brain [1]. An important issue associated with performing chemical investigations in the CNS is the time scale of these events. Chemical signaling in the brain covers a wide dynamic range **(Fig 4.1)**. The process of neurotransmission involves substance being released by one neuron and acting rapidly, briefly, and at short range on the membrane of an adjacent neuron, causing excitation or inhibition [2]. In order to measure fast dynamic changes in concentration of neurochemically active species possibly triggered by disease state or by a therapeutic drug, the temporal resolution of analysis is an important consideration.

There has been considerable interest in the literature in coupling microdialysis sampling with capillary electrophoresis (CE) to generate integrated online systems capable of performing *in vivo* analysis with high temporal resolution. Temporal resolution of an analytical system can be defined as the shortest interval of time that is necessary between repeated analyses. The limiting factors that affect temporal resolution in online systems can be scrutinized in terms of properties of the microdialysis probe as well as capabilities of the analysis method. For monitoring compounds with high endogenous concentration (glutamate, aspartate) in an online separation based system, the temporal resolution is dictated by the analysis time.

One of the major concerns associated with microdialysis is analyte recovery (i.e. dialysate concentration compared to the external concentration). In order to

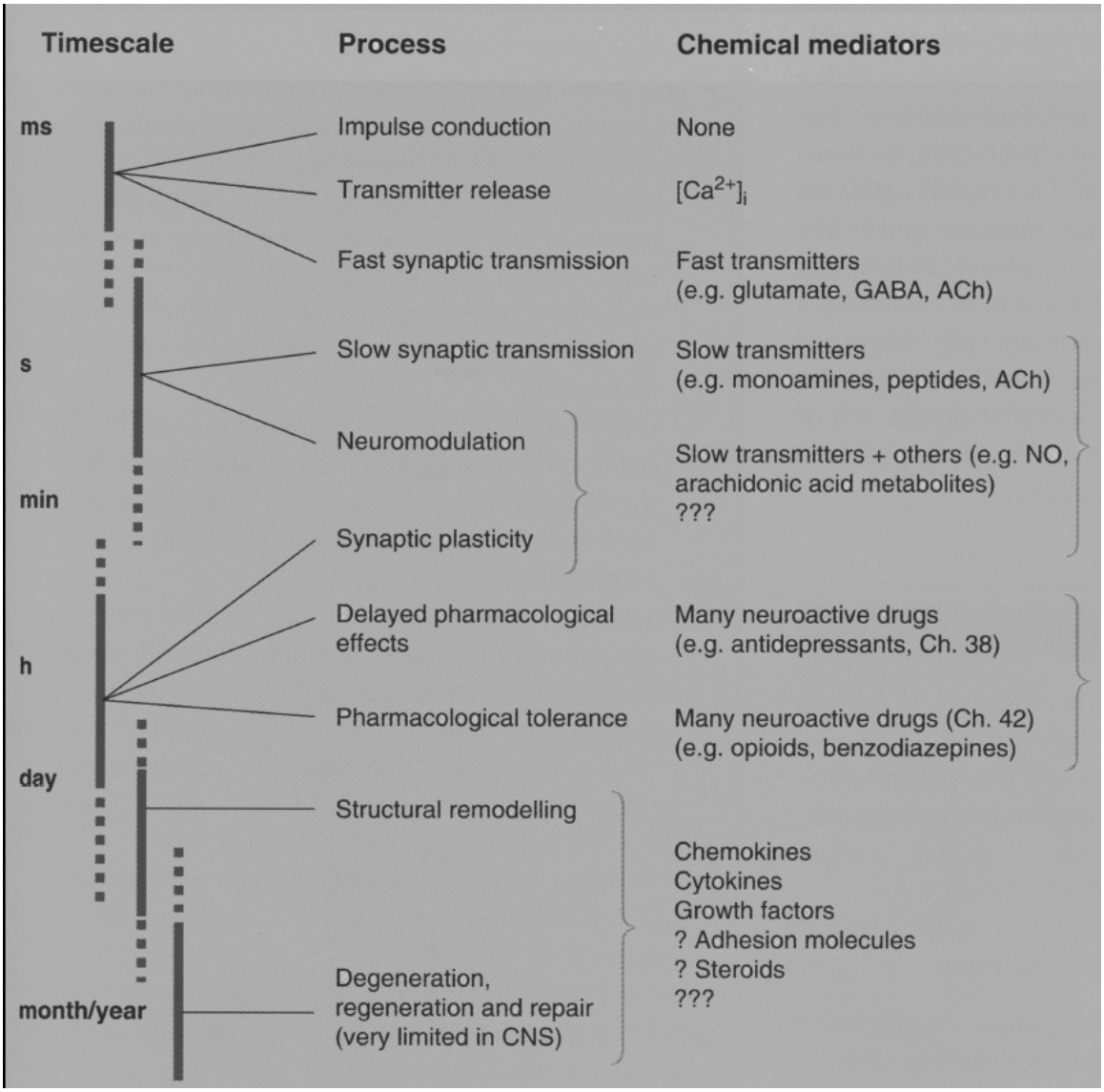


Fig 4.1: Timescale of chemical signaling in the nervous system [13].

precisely measure rapid concentration changes occurring in the extracellular space, the analyte concentration in the perfusate must be in equilibrium with the external concentration. However, since microdialysis is a dynamic method where perfusate is constantly being removed, such equilibrium is not reached. The concentration difference between the analyte recovered in the perfusate and the analyte in the extracellular space is dependent on perfusion flow rate, probe size, molecular weight (MW) cutoff of the membrane, temperature, analyte properties and composition of the external matrix [3]. The relative recovery of analyte increases exponentially with decreased perfusion rate. A high precision syringe pump is necessary for microdialysis applications to ensure changes in recovery are due to changes in analyte concentration in the brain and not due to alterations in concentration gradient produced by flow rate changes from the pump [4]. Increasing membrane size (length or diameter), while increasing recovery, will decrease spatial resolution and induce more tissue damage. Therefore, the consistency of probe recovery with alterations in brain chemistry is necessary to perform meaningful high temporal resolution analysis.

The analysis system needs to possess certain features to perform high temporal resolution studies. First, the detection scheme should be sensitive enough to detect analytes of interest in the microdialysis sample. Second, the system should be able to perform injections and separations as frequently as possible. The rate of perfusion through the microdialysis probe and the sensitivity of the detection system are the interrelated factors that affect the value of temporal resolution. For instance, low flow rates (low μL to nL) through microdialysis probe leads to higher mass

recovery of analytes. However, these low flow rates also yield less volume of sample in a given period of time. In some cases, the analysis system must wait for the required length of time to obtain enough sample for injection. On the other hand, high flow rates can produce required requisite sample volumes faster but will be more dilute due to lower relative recovery rates compared to the previous case. In such cases, the sensitivity of the detection system needs to be high so that it is capable of detecting low concentration analytes in the microdialysate. To mitigate detection issues, fluorescence is the most popular detection mode for online systems due to its high sensitivity.

Microchip electrophoresis is an attractive platform for online pairing of microdialysis with electrophoresis separations because of its small sample volume requirements (nL), small footprint, ability to perform fast, highly efficient separations and easy integration of fluorescence detection. Also, chips can be custom-designed based on application and additional sample prep steps (such as derivatization reaction for fluorescence detection) can be built in. Our group has previously reported flow through devices with short separation channels for analysis of amino acids and peptides as well as serpentine channels for the detection of amino acid neurotransmitters *in vivo* brain microdialysate samples (chapter 3).

The present study is targeted towards the development of a microchip device with high temporal resolution capabilities for monitoring neuroactive amino acids. These chips incorporated a precolumn derivatization step with NDA and CN^-. In this chapter, flow-through sampling techniques (includes chip fill up, sample flow,

derivatization, gated injection and separation) were systematically optimized on chip [5] to render the chip more practical for in vivo analysis. The chip device was fabricated from poly(dimethylsiloxane) (PDMS) and incorporated straightforward method for buffer refill, equilibration and bubble removal without disturbing the microdialysis flow. The chip was characterized in terms of reagent mixing with microdialysate by hydrodynamic flow (for online derivatization) and high temporal resolution analyses were performed with amino acid standards. Online concentration change experiments were also performed to demonstrate the suitability of the chip for *in vivo* measurements.

4.2 Materials and Methods

Chemicals and materials: The following chemicals and materials were used: boric acid, SDS, acetonitrile, disodium fluorescein, amino acid standards, sodium cyanide, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate and disodium phosphate were purchased from Sigma (St. Louis, MO). Naphthalene 2,3 dicarboxaldehyde was purchased from Molecular Probes (Eugene, OR). All aqueous buffers and solutions were prepared using Milli-Q water and filtered using .22 um Cameo Teflon syringe cartridges from Osmonics (Minnetonka, MN). Brain probes (CMA 12) obtained from CMA (North Chelmsford, MA).

Chip Fabrication: All microchip devices were fabricated from PDMS obtained from Ellsworth Adhesive (Minneapolis, MN). Utilizing standard photolithographic

techniques, channel molds were prepared on silicon wafers (Telic Co.) using SU8-10 photoresist (Newton, MA) and a high resolution negative transparency (IGI, Minneapolis, MN) containing the chip design. Then, approximately a 5 mm thick layer of a mixture of PDMS and curing agent (10.5:1.5) was poured onto the channel mold followed by curing for 25-30 minutes at 90^{o}C in a convection oven. Following this, the reservoirs were produced with biopsy punches (4 mm diameter) and inlets for hydrodynamic flow were produced using a 20 gauge luer stub. Another thin PDMS layer (approximately 1 mm) was semi cured in the oven for 15-18 minutes at 90^{o}C until it was tacky to touch. Then the channel layer was conformally contacted with the semi cured layer and cured in the oven at 85^{o}C overnight for semi-permanent contact. The chip was interfaced with hydrodynamic flow using steel pins that were embedded in the flow inlets. The other end of the pin was connected to the outlet of a pump or that of a microdialysis probe via FEP tubing.

In vitro experiments: For these studies, a brain microdialysis probe (4mm membrane length, CMA, Sweden) was placed in the vial containing the amino acids of interest. The inlet of the probe was connected to 1mL syringe pump containing water via FEP tubing, and outlet of the probe was connected to microfluidic device.

Derivatization reactions: For offline analysis, amino acid standards or microdialysates were derivatized with equal parts by volume of 7 mM NDA (dissolved in acetonitrile) and 10 mM NaCN (dissolved in 50 mM boric acid buffer, pH 9.2). For online derivatization, 7 mM NDA (dissolved in 1:1 acetonitrile:H_2O)

and 10mM NaCN (50 mM boric acid buffer, pH 9.2 and 5% acetonitrile) was used. The flow rate for each inlet was optimized at 1μL/min (total flow = 3 x 1μL/min).

Chip operation: For offline studies, a voltage of 2.7 KV was applied at the sample reservoir (SR), -5KV was applied at the sample waste (SW) with zero voltage at buffer reservoir (BR). For separation, the same voltages were applied except that 5 KV was applied at BR **(Fig 4.2A)**. For online analysis, voltages of +5KV and -5KV were applied to the side channel and end of separation channel respectively. For sample injection, the separation voltage was stopped during the period of injection (0.5s).

LIF detection system: The detection system consisted of an epi-fluorescent microscope (Nikon Ti series). The chip device was placed on a glass microscope slide (Gold Seal cover glass #3334) and positioned on the microscope stage with clamps. Fluorescent light (mercury lamp source connected to the microscope by fiber optic cable) was focused at a point of the separation channel usually at a distance of 0.2 cm from the waste reservoir through a 40X objective. Filter cubes housing the appropriate excitation/emission filters and dichroic mirrors were installed inside the carousel. Fluorescein studies were performed using FITC filter cube obtained from Nikon. For analysis of NDA derivatives, a custom built cube was purchased from Chroma (25 mm diameter z442/10X clean-up filter, 25 mm diameter 510hq/50m bandpass emission filter and 25.5x36mm laser dichroic filter and transmit hq510/50m). A photomultiplier tube (Hamamatsu) was aligned to the side port of the microscope to collect the emission from the sample. Data was collected in a computer

that was coupled to the PMT by means of a preamplifier (Standford Research Systems) and data acquisition (Chromgraph Interface) module. Electropherograms were analyzed using Origin software.

