FLOW-GATED CAPILLARY ELECTROPHORESIS FOR RAPID ANALYSIS OF BIOLOGICAL SAMPLES

A Dissertation by

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Submitted to the Department of Chemistry and the faculty of the Graduate School of Wichita State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

July 2016
FLOW-GATED CAPILLARY ELECTROPHORESIS FOR RAPID ANALYSIS OF BIOLOGICAL SAMPLES

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DEDICATION

To my family and my dear friends

For your love and support
EPIGRAPH

10.1

To see a world in a grain of sand,

And a heaven in a wild flower,

Hold infinity in the palm of your hand,

And eternity in an hour.
ACKNOWLEDGMENTS

I really appreciate the assistance and contributions from various individuals during my Ph.D. study. First, I would like to express my profound gratitude to my research advisor and mentor, Dr. Maojun Gong, for giving me the opportunity to work with him as a Ph.D. student and for his guidance throughout the entire process. I attribute my professional growth to his mentoring, which has led to my development as a creative and enthusiastic scientist. I greatly appreciate his pushing me out of my comfort zone, telling me that I could do better, and setting up a role model for my professional career.

I would like to extend my gratitude to members of my committee, Dr. Douglas S. English, Dr. Kandatege Wimalasena, Dr. Katie Mitchell-Koch and Dr. Ramazan Asmatulu, for their helpful comments and suggestions on all stages of this dissertation.

I would like to thank Dr. Katie Mitchell-Koch and Dr. Jeremy Mitchell-Koch for giving me valuable suggestions during my M.S. and Ph. D and for presenting chemistry as a useful and interesting subject; without these two educators it would be unlikely that I have pursued an advanced degree in chemistry.

I would like to thank Dr. Kevin Langenwalter for providing our group sufficient aids on the instrumental facilities. I also acknowledge Dr. Alexander Chong for teaching me basic LabVIEW programming.

I am thankful to the members of Gong group for their participation and encouragement. In particular, Dr. Qingfu Zhu, Naveen Maddukuri, Ning Zhang and Shan Shi worked with me on various projects. I thank Archana Mishra for her thoughtful help and valuable peer support during the past four years. I also thank Anushka Chathuranga, Pathum Weerawarna, Matthew Baird, Jon Ellis, Lava Raj Kadel, Sireesha Mamillapalli,
Sumudu Tharangani Mapa, Vishnu C. Damalanka and Ravi Vattepu for their help and support during my journey in graduate school, and Susan McCoy and Mary Cambridge for their assistance in the chemistry stockroom.

Many thanks go to my family and friends for guidance and advice along the way. Their encouragements gave me so much strength to complete my study.

I also gratefully acknowledge the financial support from various sources including Gene Zaid Summer Fellowship in Chemistry, Chemistry Department at Wichita State University, Kansas IDeA Network of Biomedical Research Excellence (K-INBRE) and the National Institutes of Health (NIH) of U. S.
ABSTRACT