4.3 Results and discussions

4.3.1 PDMS-based microchip device.

The chip devices used in this study were fabricated from PDMS. Most reports for online microdialysis microchip setups have utilized glass devices for analysis [6,7]. However, glass microchip fabrication can be quite challenging due to problems associated with thermal bonding of the glass plates containing etched channels. Other bonding methods (Calcium-assisted bonding) have proved to be quite unreliable in our lab setting [8]. Although PDMS suffers from drawbacks compared to glass for electrophoresis separation in terms of hydrophobic analyte adsorption and surface modification leading to slow deterioration of EOF, there have been an extensive number of publications that use PDMS because of ease of construction and reproducibility. The present study makes use of simple and standard fabrication steps to construct the PDMS devices. Also, a trouble-free, robust and reproducible interface using steel tubing was used for coupling hydrodynamic flow to the chip. This did not require expensive nanoports (Upchurch) which are generally thought to be unsuitable for PDMS based chips due the epoxy-based adhesive that must be employed to mount them.

4.3.2 Optimization of gated injection in a serpentine chip (offline analysis).

For the development of a microdialysis microchip electrophoresis system for high temporal resolution, rapid injection and separation of samples on chip device is essential. Electrokinetic gating is the easiest method to implement ultrafast injections that does not require intricate fabrication steps (e.g. soft lithographic valving). Gated injection in serpentine microchips has been employed in a few published reports [6,9,10].

One such device, based on a report by Sandlin *et al.*, is shown in **Figure 4.2A** [6]. In our chip based on this design, a voltage was applied to create EOF which would push the sample from the top reservoir and simulate the hydrodynamic course for flow-through injection. Offline analysis with fluorescein was performed on this chip (electropherogram not shown). Acceptable RSD values for peak heights at 4.2% (N = 7 inections) were obtained for fluorescein. Analysis of NDA derivatized glutamate, aspartate and OPS (internal standard) also exhibited good precision; peak height RSD values for glutamate, aspartate and OPS were calculated to be 2.98%, 3.61% and 5.90% based on 5 injections **(Fig 4.2B)**.

4.3.3 Optimization of mixing in reaction channels.

Huynh *et al.* reported the development of flow-through sampling with on-chip derivatization [11]. The first goal of this study was to develop an efficient precolumn mixer for derivatization reagents and analytes prior to injection and separation on

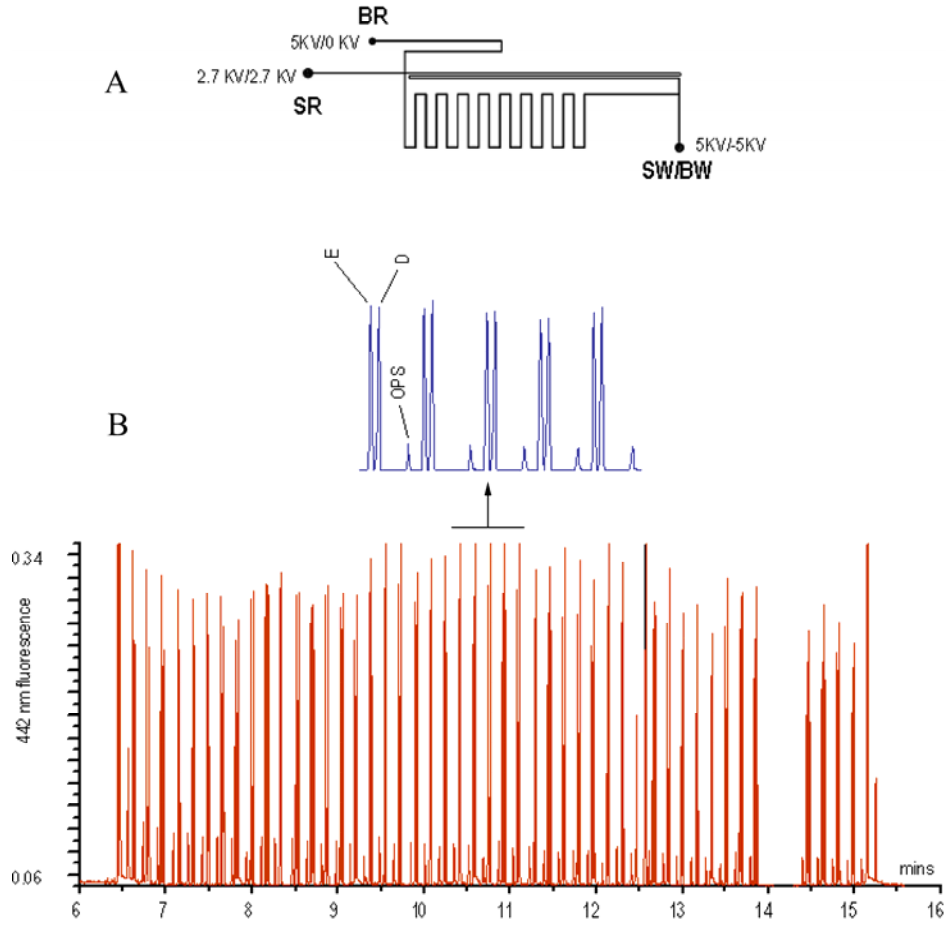


Fig 4.2: A. Chip design and injection for offline simulated flow-through studies [6]. Sample was injected from SR electrokinetically with SR at 2.7KV, BR at 0KV and SW/BW at -5KV. Separation was performed with SR at 2.7KV, BR at 5KV and SW/BW at -5KV. B. Separation of 7μM glutamate, aspartate and ortho-phosphoserine (OPS, an internal standard) with temporal resolution of 15s. The continuous set of electropherograms in the bottom panel shows a set of continuous injections and separations. Separation buffer: 20mM borate, 10mM SDS, 10% acetonitrile, pH 9.2.

chip. Chiefly, two mixing channel designs were evaluated. These were designs based on reports by Ismagilov *et al.* (called Design 1 in this paper) [12] and dolomite micromixer chip from Dolomite Microfluidics (Design 2).

First, the flow rate of the analyte and reagent delivery into the chip was optimized for each design. Since efficient derivatization would require the delivery of all three reagents at approximately equal volumes, the stream width of the individual flows were assessed with food color to get an idea of uniformity of liquid delivery. In design 2, (**Fig 4.3**-panel A) uniform delivery was accomplished at 0.5 μL/min for each flow. Micrographs taken at a few other areas (**Fig 4.3**-panels B and C) further down the reaction channel showed evidence of mixing by splitting up and reuniting the streams. This design was further tested for NDA/CN^- reaction by observing the actual fluorescence generated by the reaction. Bright fluorescence was observed with standard solution of aspartate (1mM) indicative of its online reaction with NDA/CN^- (**Fig 4.4** – panels B and C).

Parallel studies were performed with design 1 (**Fig 4.3**D), where the channel joins the flow stream later and was intended to simulate addition of NDA following mixing of amino acids and NaCN. However, with this design we were not able to generate reproducible flow patterns after several trials at flow rates 0.5-1 μL/min. Also superior mixing was demonstrated by design 2. Hence further studies were carried out with design 2 for flow through sampling and analysis.

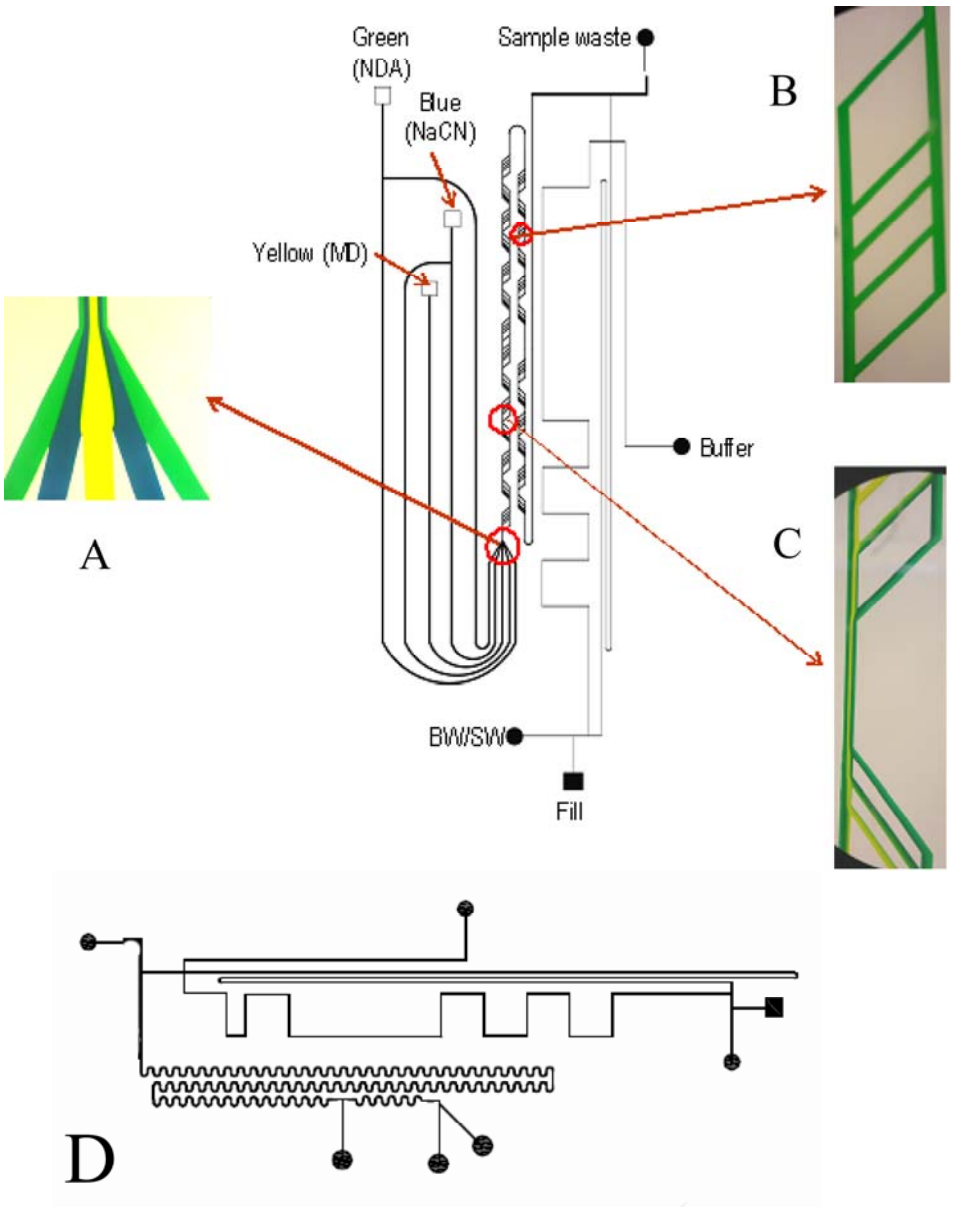


Figure 4.3: A-C: Design and characterization of dolomite micromixer-based chip. The micrographs with colored dyes show the flow and mixing pattern in various locations of the derivatization channel. Panel C shows segregated flow streams whereas D demonstrates a uniform color pattern indicating mixing. D: Microchip design based on reference [12] .

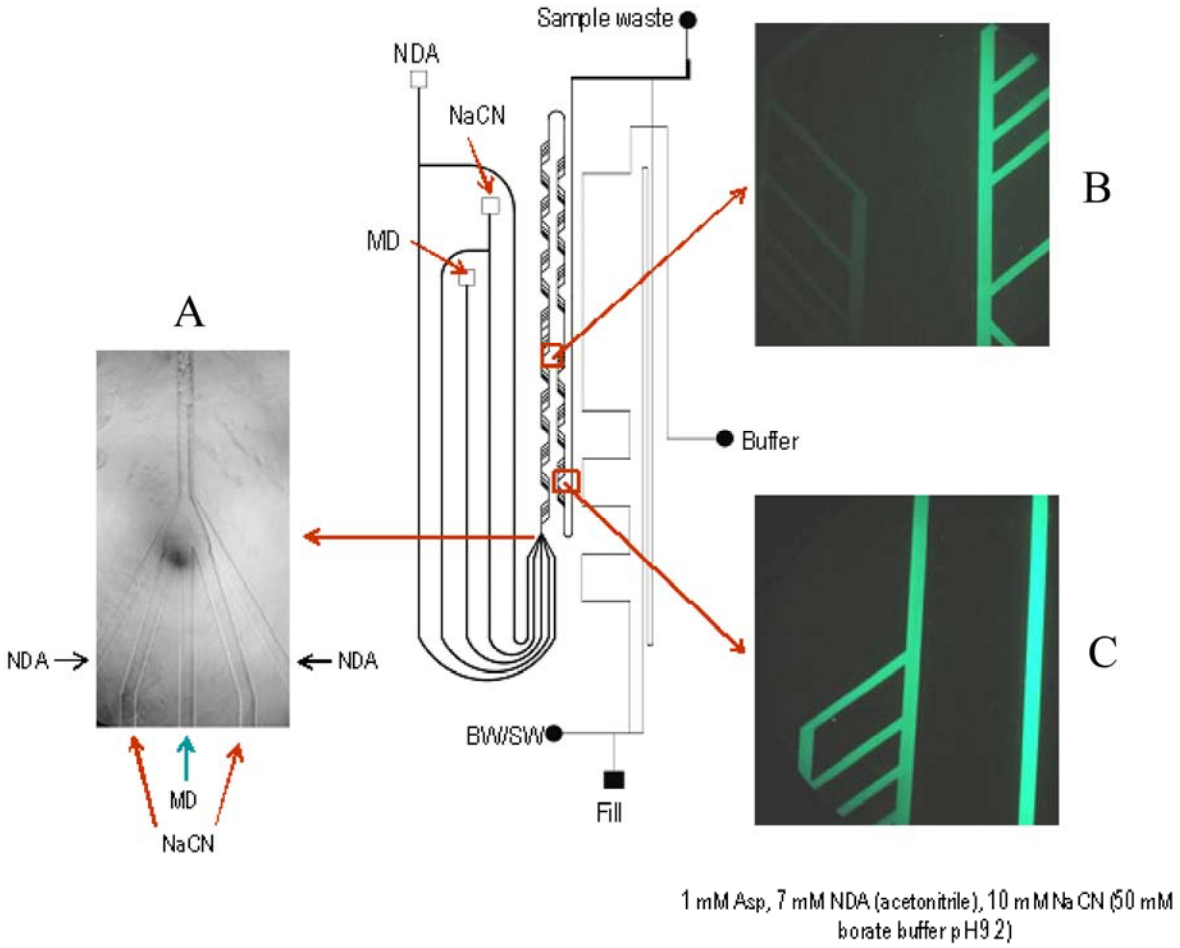


Figure 4.4: Characterization of micromixer chip with 1 mM glycine, 7mM NDA in acetonitrile, 10mM NaCN in 50mM borate buffer at pH 9.2. Panel A shows the flow profile at the junction where NDA, CN^- and microdialysate flows converge. The micrographs (B, C) demonstrate the fluorescence in the derivatization channels.

4.3.4 Chip modifications for flow through analysis.

A flow through design was reported by the Chen group [5] for nonstop injection and sample analysis from an uninterrupted flow stream. The present design has added increased complexity with respect to addition of a derivatization channel, a serpentine separation channel and use of hydrophobic PDMS as the chip substrate. The first challenge that needed to be overcome with such a design was to be able to fill up the appropriate channels of the chip reproducibly and easily with background buffer and analyte with reagents without affecting each other. Also the microdialysis and buffer solutions needed to be equilibrated within the PDMS microchannels for better chip performance (e.g. resolution and reproducibility). The addition of an inlet for easy buffer fill with a syringe pump from the bottom of the chip proved to be a key solution for several problems. After the start of analyte (amino acid) and reagent (NDA/CN^-) flow through the derivatization channels, air pockets were created in the unfilled spaces within the PDMS channel. Over time the air pockets get pushed into the separation channel and ultimately impede the sample injection and separation. Subsequent introduction of the separation buffer at a high flow rate (20-30 μL/min) not only directed the bubbles exclusively towards the sample waste but also prevented the derivatized analyte from filling up the separation channel. This also ensured that all the streams were being maintained in the appropriate channels for equilibration without bubble creation. Once the chip was equilibrated in such a manner, injection and separation could be carried out reproducibly without any problem.

4.3.5 Continuous analysis of offline derivatized amino acids.

A mixture of 5μM amino acids, that were derivatized offline, was analyzed online on chip **(Fig 4.5)** to evaluate injection from continuous flow. The peak height RSD values for serine, GABA, glutamate, aspartate and OPS were calculated to be 2.78%, 3.26%, 3.04%, 2.89%, and 3.17% (<5% for all amino acids) respectively. Also, a concentration change experiment was performed with glycine, glutamate and aspartate **(Fig 4.6)** where the concentration change was made at the microdialysis probe vial from 1 μM to 4 μM for all amino acids. The rise time was calculated to be approximately 2 mins for the setup.

4.3.6 Online derivatization and analysis.

To demonstrate the performance of the chip after initial design and flow optimization studies, online derivatization of amino acids followed by analysis was performed. First, injection was optimized for glutamate and aspartate **(Fig. 4.7)**. An injection time of 0.1s was chosen as optimum based on the separation resolution. The online derivatized amino acids (namely glutamate and asp) exhibited RSD values of less than 5% (N = 15 injections). Based on an injection plug size of 10μm length with amino acid concentration of 10μM, approximately 80 fmoles of each amino acid was injected. LOD studies were not performed with the precolumn derivatization approach used here. In the literature, LODs of 0.08, 0.09 fmol for glu and asp respectively have been reported for precolumn detection with OPA and β-mercaptoethanol [6]. Subsequently, a concentration change experiment was

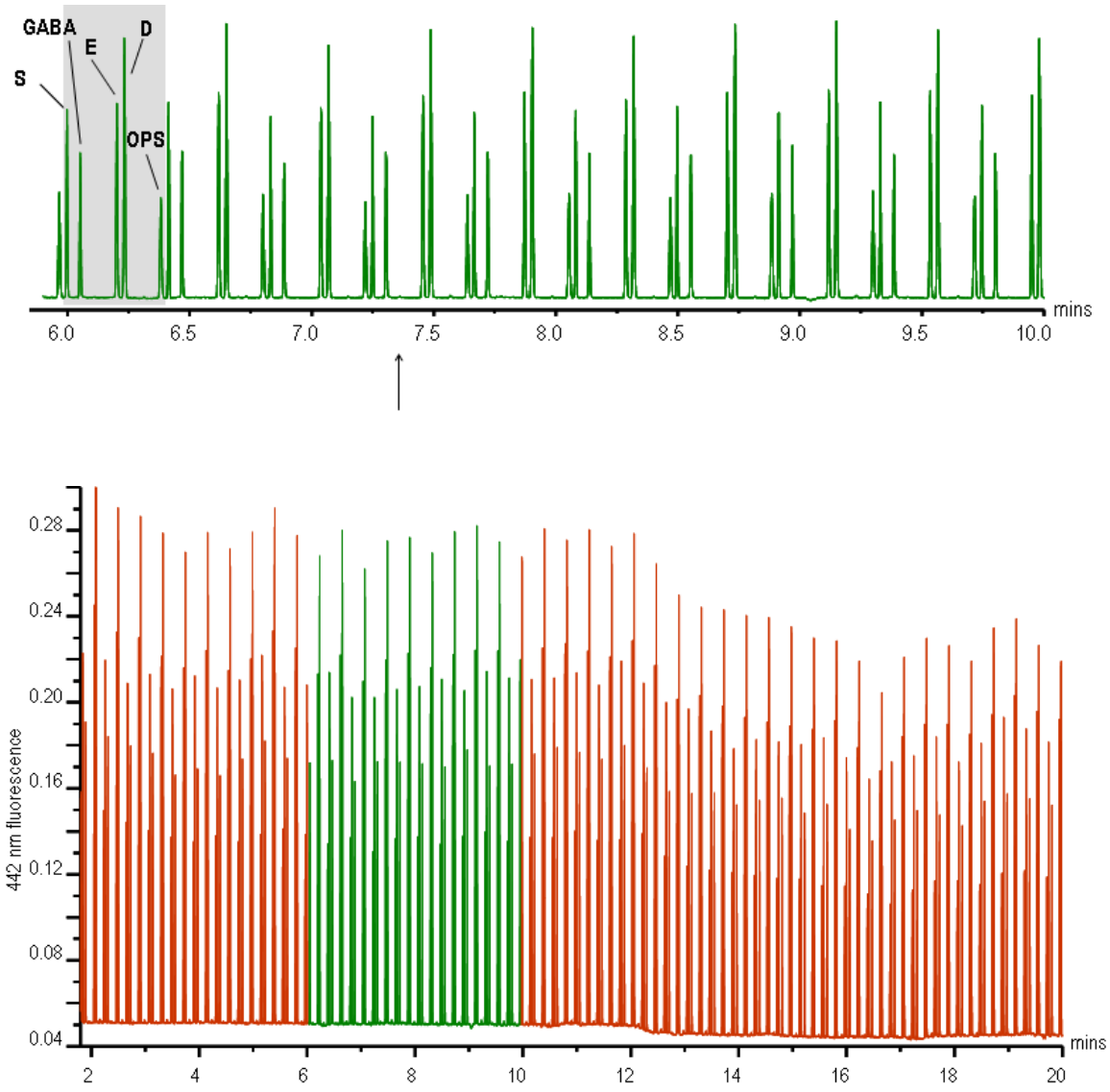


Figure 4.5: A. Online flow through sampling analysis with prederivatized amino acids 5mM serine, GABA, glutamate, aspartate, ortho-phosphoserine (OPS, internal standard) with temporal resolution of 25s. Separation buffer: 20mM borate, 10mM SDS, pH 9.2, injection time 0.5s, 500V/cm separation voltage. The micromixer chip was used with 1 inlet for prederivatized amino acids.