Capillary electrophoresis (CE) and related technologies have been utilized with great success for a variety of bioanalytical applications. CE has the advantages of decreased analysis time, integrated sample processing, high portability, high throughput, and minimal reagent consumption. Microfabricated fluidic devices have also gained considerable interests due to its miniaturization and high level of integration. The goal of my research projects was to develop an integrated microfluidic system and technologies to perform in vitro and in vivo analysis of biological samples via electrophoretic separations. This dissertation focuses on the construction of flow-gated capillary electrophoresis systems and the development of related technologies coupled with laser induced fluorescence detection (CE-LIF) for rapid separation and sensitive detection of important components in biological samples. This dissertation first presents a custom-made PDMS-interconnected flow-gated CE system targeting on measurements of essential neurotransmitters. Experimental results show that this system was capable of performing long-term measurements with reproducibility, accuracy, sensitivity and robustness. Then, a novel and simple on-line sample preconcentration method was developed exclusively for enriching fluorescent derivatives of catecholamines, including dopamine and norepinephrine. The detection enhancement of over 100 folds was obtained and the limits of detection were lowered to the pico-molar range. Finally, a rapid and sensitive method was developed for the determination of cyanide ions in human urine, which is suitable for real-world applications, such as early diagnosis of cyanide exposure.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CHAPTER 1: INTRODUCTION</td>
</tr>
<tr>
<td></td>
<td>1.1. Dissertation Overview</td>
</tr>
<tr>
<td></td>
<td>1.2. Capillary Electrophoresis</td>
</tr>
<tr>
<td></td>
<td>1.2.1. Modes of CE</td>
</tr>
<tr>
<td></td>
<td>1.3. Electroosmotic flow and electrophoretic mobility</td>
</tr>
<tr>
<td></td>
<td>1.3.1. Instrument</td>
</tr>
<tr>
<td></td>
<td>1.3.2. EOF measurement methods</td>
</tr>
<tr>
<td></td>
<td>1.3.3. Efficiency</td>
</tr>
<tr>
<td></td>
<td>1.3.4. Resolution</td>
</tr>
<tr>
<td></td>
<td>1.3.5. Dispersion</td>
</tr>
<tr>
<td></td>
<td>1.3.6. Injection</td>
</tr>
<tr>
<td></td>
<td>1.3.7. Joule heating</td>
</tr>
<tr>
<td></td>
<td>1.3.8. Electromigration</td>
</tr>
<tr>
<td></td>
<td>1.3.9. Detection methods</td>
</tr>
<tr>
<td>2.</td>
<td>CHAPTER 2: PDMS-INTERCONNECTED MICROFLUIDIC SYSTEMS FOR RAPID SEPARATIONS OF NEUROTRANSMITTERS</td>
</tr>
<tr>
<td></td>
<td>2.1. Introduction</td>
</tr>
<tr>
<td></td>
<td>2.2. Experimental</td>
</tr>
<tr>
<td></td>
<td>2.2.1. Materials and reagents</td>
</tr>
<tr>
<td></td>
<td>2.2.2. Preparation of molds</td>
</tr>
<tr>
<td></td>
<td>2.2.3. Prototyping</td>
</tr>
<tr>
<td></td>
<td>2.2.4. Instrumentation</td>
</tr>
<tr>
<td></td>
<td>2.2.5. Amino acid separation</td>
</tr>
<tr>
<td></td>
<td>2.3. Results and Discussion</td>
</tr>
<tr>
<td></td>
<td>2.3.1. Interface fabrication and capillary arrangement</td>
</tr>
<tr>
<td></td>
<td>2.3.2. Flow-gated injection</td>
</tr>
<tr>
<td></td>
<td>2.3.3. Performance of interconnected system</td>
</tr>
<tr>
<td></td>
<td>2.4. Conclusions</td>
</tr>
<tr>
<td>3.</td>
<td>CHAPTER 3: DETECTION OF CATECHOLAMINES IN CEREBOROSPINAL FLUID USING AN ON-LINE PRECONCENTRATION METHOD</td>
</tr>
<tr>
<td></td>
<td>3.1. Introduction</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2. Experimental</td>
<td>51</td>
</tr>
<tr>
<td>3.2.1. Chemicals and reagents</td>
<td>51</td>
</tr>
<tr>
<td>3.2.2. Instrumentation</td>
<td>52</td>
</tr>
<tr>
<td>3.2.3. Experimental conditions</td>
<td>53</td>
</tr>
<tr>
<td>3.3. Results and Discussion</td>
<td>54</td>
</tr>
<tr>
<td>3.3.1. Mechanism of borate-assisted CA preconcentration</td>
<td>54</td>
</tr>
<tr>
<td>3.3.2. pH effect on CA-CBI focusing</td>
<td>57</td>
</tr>
<tr>
<td>3.3.3. Borate concentration effect</td>
<td>61</td>
</tr>
<tr>
<td>3.3.4. Sample and BGE conductivity effect</td>
<td>64</td>
</tr>
<tr>
<td>3.3.5. Other factors on CA focusing</td>
<td>65</td>
</tr>
<tr>
<td>3.3.6. Separation of DA/NE-CBI</td>
<td>68</td>
</tr>
<tr>
<td>3.3.7. Concentration enhancement and LODs</td>
<td>69</td>
</tr>
<tr>
<td>3.3.8. Calibration curve</td>
<td>70</td>
</tr>
<tr>
<td>3.3.9. CSF sample with spiking</td>
<td>71</td>
</tr>
<tr>
<td>3.4. Conclusions</td>
<td>72</td>
</tr>
<tr>
<td>4. CHAPTER 4: RAPID DETERMINATION OF CYANIDE IN HUMAN URINE BY CE-LIF</td>
<td>80</td>
</tr>
<tr>
<td>4.1. Introduction</td>
<td>80</td>
</tr>
<tr>
<td>4.2. Experimental</td>
<td>82</td>
</tr>
<tr>
<td>4.2.1. Materials and reagents</td>
<td>82</td>
</tr>
<tr>
<td>4.2.2. Derivatization of cyanide</td>