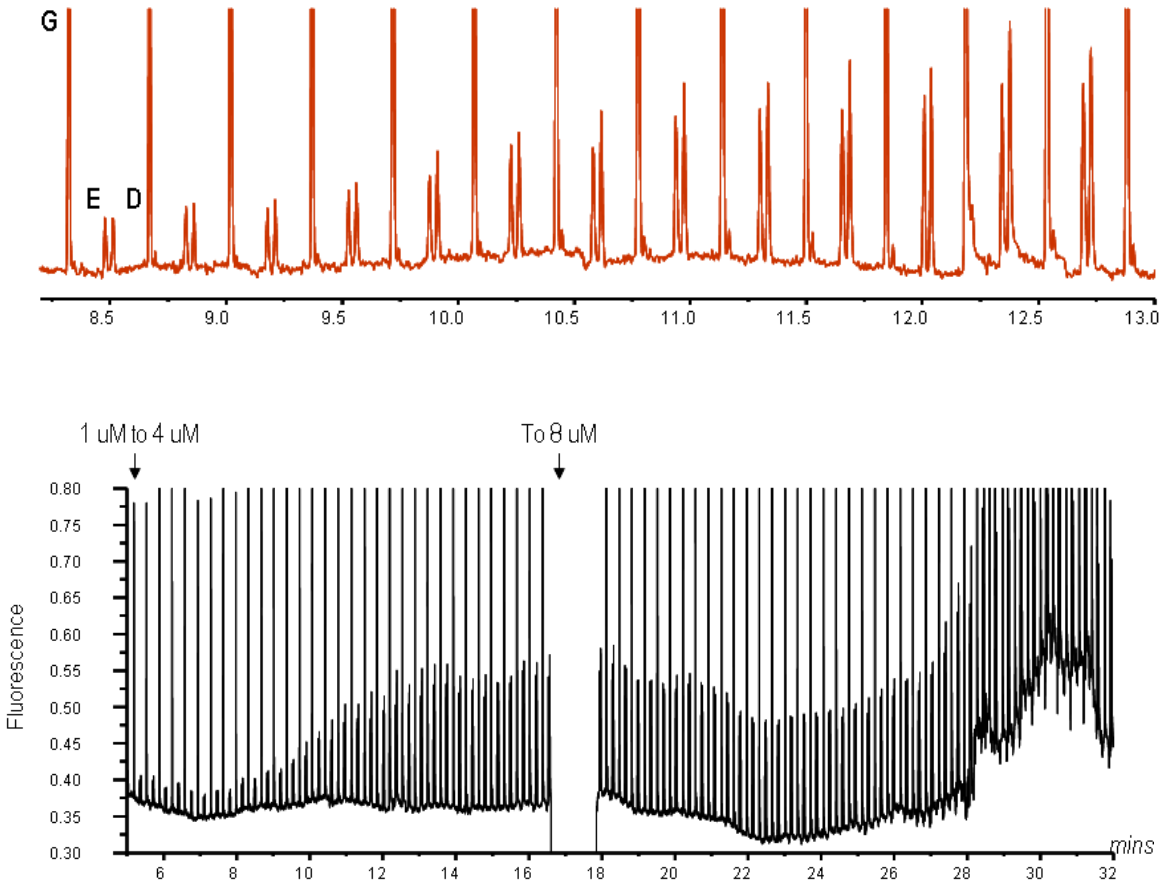


Figure 4.6: Concentration change experiment with prederivatized glycine, glutamate and aspartate. The top panel shows a snapshot of the rise time (from 9 mins to 12 mins). The bottom panel demonstrates the continuous injections and separations with concentration changes at 2 points (1μM to 4μM and then to 8μM indicated by the arrows). Buffer conditions: 20mM borate, 10mM SDS at pH 9.2. The amino acids were derivatized with 7mM NDA in acetonitrile, 10mM NaCN in 50mM borate buffer at pH 9.2. Injection time is 0.5 s with a separation potential of 500V/cm.

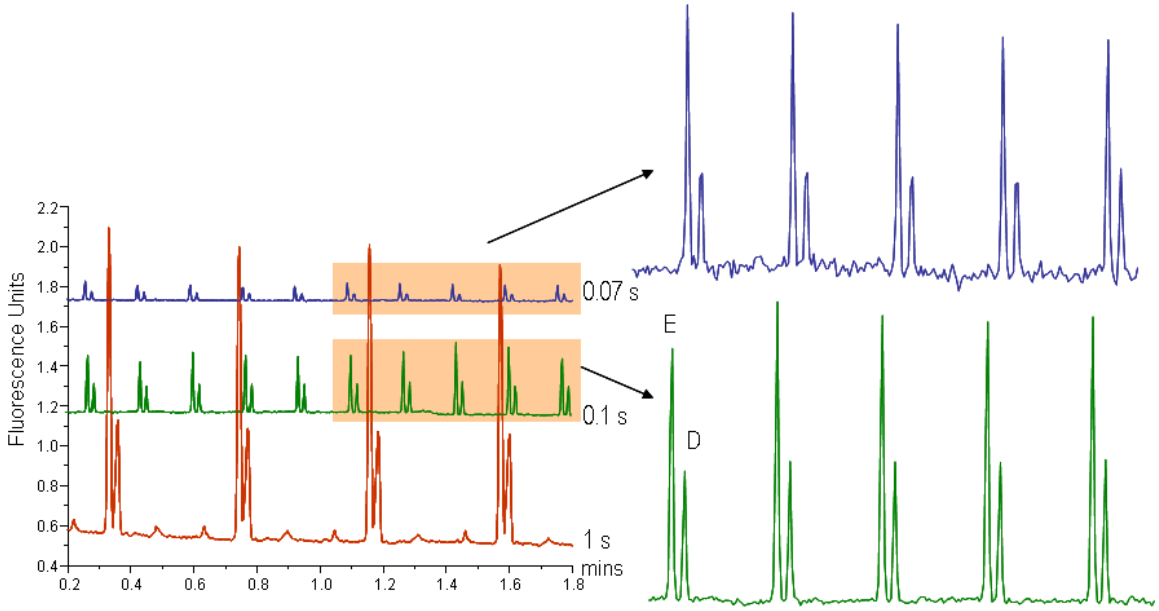


Figure 4.7: Online derivatization of 10μM of glutamate and aspartate. Injection times (1s, 0.1s and 0.07s) were optimized for baseline separation of the analytes. Separation buffer: 20mM borate, 10mM SDS, pH 9.2. Online derivatization was performed with 7mM NDA in acetonitrile, 10mM NaCN in 50mM borate buffer at pH 9.2. The separation potential was 500V/cm.

performed where the concentration of glutamate was increased from 3 μM to 10 μM **(Fig 4.8)**. The rise time in this case was noted to be 5 mins compared to 2 mins in case of prederivatized sample. It has been reported that NDA and NaCN takes 3 to 5 mins for complete reaction with primary amines. However, the underlying assumption is that all the 3 analytes are added to a single vial followed by agitation. In the case of online reaction, the lack of active vigorous mixing phenomena in the reaction channels possibly lengthened the reaction time. Therefore, when the concentration changes, the varying analyte mass takes a longer period of time to meet the NDA and CN^- molecules for efficient reaction. However, in case of prederivatized amino acids, the only limiting factor for the concentration change was the probe recovery. Another factor that may have affected the change is concentration is Taylor dispersion along the length of the derivatization channel.

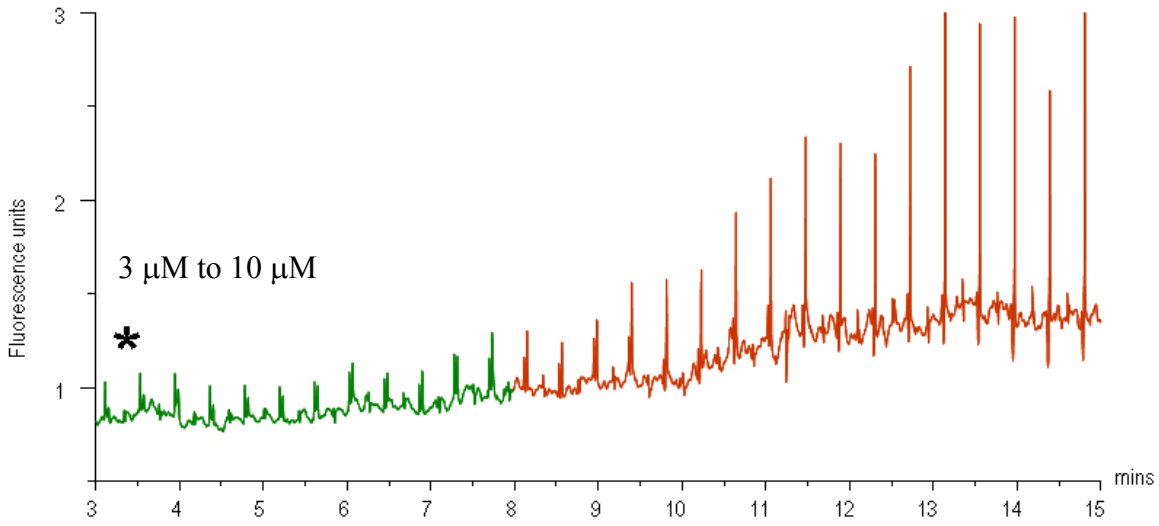


Figure 4.8: Online derivatization with concentration change of glutamate demonstrating the rise time (8 mins to 13 mins) of approximately 5 mins. Separation buffer: 20mM borate, 10mM SDS, pH 9.2; separation potential 500V/cm. Derivatization was performed with 7mM NDA in acetonitrile, 10mM NaCN in 50mM borate buffer at pH 9.2.

4.4 Conclusion

The primary goal of this study was to develop an easily fabricated microchip coupled to microdialysis to perform high throughput sampling with precolumn derivatization. Designs were optimized in this study for such a system followed by characterization with final online *in vitro* analysis of amino acids. Additional features were integrated into the device for practical consideration of fluid flow. The ultimate application of this device is for high temporal resolution *in vivo* monitoring of amino acid neurotransmitters.

4.5 References:

[1] J.A. Bourne, Intracerebral microdialysis: 30 years as a tool for the neuroscientist, Clin. Exp. Pharmacol. Physiol. 30 (2003) 16-24.

[2] H.P. Rang, M.M. Dale, J.M. Ritter, P.K. Moore, Amino acid transmitters, Pharmacology (5th Ed.) (2003) 462-473.

[3] S. Menacherry, W. Hubert, J.B. Justice, Jr., In vivo calibration of microdialysis probes for exogenous compounds, Anal. Chem. 64 (1992) 577-83.

[4] C.A. Connelly, Microdialysis update: optimizing the advantages, J Physiol 514 (Pt 2) (1999) 303.

[5] Y.H. Lin, G.B. Lee, C.W. Li, G.R. Huang, S.H. Chen, Flow-through sampling for electrophoresis-based microfluidic chips using hydrodynamic pumping, J. Chromatogr. A. 937 (2001) 115-25.

[6] Z.D. Sandlin, M. Shou, J.G. Shackman, R.T. Kennedy, Microfluidic electrophoresis chip coupled to microdialysis for in vivo monitoring of amino acid neurotransmitters, Anal. Chem. 77 (2005) 7702-8.

[7] B.H. Huynh, B.A. Fogarty, R.S. Martin, S.M. Lunte, On-line coupling of microdialysis sampling with microchip-based capillary electrophoresis, Anal. Chem. 76 (2004) 6440-7.

[8] P.B. Allen, D.T. Chiu, Calcium-assisted glass-to-glass bonding for fabrication of glass microfluidic devices, Anal. Chem. 80 (2008) 7153-7.

[9] J.D. Ramsey, S.C. Jacobson, C.T. Culbertson, J.M. Ramsey, High-efficiency, two-dimensional separations of protein digests on microfluidic devices, Anal. Chem. 75 (2003) 3758-64.

[10] G.T. Roman, K. McDaniel, C.T. Culbertson, High efficiency micellar electrokinetic chromatography of hydrophobic analytes on poly(dimethylsiloxane) microchips, Analyst 131 (2006) 194-201.

[11] B.H. Huynh, B.A. Fogarty, P. Nandi, S.M. Lunte, 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, J. Pharm. Biomed. Anal. 42 (2006) 529-34.

[12] H. Song, D.L. Chen, R.F. Ismagilov, Reactions in droplets in microfluidic channels, Angew. Chem., Int. Ed. 45 (2006) 7336-7356.

[13] H.P. Rang, M.M. Dale, J.M. Ritter, P.K. Moore, Chemical transmission and drug action in the central nervous system, Pharmacology (5^{th} Ed.) (2003) 456-460.