<td>82</td>
</tr>
<tr>
<td>4.2.3. Instrumentation</td>
<td>83</td>
</tr>
<tr>
<td>4.3. Results and Discussion</td>
<td>84</td>
</tr>
<tr>
<td>4.3.1. Fluorogenic derivatization of cyanide ions</td>
<td>84</td>
</tr>
<tr>
<td>4.3.2. Optimization of conditions</td>
<td>87</td>
</tr>
<tr>
<td>4.3.3. Potential interferences</td>
<td>89</td>
</tr>
<tr>
<td>4.3.4. Cyanide measurements of smokers and non-smokers</td>
<td>90</td>
</tr>
<tr>
<td>4.4. Concluding Remarks</td>
<td>92</td>
</tr>
<tr>
<td>5. CHAPTER 5: OVERALL CONCLUSION</td>
<td>96</td>
</tr>
<tr>
<td>5.1. Summary</td>
<td>96</td>
</tr>
<tr>
<td>5.2. Future Work</td>
<td>97</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Capillary zone electrophoresis</td>
<td>3</td>
</tr>
<tr>
<td>1.2. Micellar electrokinetic chromatography with anionic micelles</td>
<td>4</td>
</tr>
<tr>
<td>1.3. Capillary flow profiles</td>
<td>5</td>
</tr>
<tr>
<td>1.4. Schematic diagram of a CE instrumental setup</td>
<td>7</td>
</tr>
<tr>
<td>1.5. Principle of current monitoring method for EOF determination</td>
<td>9</td>
</tr>
<tr>
<td>1.6. Effect of electromigration on the separation of cations</td>
<td>16</td>
</tr>
<tr>
<td>2.1. Image of a machined flow gate with polycarbonate</td>
<td>28</td>
</tr>
<tr>
<td>2.2. Schematic diagram showing the preparation procedure of molds for PDMS casting of the flow gate and the 4-way mixing interface</td>
<td>31</td>
</tr>
<tr>
<td>2.3. Images of the flow gate and tubing assembly</td>
<td>32</td>
</tr>
<tr>
<td>2.5. Images of 4-way mixing capillary assemblies</td>
<td>35</td>
</tr>
<tr>
<td>2.6. Images of tube-to-tube connectors</td>
<td>36</td>
</tr>
<tr>
<td>2.7. Flow-gated injection process in the assembled PDMS interface</td>
<td>37</td>
</tr>
<tr>
<td>2.8. Gating by using different flow rates and various distances between the capillary tips</td>
<td>39</td>
</tr>
<tr>
<td>2.9. Performance of interconnected system</td>
<td>41</td>
</tr>
<tr>
<td>2.10. Separation of 20 analytes by MEKC</td>
<td>42</td>
</tr>
<tr>
<td>3.1. Schematic procedure showing on-line DA/NE-CBI preconcentration and separation</td>
<td>55</td>
</tr>
<tr>
<td>3.2. Focusing comparison</td>
<td>60</td>
</tr>
<tr>
<td>3.3. Comparison of NE-CBI focusing results at various concentrations of borate in sample mixtures and at various injection times</td>
<td>61</td>
</tr>
<tr>
<td>3.4. BGE borate effect on NE-CBI focusing</td>
<td>63</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>3.5. Sample and BGE conductivity effect</td>
<td>64</td>
</tr>
<tr>
<td>3.6. Oxidation of DA and NE in various solvents with different pH values</td>
<td>67</td>
</tr>
<tr>
<td>3.7. Typical electropherogram showing the separation of DA-CBI and NE-CBI</td>
<td>68</td>
</tr>
<tr>
<td>3.8. DA-CBI peak sizes increase with DA concentrations.</td>
<td>69</td>
</tr>
<tr>
<td>3.9. CSF sample with spiking.</td>
<td>70</td>
</tr>
<tr>
<td>4.1. NDA and the primary amine react to produce imines without the present of cyanide ions</td>
<td>85</td>
</tr>
<tr>
<td>4.2. Different organic solvents for dissolving NDA. Peak heights of the derivative by NDA, glycine and CN^−</td>
<td>86</td>
</tr>
<tr>
<td>4.3. Optimization of conditions</td>
<td>88</td>
</tr>
<tr>
<td>4.4. Interference effect on CN^− derivative peak height.</td>
<td>89</td>
</tr>
<tr>
<td>4.5. Electropherograms of urine with standard additions</td>
<td>91</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. LODs for common CE detectors</td>
<td>17</td>
</tr>
<tr>
<td>3.1. Mobilities determined in the sample solution during injections.</td>
<td>65</td>
</tr>
<tr>
<td>4.1. Summarized Results of Smokers and Non-Smokers</td>
<td>90</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>affinity capillary electrophoresis</td>
</tr>
<tr>
<td>APCE</td>
<td>affinity probe capillary electrophoresis</td>
</tr>
<tr>
<td>BGE</td>
<td>background electrolyte</td>
</tr>
<tr>
<td>CA</td>
<td>catecholamine</td>
</tr>
<tr>
<td>CAC</td>
<td>critical aggregation concentration</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CD</td>
<td>hydroxypropyl-beta-cyclodextrin</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary electrochromatography</td>
</tr>
<tr>
<td>CGE</td>
<td>capillary gel electrophoresis</td>
</tr>
<tr>
<td>CIEF</td>
<td>capillary isoelectric focusing</td>
</tr>
<tr>
<td>CITP</td>
<td>capillary isotachophoresis</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>CZE</td>
<td>capillary zone electrophoresis</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>EC</td>
<td>electrochemical</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EOF</td>
<td>electroosmotic flow</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein-5-isothiocyanate</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS (continued)