Chapter 5

Future Directions

5.1 Summary

Over the previous data chapters, the evolution of the application of online microdialysis-microchip electrophoresis systems from the monitoring of a simple *in vitro* reaction to the much more complicated *in vivo* applications with precolumn derivatization has been demonstrated. In the second chapter, a simple-T (3cm separation) hybrid PDMS-glass based chip system was developed for on-column derivatization of amino acids and peptides with NDA/β-mercaptoethanol as the derivatization reagents. Chapter 3 focused on the development of a system that was applied for *in vivo* applications. A serpentine separation channel (20 cm) was incorporated into the chip to obtain a higher plate number for better resolution of glutamate and aspartate from other amino acids in brain microdialysis samples. Also, a simple scheme for the precolumn derivatization of primary amines with NDA/CN^- was added on-chip that allowed injection and separation of plugs of microdialysate containing the analytes of interest (amino acid neurotransmitters). This was demonstrated by monitoring aspartate and glutamate in the microdialysis sample of an anesthetized Sprague-Dawley rat. Fluorescein, a passive permeability marker at the blood brain barrier, was also incorporated in the analysis to allow possibility to simultaneously monitor the levels of excitatory neurotransmitters and integrity of the blood brain barrier. In Chapter 4, the microchip system was further modified to perform continuous injections and separations for high temporal resolution analysis. A more complex mixing scheme for precolumn derivatization was incorporated into the chip design and was characterized for derivatization efficiency. The chip was

evaluated for the separation of prederivatized as well as on-chip derivatization (with NDA/CN^-) of amino acids.

5.2 Short-term future directions

An important *in vivo* application of an online microdialysis-microchip system is the ability to monitor fast *in vivo* concentration changes. We demonstrated the effect of high K^+ on brain glutamate levels by offline analysis on chip (Chapter 3). Therefore, one of the immediate goals is to use the setup to monitor *in vivo* changes in neurotransmitters (glutamate). This can then be extended to other analytes of interest such as aspartate, taurine and γ-aminobutyric acid (GABA, an inhibitory neurotransmitter). For such applications, the online system developed in Chapter 4 can be employed to achieve high temporal resolution.

Most of the work done in this thesis concerns proof-of-concept studies towards the development of microdialysis-microchip devices for *in vivo* sampling. However, a more thorough characterization of various chip factors is required that include (i) effect of background electrolyte (e.g. aCSF containing high concentration of salts) in case of electrokinetic injection of multiple analytes (injection bias), (ii) adsorption of CBI derivatives by PDMS, (iii) the precise length of separation required for adequate resolution and (iv) effect of turn geometry (tapered vs. non-tapered) on band broadening. Such studies will help identify the proper design/fabrication modifications of the chip for improvement in analytical performance. Also,

evaluation of such parameters will help in extracting reliable quantitative information from chip analysis.

In this thesis, the derivatization channel designs were approached in a simple manner in terms of building serpentine channels (Chapter 3) and a slightly more complex "ladder design" (Chapter 4). However, more thorough studies need to be done for fast and efficient mixing. A simple solution is the incorporation of "wedges" or "posts" in the mixing channels to induce high turbulence.

With respect to the microchip analysis systems, efforts should be directed towards making the detection spot size (approximately 0.5 mm in diameter currently) smaller; this can be achieved by integrating a "pinhole" in the current setup or by setting up a microscope system incorporating a laser source that can be focused to a spot by using motorized irises. Detection can also be performed with an avalanche photodiode (APD) instead of a photomultiplier tube (PMT) to increase the sensitivity of detection.

5.3 Long-term future directions

The temporal relationship between the release of excitatory neurotransmitters (glutamate) and loss of blood brain barrier (BBB) integrity is an ideal *in vivo* system to apply the online microdialysis-microchip setup with high temporal resolution analysis. Glutamate excitotoxicity has been associated with the breakdown of the BBB during ischemic stoke [1]. The microdialysis-microchip system could be employed to obtain direct evidence that NMDA receptor stimulation causes loss of

BBB integrity. For such investigations, microdialysis probes will be implanted in the rat striatum and focal cerebral ischemia will be induced in the animal using accepted methods (Rose Bengal) [2]. At the same time, sodium fluorescein will be administered continuously by IV infusion. After the induction of stroke, it is expected that a sudden increase in the concentration of glutamate would be observed as well as an increase in the concentration of fluorescein in the brain probe. The increase of fluorescein levels in the brain correspond to the opening of the BBB. Also, the near real-time monitoring will give us an idea of the time interval between stroke and opening of the BBB in the rat stroke model.

In order to determine if NMDA receptor stimulation by glutamate is directly responsible for BBB opening, an NMDA receptor agonist (NMDA) can be delivered via the microdialysis probe to the brain interstitial space. The agonist will prevent any interaction of glutamate with the NMDA receptor. If such receptors are involved in the augmentation of BBB permeability, then this should be inhibited by the agonist. In a separate experiment, a competitive inhibitor of NMDA receptor (AP-5) will be delivered [3]. In this case, some glutamate will still be available to interact with the receptor and less disruption of the BBB should be observed if glutamate is directly involved in the opening of the BBB (i.e. the concentration of fluorescein should be less compared to the previous case). Based on such experiments, further studies can be conducted to elegantly pinpoint the exact NMDA receptor subtypes responsible for opening of BBB.

The online microdialysis-microchip system can also be used to monitor matrix metalloproteinase (MMP) and its relation to BBB opening. MMPs are responsible for the breakdown of extracellular protein matrix. Therefore it has been suggested that following stroke, MMPs are activated and become responsible for slow disintegration of the BBB [4]. Also, the time scale between the onset of enzyme action and loss of BBB integrity is not fully understood. The experiment can be designed in the following manner. First, a fluorogenic substrate of MMP can be delivered using the microdialysis probe with a suitable molecular weight cutoff. The advantage of online derivatization system is that *in vivo* MMP activity can be monitored even in the absence of fluorescent substrates for LIF detection. Using the online microdialysis microchip electrophoresis system, it may be feasible to simultaneously monitor the activity of the MMPs and the integrity of the BBB (monitored by fluorescein). It is also imperative that *in vitro* separation method development be performed for the metabolite of the MMP substrate and fluorescein before performing *in vivo* experiments.

The near-real time high temporal resolution monitoring capability of microdialysis-microchip system will help in understanding the sequence of biological events following ischemia including glutamate excitotoxicity and the activation of MMPs, all of which lead to the disintegration of the BBB. Overall, this can provide scientists with valuable information regarding the time course of events which will help to better establish the time window available for critical therapeutic measures in stroke patients.

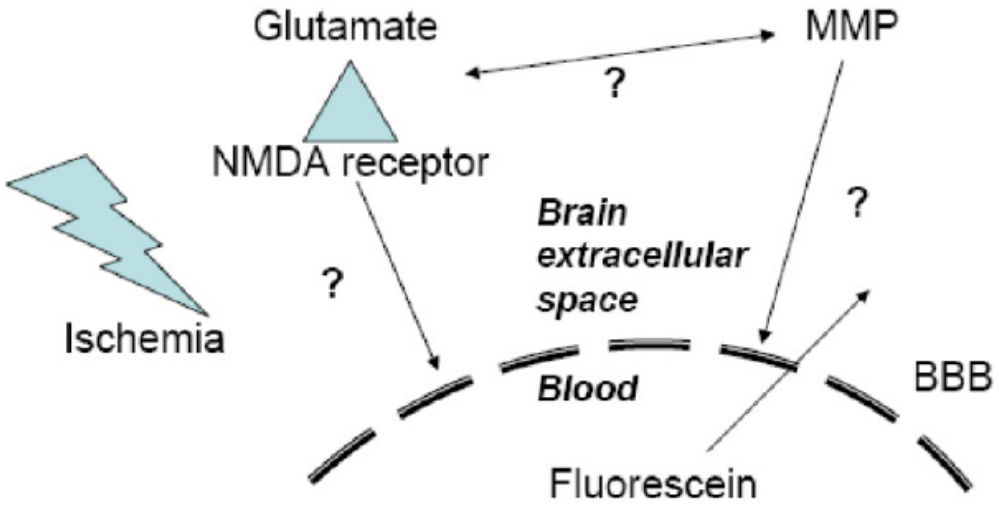


Figure 5.1: Schematic showing interaction between different compounds and opening of BBB. ("?" indicates possible interactions that may be elucidated through studies discussed in section 5.3)

5.4 References:

[1] H.P. Rang, M.M. Dale, J.M. Ritter, P.K. Moore, Neurodegenerative disorders, Pharmacology (5th Ed.) (2003) 490-502.

[2] A. Durukan, T. Tatlisumak, Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia, Pharmacol. Biochem. Behav. 87 (2007) 179-97.

[3] H.P. Rang, M.M. Dale, J.M. Ritter, P.K. Moore, Amino acid transmitters, Pharmacology (5th Ed.) (2003) 462-473.

[4] Y. Gasche, J.-C. Copin, P.H. Chan, The role of metalloproteinases on blood-brain barrier breakdown after ischemic stroke, Inflammation Stroke (2001) 265-274.

Chapter 6

Analysis of Oxidized Protein Species by Micellar Electrokinetic Chromatography (MEKC)

6.1 Introduction

Proteins play a vital role in the regulation of various processes in the body. Many proteins are metabolic enzymes that can catalyze biochemical processes in the body. Proteins serve an important role in the formation of receptors and channels in membranes and provide structural and mechanical support for the cells and organs. Proteins are also important in cell signaling and generation of immune responses. Proteins are composed of an amino acid sequence that is encoded in the genetic code. During the synthesis or shortly after synthesis, proteins can undergo chemical modifications (post-translational modification) such as phosphorylation, acylation, glycosylation which alter the structure and activity of the protein [1].

In recent years, a lot of focus has been placed on developing protein-based pharmaceuticals because of certain advantages offered by such compounds over small molecule drugs. Protein therapeutics are generally capable of performing highly specific and complex set of functions without interfering with normal biological processes resulting in less side effects. Protein molecules are endogenous to the body and therefore there is a greater chance of tolerance of such protein pharmaceuticals without producing an immune response [1]. Protein pharmaceuticals are prone to several post-translational modifications that affect their efficacy and shelf life and in certain cases can also trigger deadly immune responses against the protein. The modifications that are of importance include glycosylation, misfolding and aggregation, proteolytic cleavage, deamidation of asparagine and glutamine and oxidation of methionine [2].

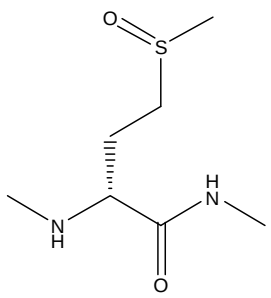
Oxidation is a one of the major problems associated with the chemical degradation of protein and peptide formulations. This degradation process or reaction can arise during any of the processing steps (protein isolation, purification) as well as storage. Oxidation of protein formulations can lead to alterations in tertiary structure (conformation) causing immunogenicity and loss of therapeutic efficacy. Oxidation can occur due to the presence of contaminants, such as peroxide present in various formulation additives (polyethylene glycol, Polysorbate 80). Autooxidation is mentioned as another pathway through which proteins can undergo oxidation; such reactions are catalyzed by the presence of trace metal ions which can originate from buffer(s) used during processing and formulation. Oxidation can also be induced by light and this is a concern for photosensitive formulations. Strategies to minimize oxidation include chemical methods such as site directed mutagenesis where the reactive amino acid residues are either changed to an alternative less reactive or unreactive amino acid or the reactive site of the amino acid is blocked through chemical modifications. Physical methods to increase oxidation stability include lyophilization (freeze drying) or addition of chemical additives in the formulation such as antioxidants and chelating agents. Altering the solution pH, changing the buffer species, reducing levels of molecular oxygen or the addition of oxygen scavengers have also been proposed for enhancing stability against oxidation [3].