IS internal standard
ITP isotachophoresis
LC liquid chromatography
LIF laser-induced fluorescence
LOD limit of detection
MEKC micellar electrokinetic chromatography
MS mass spectrometry
NDA naphthalene-2,3-dicarboxaldehyde
PDMS polydimethylsiloxane
pI isoelectric point
PMT photomultiplier tube
RSD relative standard deviation
SDS sodium dodecyl sulfate
SPE solid phase extraction
TOF time of flight
Tris tris(hydroxyaminomethane)
UV ultraviolet
VIS visible
ζ zeta potential
ε dielectric constant
η viscosity
CHAPTER 1: INTRODUCTION

1.1. Dissertation Overview

Capillary electrophoresis (CE) is a special separation method which utilizes electrical field to separate the components of a mixture [1,2]. CE carries out separations within the confines of a narrow tube, which is differentiated from other forms of electrophoresis [3], such as gel electrophoresis. Stellan Hjérten preformed the first work in CE in 1967, using millimeter-diameter capillary tubes [4]. Since the early 1980s, with its renaissance especially attributed to Jorgenson’s pioneering work, CE has attracted wide attention in scientific community and industry [5-7]. In the past thirty years, CE has been developed into a family of related techniques that employ narrow-bore (5-200 μm ID) capillaries to perform highly efficient separations of both large and small molecules, which makes CE an attractive separation tool, especially for the analysis of biological samples [8,9]. The CE family includes capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), micellar electrophoresis chromatography (MEKC) and capillary gel electrophoresis (CGE) [10-14]. Microchip capillary electrophoresis (MCE) has been established as an important miniaturization of traditional CE and has found widespread use in academic laboratories and more recently somewhat use in industry [15-17]. Compared with conventional CE, MCE has numerous advantages such as smaller sizes, further reduced consumption of reagents and analytes, ease for integration, and rapid analysis [18-20].

My goal is to develop an integrated microfluidic system coupled with electrophoretic separations for rapid determination of target analytes with integrated sample processing, high throughput, minimal reagent consumption, and low analysis cost.
The ultimate goal is to develop a lap-on-a-chip system with high portability and highly efficient separations. The second chapter of this dissertation presents the development of a rapid prototyping method to fabricate monolithic poly(dimethylsiloxane) (PDMS) interfaces for tube-to-tube connection, online reagent mixing and flow-gated injection in an integrated CE system [21]. The third chapter focuses on the improvement of the detection sensitivity for catecholamine with the integrated microfluidic CE system coupled with sequential procedures including microdialysis, on-line mixing and fluorogenic derivatization [22]. The fourth chapter discusses the development of a novel detection method for cyanide ions by using CN⁻ fluorogenic derivatization with NDA at the existence of a primary amine [23].

1.2. Capillary Electrophoresis

CE is an analytical technique that separates compounds based on their electrophoretic mobilities under an applied voltage. In 1981, Jorgenson and Lukacs first described the use of a fused silica capillary column and aqueous buffer to separate charged compounds, indicating the potential of CE as a new analytical separation technique [5,6]. CE exhibits the capability to efficiently separate both charged and neutral molecules in a short analysis time (minutes) while using small sample and reagent volumes (μL). These attractive features make CE a competitive alternative to the traditional techniques such as high pressure liquid chromatography (HPLC) or gas chromatography (GC).
1.2.1. Modes of CE

As a rapidly growing separation technique, CE has diverse applications, including food analysis [24,25], pharmaceutical analysis [26,27], bioanalysis [28], and environmental analysis [29-30]. The versatility of CE is partially originated from its various modes of operation. Based on the separation mechanism, the main modes of CE can be classified as follows: capillary zone electrophoresis (CZE) (separating analytes via their different electrophoretic mobilities) [31,32] (Figure 1.1), micellar electrokinetic chromatography (MEKC) (separation of compounds based on their partitioning between the background electrolyte (BGE) and the micelles that serve as a pseudo-stationary phase) [33,34] (Figure 1.2), capillary gel electrophoresis (CGE) (the adaptation of traditional gel electrophoresis into the capillary using polymers in solution to create a

**Figure 1.1.** Capillary zone electrophoresis. The negatively charged silanol groups on capillary walls attract cations from buffer, creating an electric double layer. Once applying a voltage, an electroosmotic flow is generated causing the bulk flow along the capillary. In a basic buffer, all analytes are normally carried with the buffer solution towards the cathode. Based on their differing mobilities or velocities, cations come out first, and anions later, while all neutral compounds co-elute with EOF without any resolution.
molecular sieve) [35,36], capillary isoelectric focusing (CIEF) (a high-resolution technique for protein separation based on differences in isoelectric points (pI) [37,38], capillary isotachophoresis (CITP) (a “moving boundary” electrophoretic technique in which sample components condense between leading and terminating constituents, producing a steady-state migrating configuration composed of consecutive sample zones [39], and capillary electrochromatography (CEC) (an emerging hybrid separation technique that combines advantages of both electrophoretic and chromatographic processes for the separation of compound mixtures in columns packed with a chromatographic stationary phase) [40,41].

![Diagram of micellar electrokinetic chromatography](image)

**Figure 1.2.** Micellar electrokinetic chromatography with anionic micelles. During separation, compounds are separated according to their differing affinities for the micelles, which are formed by adding a surfactant to BGE above its critical micellar concentration (CMC).

CZE is the most widely used mode because it is applicable to separations of both anions and cations, and from small ions to large ones. The development of MEKC is a major advancement in CE because it has provided a method for separation of electrically neutral compounds. Schematic representations of the separation mechanism of CZE and
MEKC are shown in Figure 1.1 and Figure 1.2 since these two separation modes were used in the following chapters.

A. Electroosmotic flow

B. Pressure-driven flow

**Figure 1.3.** Capillary flow profiles. (A) Plug-like electroosmotic flow through a capillary. (B) Pressure-driven parabolic flow through a capillary.

### 1.3. Electroosmotic flow and electrophoretic mobility

EOF plays a vital role in CE and MCE [42]. A zeta potential is generated at the surface-buffer interface in a capillary or a microfabricated channel, where an electrical double layer is formed by attracting mobile cations in BGE to the negatively charged inner wall of the capillary [31]. Upon a voltage applied across the capillary, the cations in the diffuse portion of double layer migrate towards the cathode, carrying water with them, and finally resulting in a net flow of bulk BGE moving to the same direction. Since the EOF is generally greater than the electrophoretic velocities of analytes, all analytes are carried
with BGE towards the cathode. In the case of the positively charged surface of the capillary, anions associate with the capillary wall and the resulting EOF moves to the anode. The flat flow profile (Figure 1.3) for EOF results in high-efficiency separation with significantly less deleterious dispersive effects than the parabolic one encountered in HPLC, which relies on high pressure pumps to induce flow [43-46]. The electroosmotic mobility ($\mu_{eof}$) is defined in Equation 1.1, and is related to the zeta potential ($\zeta$) (governed by the charge on the capillary surface) across the double layer, the viscosity ($\eta$) and the dielectric constant ($\varepsilon$) of the BGE as shown in Equation 1.2. The magnitude of EOF will change due to changes in surface charge density, in the pH and BGE composition, and changes in temperature [44,47-49].

$$\mu_{eof} = \frac{V_{eof}}{E}$$ (1.1)

where, $V_{eof}$ is the linear velocity of EOF, and $E$ is the electrical field strength.

$$\mu_{eof} = \frac{\varepsilon\zeta}{4\pi\eta r}$$ (1.2)

where $r$ is the capillary inner radius.