Methionine is the primary amino acid residue vulnerable to oxidation. Several reports have indicated alterations in biological functions of proteins following methionine oxidation [4-5]. The various reaction mechanisms through which

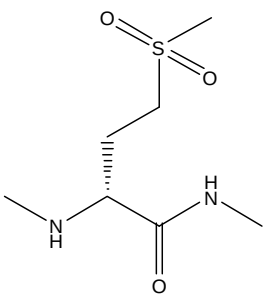
Methionine can be oxidized are demonstrated in **Figure 6.1**; cysteine, histidine, tyrosine and tryptophan can also undergo oxidation by various mechanisms.

The addition of hydrogen peroxide (H_2O_2) is a convenient approach to study protein oxidation as the identity and concentration of initial oxidant is known and can be controlled [6]. Traditionally, hydrogen peroxide was found to react with those residues that are easily accessible on the surface of the protein rather than the residues that are buried within the protein; however, recent studies have shown that both exterior and masked residues in proteins are affected by H_2O_2 [7].

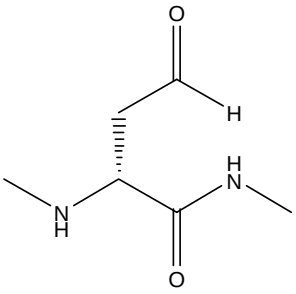
Antibodies or immunoglobulins (Ig), first identified in 1890, can be classified as an important group of protein therapeutics. Immunoglobulin G (IgG), a 150 kDa glycoprotein, is the most abundant antibody found in the serum and is the most popular for the development of therapeutics. Such proteins have antigen recognition sites which are generally exploited to guide the immune system for targeted destruction of molecules or cells. Another mechanism of action of antibodies can be the occupation of the functionally important region of the molecule. IgGs consist of four polypeptide chains, two identical "heavy chains" (50-55 kDa) and two identical "light chains" (approximately 25 kDa). The chains are held together by disulfide bonds. **Figure 6.2** depicts the various parts of the molecule which is comprised of antigen-binding fragment (F_{ab}) and the constant region (F_c). The constant regions stay common for all IgGs and the variable domains are unique [8]. Each IgG has two methionine residues, Met 252 and Met 428 (both based on intact heavy chain sequence) [9]. Investigations involving methionine oxidation in such proteins have



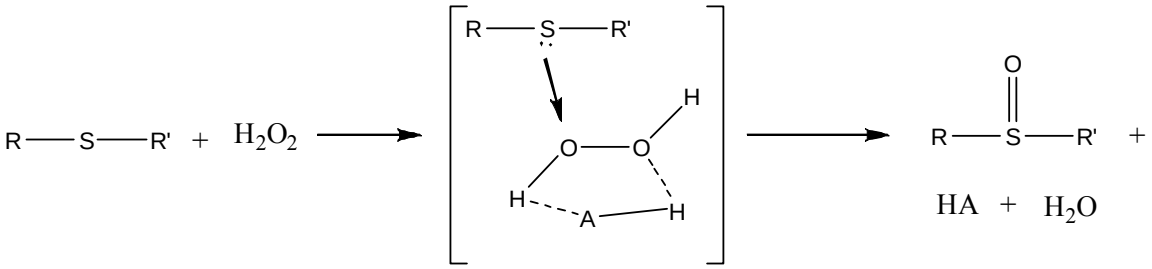
A. Sulfoxide



B. Sulfone



C.Aspartyl semialdehyde



D. Formation of sulfoxide (acid catalyzed)

Figure 6.1: Mechanisms of oxidation of methionine in presence of H_2O_2 to form sulfoxide [3].

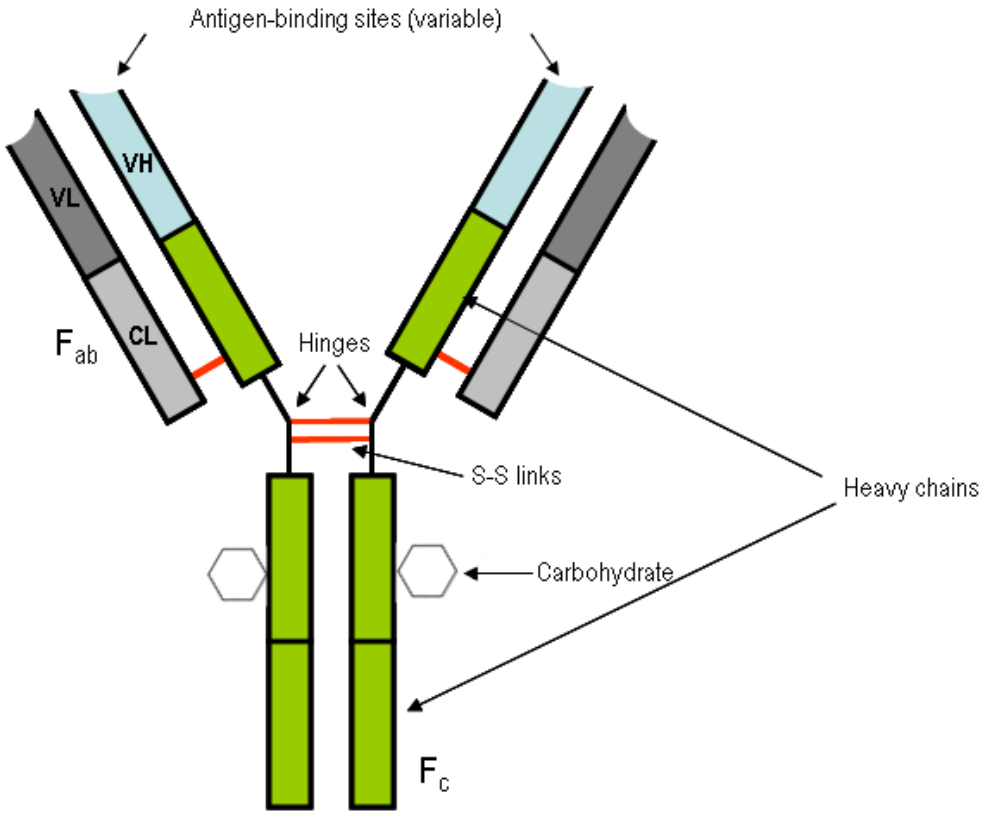


Figure 6.2: Cartoon of IgG antibody structure. Antibodies bind to the antigen via the variable region (VL and VH). L stands for light chain, H for heavy chain.

revealed that the methionine residues in the Fc region are susceptible to oxidation during storage and exposure to active oxygen species [10-11]. Also, It has been found that oxidation of methionine residues accelerate protease cleavage on the C_H2 domain [9] . Therefore, study of methionine oxidation has become an important area of research in the biopharmaceutical industry, especially with the advent of a large variety of new therapeutic proteins produced by recombinant DNA technology.

Capillary electrophoresis (CE) and more specifically, micellar electrokinetic chromatography (MEKC) is a powerful separation technique for proteins, and is being used routinely in the industry for preformulation and Quality Control (QC) characterization of protein formulations [12]. CE offers the advantages of fast analysis times, high resolution, separation efficiency, easy quantitation compared to SDS-PAGE gel electrophoresis, easy automation and ability to perform LIF detection for high sensitivity analysis.

CE separations of proteins are mainly based on charge-to-mass ratio, size, ligand interactions and hydrophobic/hydrophilic interactions. The presence of negatively charged silanol groups on fused silica capillaries can lead to protein adsorption and consequently gives rise to poor resolution/reproducibility, broad peak profiles and decreased sensitivity. Another consequence of protein adsorption on capillary walls is the nonuniform shielding of negative charge giving rise to disorderly EOF patterns within the capillary that contributes to the analytical problems discussed previously. Options to overcome such issues are either to utilize capillaries that have been covalently bonded with proprietary polymer coatings or use

a polymer solution that uniformly adsorbs on the capillary wall to create a dynamic coating. Dynamic coating provides a simple, fast and inexpensive method to modify fused silica capillaries for protein/peptide separations using known and characterized polymers such as poly(vinyl alcohol) (PVA), cellulose acetate, hydroxypropylmethyl cellulose (HPMC), hydroxyethylcellulose (HEC) [13-15]. Although covalently bonded capillaries can produce excellent data for very specific applications, use of such capillaries for non critical method development can be very expensive and therefore impractical [16].

Digestion of proteins is a popular method to characterize protein structure. The steps include digestion of the protein with an enzyme resulting in the formation peptide fragments which can be separated using an appropriate analysis technique. The present study aims to investigate the possible use of CE as an alternative to HPLC for quantitative characterization of the oxidation of a protein formulation. Also, the use of triethanolamine dextran (Bio-Rad CE-SDS gel) as a cheaper alternative to commercially coated capillaries for protein analysis was also explored. A method was developed to separate the peptide fragment with Met from other fragments after Lys-C digestion of the protein. LysC specifically cleaves proteins at at the lysine C-terminus which produces lesser number of fragments leading to simplified separation compared to Trypsin digest that is most popular in protein characterization studies. Also, for oxidation studies involving methionine residues, larger peptides lead to improved quantification using UV detection scheme [17]. LysC has also been reported to be a robust enzyme capable of withstanding high

concentration of denaturants without significant loss of activity. Subsequently, studies with protein formulation were presented to compare the performance of covalent coated capillaries and dynamic gel coating.

6.2 Materials and Methods

All reagents were used as received. Boric acid, sodium phosphate, sodium dodecyl sulfate (SDS) were received from Sigma (St. Louis, MO). The monoclonal antibody reference standards and the formulations were received from Pfizer (St. Louis, MO). The neutral covalently coated capillaries were obtained from Beckman (Fullerton, CA). CE-SDS gel buffer was obtained from Bio-Rad (Hercules, CA). Endoproteinase Lys-C was obtained from Roche Diagnostics (Manheim, Germany). Hydrogen peroxide (30% in water) solution was obtained from Sigma Chemicals (St. Louis, MO).

All experiments were performed using Beckman PA800 or MDQ systems. Sample was introduced into the capillary by utilizing a hydrodynamic injection (5 sec at 20psi). Separation was carried out in the reverse polarity mode (negative and positive voltage at the injection and detection end respectively). For dynamic coating (EOF suppression), the Bio-Rad CE-SDS gel containing triethanolamine dextran as a dynamic coating was pressure flushed through 75μm i.d. capillary at 30psi for 30mins and then equilibrated for 2 hours. Following this, the capillary was flushed with water and buffer to remove excess gel after which analysis was performed.

LysC digestion protocol: Digestion buffer consisting of 25mM Tris HCl, pH 8.5 and 1mM EDTA was prepared. The lyophilized endoproteinase Lys-C was diluted with 5 μL of double distilled water to reach an enzyme concentration of 1 mg/mL. The monoclonal antibody reference standard was diluted appropriately with digestion buffer to reach a concentration of 2 mg/mL. Lys-C (5 μL) was added to 150 μL of protein solution and incubated for 16 hrs.

Oxidation studies: Oxidation studies were performed with a final concentration of 0.1% H_2O_2 in the protein digest solution.