In CZE with normal polarity, charged analytes are separated in the BGE according to their individual electrophoretic mobilities in an electrical field. The resulting electrophoretic velocities in this field are described by Equation 1.3 in which velocity ($v_{ep}$) is equal to the intrinsic electrophoretic mobility of an analyte ($\mu_{ep}$) multiplied by the field strength ($E$).

$$v_{ep} = \frac{q}{f}E = \mu_{ep}E$$ (1.3)

Electrophoretic mobility ($\mu_{ep}$) is governed by the analyte’s charge ($q$) and frictional coefficient ($f$) as defined by the Stokes equation (Equation 1.4).
\[ f = 6\pi \eta r \]  

(1.4)

This equation describes the frictional coefficient \( f \) for a spherical particle having a hydrodynamic radius \( r \) in a solution with viscosity \( \eta \).

\[ v_{\text{app}} = \mu_{\text{app}} E = (\mu_{\text{ep}} + \mu_{\text{eof}}) E \]  

(1.5)

In the presence of EOF, the apparent velocity \( v_{\text{app}} \) is proportional to the sum of both the electrophoretic \( \mu_{\text{ep}} \) and electroosmotic mobilities \( \mu_{\text{eof}} \) as described in Equation 1.5.

**Figure 1.4.** Schematic diagram of a CE instrumental setup.

### 1.3.1. Instrument

A typical flow-gated CE system used in this dissertation is shown in Figure 1.4. A high voltage is applied across the capillary through platinum electrodes in buffer. Sample injection and separation are performed by applying a negative voltage on the capillary outlet reservoir. With this setup, a sample plug is electrokinetically injected via the flow-gated injection, and then separated. This procedure is controlled by a LabVIEW program.
For on-line mixing as shown in Figure 1.4, a 4-syringe pump is used to supply sample and derivatization reagents through individual gastight Hamilton syringes. Data are recorded and processed with a desktop computer.

1.3.2. EOF measurement methods

Given the importance of EOF, accurate and precise methods for its measurement are useful. Several EOF measurement methods have been reported for CE and MCE [50]. The method with a neutral marker is the earliest among those methods reported for measuring EOF in CE [5,51]. This method relies on the injection of an electrically neutral compound followed by recording its migration time arriving at the detector. The neutral marker has no interaction with the electrolyte and the capillary wall, and thus provides an average of EOF. As an alternative, the fluorescent marker method involves the introduction of a fluorescent agent downstream in the EOF direction and monitoring its appearance at the end of the capillary [52]. However, this method is less widely used due to the need of a bulky fluorescence detection system and the possible contamination caused by the fluorescent agent. The average EOF rate can also be measured by weighing the effluent from a capillary with an analytical balance [53,54]. A potential error with this method is the mass loss by evaporation; therefore, it is not used in microchips because of the small liquid mass transported by EOF. As the most widely used method, the current monitoring measures the electrophoretic current change as an electrolyte with different ionic strength gradually replaces the original buffer in the capillary (Figure 1.5) [55]. This current monitoring is a build-in function with the high voltage power supply and the LabVIEW program. Reported precision for average EOF measured by this method in CE and microchip CE ranges between 5% and 15% [55-57]. Based on a similar principle,
a conductivity cell monitors the change in bulk solution conductivity between two electrodes when a background electrolyte band passes through the electrode gap [58]. More reproducible EOF measurements (relative standard deviation (RSD) 1.9%) were reported using this method than the current monitoring method (RSD 5.9%) by Henry's group [59].

Figure 1.5. Principle of current monitoring method for EOF determination.