6.3 Results and Discussions:

The choice of the surfactant is the most important decision in MEKC since it is a critical factor to obtain the necessary resolution. SDS is the most common anionic surfactant used in MEKC. In general, micelles are formed by SDS at a concentration above its critical micelle concentration (around 9mM in water). Such micelles in CE are often referred to as a "pseudostationary phase" in comparison to the retention mechanism in HPLC. Small molecules partition differentially between the hydrophobic core and the hydrophilic exterior of the micelle resulting in analyte resolution. However, for protein or peptide analytes, the dimension of the micelle (3-6 nm) cannot accommodate proteins/peptides greater than 5 kDa. Therefore, it is generally believed that protein molecules form a complex with SDS by hydrophilic, hydrophobic and electrostatic interactions. The degree of complexation with SDS varies depending upon the nature of the protein/peptide which results in analyte

separation. In addition, the analyte attains a net negative charge due to the anionic SDS resulting in high electrophoretic mobility towards the anode. The present study employed coated capillaries that suppressed or eliminated EOF within the capillary [18]; hence application of reverse polarity aided in reducing the analysis time.

Preliminary method development studies were performed with a MAb reference standard from Pfizer. MEKC separations were performed to identify the peptide fragment containing the Met-252 residue which is principally responsible for oxidative degradation of the protein. Therefore following LysC digestion, we were only interested in the peptide fragment with Met-252 and its oxidized product. First the peak for the peptide fragment was identified by spiking with peptide fraction that was isolated from LC. Next, this was confirmed by the addition of 0.1% H_2O_2 ; it was observed that the peak height of the peptide fragment decreases whereas an additional peak appears that slowly increases. It was hypothesized that the peak whose height increases over time is the oxidized peptide (**Fig. 6.3**). This idea was further investigated later.

Next buffer optimization studies were performed to determine its effect on the separation of the peptide fragment from the oxidized peak. A buffer concentration of 25 mM borate in 25mM phosphate (pH 8.5) and 5% acetonitrile was found to produce the maximum resolution between those 2 species. The study was carried out in a covalently bonded capillary so that the separation was only affected by the background electrolyte and not by the surface coverage of the polymer adsorbed by dynamic coating.

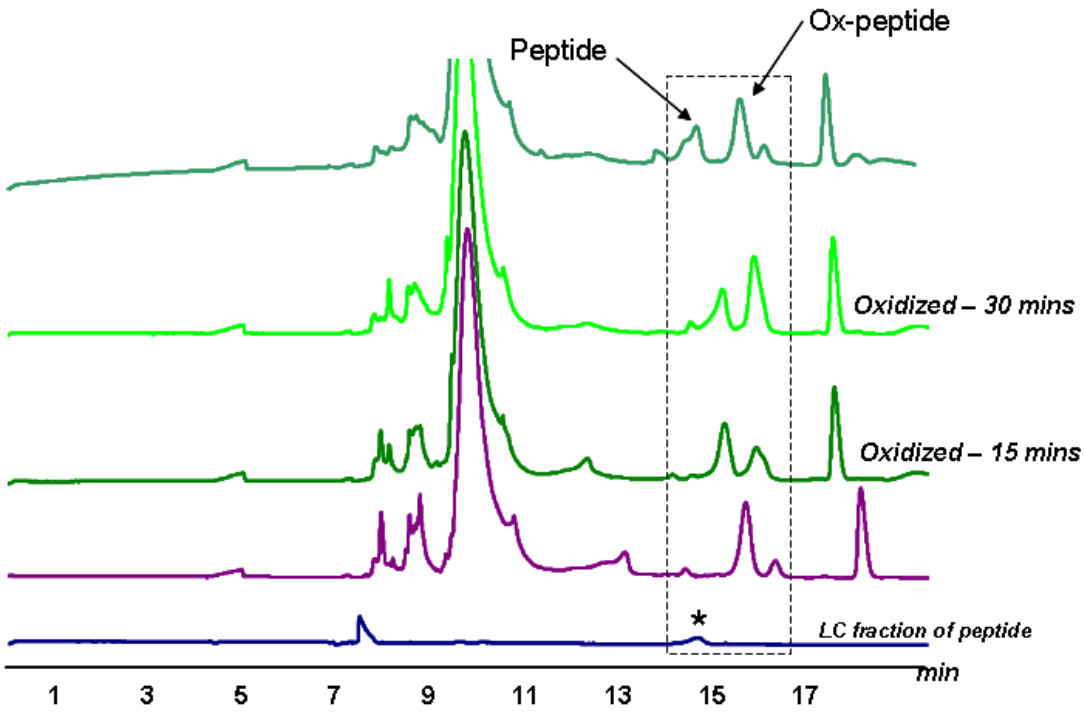


Figure 6.3: Studies with LC fraction and reference standard with covalently coated capillary. The reference standard was oxidized with 0.1% H_2O_2 and increase and decrease of peak heights were observed for proper idetification of the Met-containing peptide fragment. CE conditions are as follows: 25 mM borate-phosphate buffer pH 8.5, 25 mM SDS, 5% acetonitrile, 30 cm neutral coated capillary, UV detection at 200nm.

After establishing peak identity as well as the optimized separation conditions, the method developed in the preceding paragraph was tested with two types of capillaries i.e. covalent bonded and dynamic coated capillaries. Oxidized species were analyzed and the peaks were identified based on appearance/disappearance. (**Figs. 6.4, 6.5**). Based on the previous knowledge of the peptide fragment peak, clear trends were observed for increase/decrease of peak heights with both the covalently bonded as well as the dynamic coated capillary.

Although no quantitative measurement was done regarding the total peak area, the dynamic coating exhibited better resolution compared to neutral coated capillary. We were able to run somewhere between 10-15 runs with the covalent coating before non-reproducible data (unresolved peaks) was observed. In general, we were not able to use the same capillary for further runs after reapplication of the dynamic coating. It has been reported in the literature that some coating (methylcellulose, PVA) materials can be removed after rinsing with water [16]. Attempts were made to wash the capillary with water, HCl and 1N NaOH. However, subsequent re-coating with gel produced highly variable results. For covalent coated capillaries, the number of reproducible runs varies greatly (5-20 runs). Such variability can possibly be explained by the fact that the coating material was mentioned to be stable between a pH range of 3-8. The run buffer used in these experiments (pH 8.5) likely contributed to gradual deterioration of the coating material over a few runs.

An approach similar to the one described before for reference standards was taken to quantitate the levels of oxidation during the analysis of formulations. The

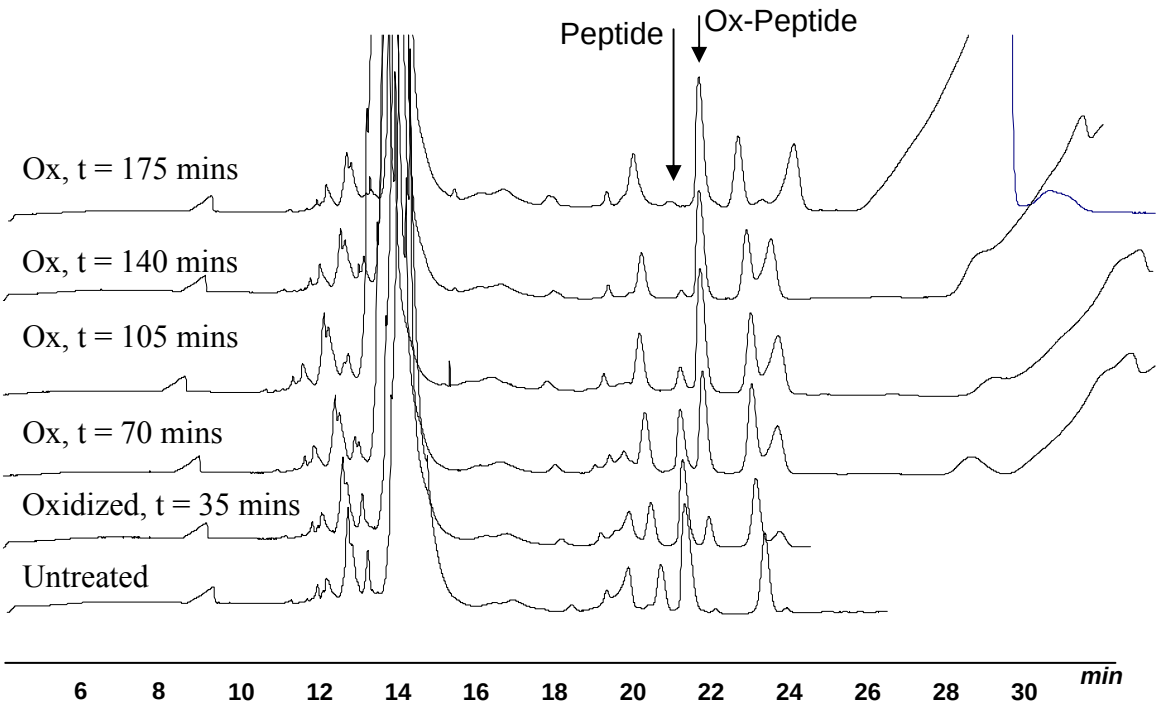


Figure 6.4: Oxidation studies with Bio-Rad gel coating. The reference standard was oxidized with 0.1% H2O2 and increase/decrease of peak heights were closely observed. The arrows indicate the peaks that appear/disappear. CE conditions: 25 mM borate phosphate buffer pH 8.5, SDS 25 mM, acetonitrile 5%, UV detection at 210 nm, 30 cm capillary with Bio-Rad gel coating.

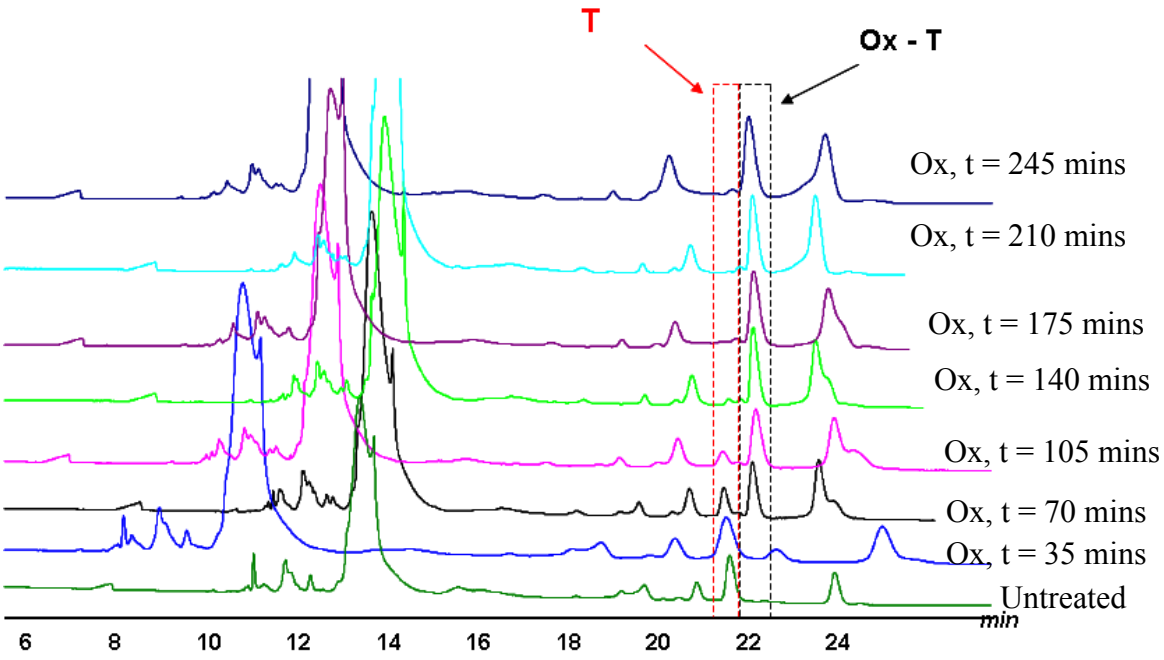


Figure 6.5: Oxidation studies with covalent-coated capillary. The reference standard was oxidized with 0.1% H_2O_2 and increase/decrease of peak heights were closely observed. The arrows indicate the peaks that appear/disappear. CE conditions: 25 mM borate phosphate buffer pH 8.5, SDS 25 mM, acetonitrile 5%, UV detection at 210 nm, 30 cm capillary with Bio-Rad gel coating.

identity of the peptide and the corresponding oxidized fragment was confirmed (**Fig. 6.6**). To detect the oxidized peak in formulations, a greater quantity of the sample needed to be injected in the capillary. This was achieved by increasing the injection time to 20 secs. Six sets of formulations were analyzed in neutral coated capillaries and with the gel coating. The fraction oxidized was calculated as percent AUC of the total AUC for the parent peptide plus the oxidized AUC (**Fig. 6.7**). The data from 2 different coating materials is summarized in Table 1.