1.3.3. Efficiency

Separation efficiency in terms of theoretical plates (N) describes the separation quality by quantifying band broadening. N is dependent on the effective capillary length (l) and the variance of the analyte concentration profile (σ²) according to the following Equation 1.6 [60].

\[ N = \frac{t^2}{\sigma^2} = \frac{l}{H} \]  

(1.6)

N can also be defined in terms of theoretical plate height (H), a constant that relates band broadening to the distance an analyte migrates in a column [61,62].

\[ H = \frac{\sigma^2}{l} \]  

(1.7)

In many separations, H resulting from the column is predicted using the van Deemter
equation, where \( u \) is the flow rate through the column and \( A, B, \) and \( C \) are constants.

\[
H = A + \left( \frac{B}{u} \right) + Cu
\]  
(1.8)

\( A, B, \) and \( C \) describe band broadening associated with multiple flow paths in a packed column, longitudinal diffusion, and mass transfer of an analyte between the stationary and mobile phases, respectively [62].

\[
H = \frac{B}{u}
\]  
(1.9)

In CZE, the column is not packed and the separation is based on the charge-to-size ratio of ions rather than differences in analyte partitioning between stationary and mobile phases; thus, \( H \) simplifies to Equation 1.9 and band broadening is theoretically limited by longitudinal diffusion. Diffusional band broadening is described by the Einstein equation with the migration time and the diffusion coefficient of the analyte represented by \( t \) and \( D \), respectively [60,63,64].

\[
\sigma_D^2 = 2Dt
\]  
(1.10)

This equation shows that analytes with long migration times have greater longitudinal diffusion and associated band broadening during a separation. An analyte’s migration time is dependent on its apparent electrophoretic mobility (\( \mu_{\text{total}} \)), the effective and total column length (\( l \) and \( L \), respectively), and the separation potential (\( V \)) [61,63,64].

\[
t = \frac{Ll}{\mu_{\text{total}}V}
\]  
(1.11)

Combining Equations 1.10, 1.14, and 1.15 results in the following expression for \( N \).

\[
N = \frac{\mu_{\text{total}}V}{2D}
\]  
(1.12)

This equation shows that the separation efficiency is proportional to the applied
voltage; thus, efficiency should increase with increasing potential if diffusion is the only
source of band broadening [61,63].

N can also be calculated directly from an electropherogram. When Gaussian peak
profiles are obtained, Equation 1.13 is utilized where \( t \) is the migration time of the analyte
and \( w_{1/2} \) is the width of the peak at half maximum [60,64].

\[
N = 5.54 \left( \frac{t}{w_{1/2}} \right)^2 \tag{1.13}
\]

N calculated from an electropherogram is often less than the theoretical plates
determined by Equation 1.12 due to other sources of band broadening resulting from
instrumental parameters or non-ideal interactions during separation.

1.3.4. Resolution

Resolution (R) describes the separation of analytes based on differential migration
times (t) and dispersion (\( \sigma \)) during a separation [61].

\[
R = \frac{t_2 - t_1}{2(\sigma_1 + \sigma_2)} \tag{1.14}
\]

In CZE, resolution can be defined in terms of N and the apparent (\( \mu_1 \) and \( \mu_2 \)) and
the average (\( \mu = (\mu_1 + \mu_2)/2 \)) electrophoretic mobilities of each analyte [60,61].

\[
R = \frac{1}{4} \sqrt{N \frac{\mu_2 - \mu_1}{\mu}} \tag{1.15}
\]

This equation is divided into components for efficiency and selectivity. R can be increased
by changing experimental parameters, such as V and T, which affect N, or by modifying
the analyte mobilities by altering pH. Changing either experimental or analyte parameters
varies the resolution, though greater improvement results from modifying analyte
separation conditions [61].
1.3.5. Dispersion

Whereas the van Deemter equation defines traditional column-based broadening, other sources are also involved. The total variance can be approximated as the sum of all variances associated with the instrument and separation.

\[ \sigma^2_{\text{tot}} = \sigma^2_D + \sigma^2_{\text{inj}} + \sigma^2_T + \sigma^2_M + \sigma^2_S \]  

(1.16)

\( \sigma^2_D, \sigma^2_{\text{inj}}, \sigma^2_T, \sigma^2_M \) and \( \sigma^2_S \) represent the variances associated with diffusion, injection, Joule heating, electromigration, and sorption, respectively [60,61,63,64].

1.3.6. Injection

In CE, analytes are loaded into the capillary using electrokinetic or hydrodynamic injection methods. For electrokinetic injection, a potential is applied across the capillary causing analytes to migrate into the capillary based on their electrophoretic velocities and/or EOF. Since EOF drives material into the capillary, the initial sample plug forms a rectangular profile with a variance based on the injection length (l), Equation 1.17.

\[ \sigma^2_{\text{inj}} = \frac{l^2}{12} \]  

(1.17)

The injection variance can be minimized by reducing the length of the sample zone.