A comparison of values obtained above with HPLC analysis demonstrated that the dynamic coating yielded more consistent data. This is surprising as commercially available coated capillaries were expected to have better results as the covalently coated neutral capillary should have better surface coverage leading to a negligible EOF. However, the long injection times may have skewed the calculated values as overloading the capillary can give rise to broad peak profiles which makes integration difficult and variable. Fluorescence detection of peptides labeled with a fluorophore after digestion can be an alternative way to perform more precise measurements to draw accurate conclusions. The high sensitivity with LIF detection will enable the researcher to inject smaller volumes of sample, yielding sharper peaks which should make the integration process less subjective.

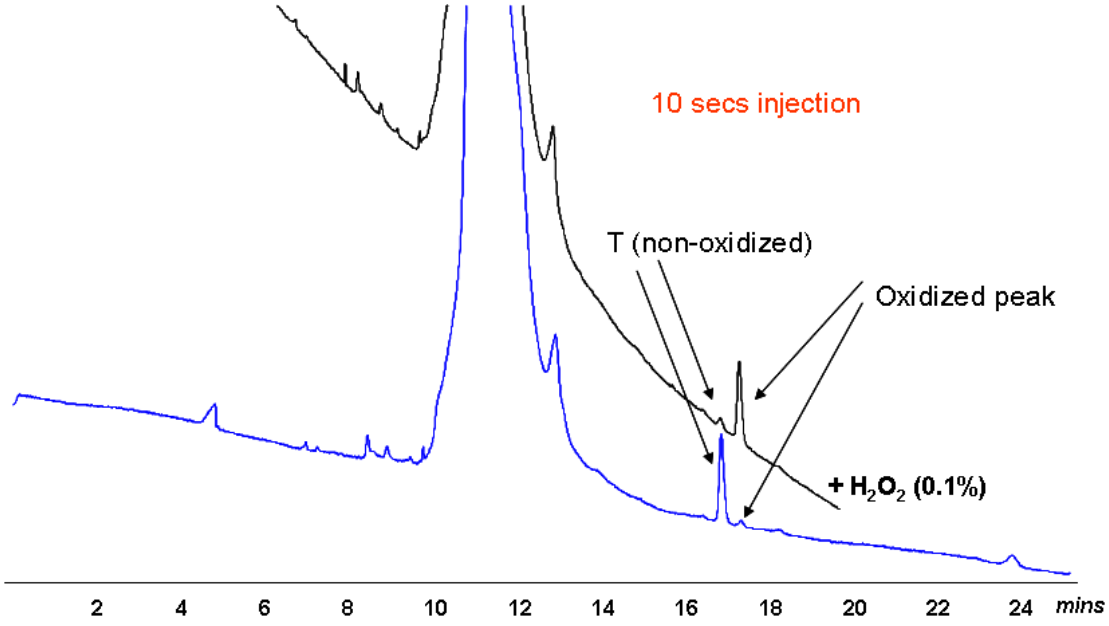


Figure 6.6: Formulation studies with neutral coated capillaries for identification of Met-containing peptide fragment. CE conditions: 25 mM borate-phosphate buffer at pH 8.5, SDS 25 mM, acetonitrile 5%, UV detection at 210 nm, 30 cm capillary.

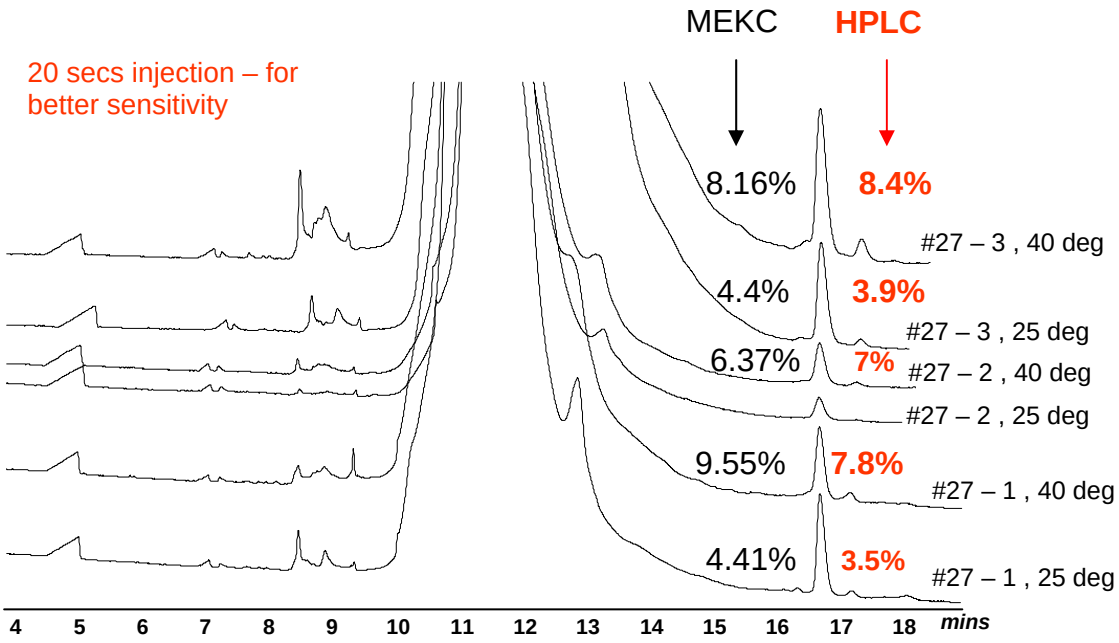


Figure 6.7: Formulation studies with neutral-coated capillaries (Beckman) showing the extent (%) of oxidation from AUC calculations. CE conditions: 25 mM borate-phosphate buffer at pH 8.5, SDS 25 mM, acetonitrile 5%, UV detection at 210 nm, 30 cm capillary.

Table 6.1: Comparison of oxidation data for 2 kinds of capillary. Calculation was done based on the formula

$$\% \text{ Ox} = (A_O/(A_O + A_P))*100$$

where,
% Ox = percent oxidation; A_O = peak area of oxidized peptide; A_P = Peak area of parent peptide.

Sample ID	HPLC (% oxidation)	Gel coating (% oxidation)	Neutral capillary (% oxidation)
MAb formulation 1 25C	3.5	2.3	4.41
MAb formulation 1 40C	7.8	4.3	9.55
MAb formulation 2 25C	4.7	1.9	Not detected
MAb formulation 2 40C	7.0	2.5	6.37
MAb formulation 3 25C	3.9	4.4	4.4
MAb formulation 3 40C	8.4	9.1	8.16

6.4 Conclusions

This study evaluated the use of coated capillaries for fast separations of peptide fragments from a protein digest. Oxidation studies were performed to identify the Met-containing peptide fragment that undergoes oxidation. Quantitative analysis of protein formulations was performed to compare the extent of oxidation calculated using CE with that of HPLC. Overall, a dynamic coating with triethanolamine dextran exhibited better performance compared to a neutral coated capillary in terms of quantitation and the number of runs that could be performed reproducibly. However, a more detailed investigation is necessary to perform a more comprehensive comparison between the two types of coating.

6.5 References:

1. B. Leader, Q. J. Baca, D. E. Golan, Protein therapeutics: a summary and pharmacological classification, Nat. Rev. Drug Discov. 7 (2008) 21-39.

2. N. Jenkins, Modifications of therapeutic proteins: challenges and prospects, Cytotechnology 53 (2007) 121-5.

3. S. Li, C. Schoneich, R. T. Borchardt, Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization, Biotechnol. Bioeng. 48 (1995) 490-500.

4. H. S. Lu, P. R. Fausset, L. O. Narhi, T. Horan, K. Shinagawa, G. Shimamoto, T. C. Boone, Chemical modification and site-directed mutagenesis of methionine residues in recombinant human granulocyte colony-stimulating factor: effect on stability and biological activity, Arch. Biochem. Biophys. 362 (1999) 1-11.

5. A. B. Sigalov, L. J. Stern, Oxidation of methionine residues affects the structure and stability of apolipoprotein A-I in reconstituted high density lipoprotein particles, Chem. Phys. Lipids 113 (2001) 133-46.

6. P. M. Bummer, Chemical considerations in protein and peptide stability, Drugs Pharm. Sci. 175 (2008) 7-42.

7. R. G. Keck, The use of t-butyl hydroperoxide as a probe for methionine oxidation in proteins, Anal. Biochem. 236 (1996) 56-62.

8. M. A. van Dijk, G. Vidarsson, Monoclonal antibody-based pharmaceuticals, Pharm. Biotechnol. (2nd Ed.) (2002) 283-299.

9. D. Liu, D. Ren, H. Huang, J. Dankberg, R. Rosenfeld, M. J. Cocco, L. Li, D. N. Brems, R. L. Remmele, Jr., Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation, Biochemistry 47 (2008) 5088-100.

10. X. M. Lam, J. Y. Yang, J. L. Cleland, Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2, J. Pharm. Sci. 86 (1997) 1250-5.

11. F. J. Shen, M. Y. Kwong, R. G. Keck, R. J. Harris, The application of tert-butylhydroperoxide oxidation to study sites of potential methionine oxidation in a recombinant antibody, Tech. Protein Chem. VII, [Symp. Protein Soc.], 9th (1996) 275-284.

12. O. Salas-Solano, B. Tomlinson, S. Du, M. Parker, A. Strahan, S. Ma, Optimization and validation of a quantitative capillary electrophoresis sodium dodecyl sulfate method for quality control and stability monitoring of monoclonal antibodies, Anal. Chem. 78 (2006) 6583-94.

13. M. Gilges, M. H. Kleemiss, G. Schomburg, Capillary Zone Electrophoresis Separations of Basic and Acidic Proteins Using Poly(vinyl alcohol) Coatings in Fused Silica Capillaries, Anal. Chem. 66 (2002) 2038-2046.

14. M. H. A. Busch, J. C. Kraak, H. Poppe, Cellulose acetate-coated fused-silica capillaries for the separation of proteins by capillary zone electrophoresis, J. Chromatogr., A 695 (1995) 287-96.

15. B. Verzola, C. Gelfi, P. G. Righetti, Quantitative studies on the adsorption of proteins to the bare silica wall in capillary electrophoresis. II. Effects of adsorbed,

neutral polymers on quenching the interaction, J. Chromatogr., A 874 (2000) 293-303.

16. M. N. Albarghouthi, T. M. Stein, A. E. Barron, Poly-N-hydroxyethylacrylamide as a novel, adsorbed coating for protein separation by capillary electrophoresis, Electrophoresis 24 (2003) 1166-75.

17. J. Krenkova, N. A. Lacher, F. Svec, Highly Efficient Enzyme Reactors Containing Trypsin and Endoproteinase LysC Immobilized on Porous Polymer Monolith Coupled to MS Suitable for Analysis of Antibodies, Anal. Chem. 81 (2009) 2004-2012.

18. M. Zhu, C. J. Siebert, Suppression of electroendosmosis during electrophoresis in gel-free polymer media by use of charged polymers. Application: WO WO, 94-US11371 9510771, 1995.