Hydrodynamic injection involves the application of a pressure resulting in shear forces and laminar flow. For hydrodynamic injection, the variance can be estimated by Taylor dispersion which is dependent on the length (L) and inner radius (r) of the capillary, pressure difference (\( \Delta P \)), injection time (t), and diffusion coefficient of the solute (D), as expressed by Equation 1.18.
\[
\sigma_{inj}^2 = \frac{r^6 \Delta p^2 t}{1536 L^2 \eta^2 D}
\]  
(1.18)

In reality, the leading edge of the sample plug shows Taylor dispersion; yet, after the sample is injected and a potential is applied to drive separation, the end of the sample plug has a flat flow profile. Thus, the variance for a hydrodynamic injection is an overestimation of the injection variance [61].

In general, the sample plug should be less than 1-2% of the capillary length to prevent sample overloading, which results in decreased resolution and distorted peak shapes due to potential conductivity mismatch between the sample and BGE [64]. Injections can be divided into two primary categories, offline and online, with online injection being more compatible for analyzing dynamic processes within chemical or biological systems.

1.3.6.1 Offline injection

Offline injection requires the capillary to be moved between a sample vial for injection and a buffer vial for separation. For hydrodynamic offline injection, sample is injected by applying a pressure to the sample vial at the capillary inlet or a vacuum to the buffer vial at the capillary outlet. Alternatively, sample can be siphoned into the capillary by raising the sample vial to a position higher than the outlet end of the capillary.

Electrokinetic offline injection is performed by applying a potential across the capillary. After injection, the potential is removed, and the capillary is moved to the buffer vial to perform separation. The molar quantity (Q) of an analyte entering the capillary during an electrokinetic injection is dependent on the voltage (V), radius (r) and length of the capillary (L), the analyte concentration (C) and its electrophoretic mobility (\(\mu_{ep}\)), the electroosmotic mobility (\(\mu_{eof}\)), and injection time (t) [63,65].
\[ Q = \frac{(\mu_{ep} + \mu_{eo})V\pi r^2 Ct}{L} \]  

Analytes migrate into the capillary based on their apparent mobilities which cause bias of sample injection. Cations with high apparent mobilities are preferentially loaded into the capillary and present greater amounts than anions[61,63,64].

Hydrodynamic injection results in somewhat reproducible quantities of injected material, but it is slow since the capillary must be moved between a sample and a buffer vials for each injection and separation. To perform fast, reproducible injections, various online injection methods have been developed.

**1.3.6.2 Online injection**

Flow gating injection was initially developed by Lemmo and Jorgenson to perform two-dimensional separations by coupling the outlet of a micro-HPLC column to the inlet of a capillary for CZE [66]. More recently, flow gating has been used to couple sample streams collected from biological systems, such as microdialysis probes, to capillaries for separation [67-74]. The sample and separation capillaries are positioned across from each other with a transverse flow passing between them. When the transverse flow is running, sample exiting the sample capillary is pushed toward a grounded waste vial. When the transverse flow is stopped, sample exiting from the sample capillary accumulates between the two capillaries and some of the material is electrokinetically injected into the separation capillary due to the applied electrical voltage across the capillary. When the transverse flow is resumed, the accumulated sample is pushed to waste and the sample in the separation capillary is separated before detection at a downstream position. This method has been used to monitor various chemical dynamics, but quantitative analyses require the use of long injection times (> 100 ms) so that slight
deviation in the transverse flow due to switching the valve does not affect the injected volume [75,76].

1.3.7. Joule heating

When current passes through a capillary, Joule heat is generated. The current (I) in a capillary is a function of the applied voltage (V), the electrolyte conductivity (κ), the capillary cross section area (S), and the length of the capillary (L) [64]. When there is significant Joule heat in the capillary, radial temperature gradients develop, resulting in higher temperatures at the center of the capillary and lower temperatures at the edges where heat is dissipated to the surrounding environment. A temperature change of 1 °C could result in a 2–3% change in solution viscosity, yielding a 2–3% change in analyte mobility associated with the change in EOF (Equation 1.7). Thus, a radial temperature gradient within the capillary alters the flat flow profile and increases the band broadening during separation [60,64].

1.3.8. Electromigration

Analyte migration is affected when the conductivity of the analyte zone differs from the buffer. This conductivity difference causes analytes to experience different field strengths as they migrate between zones, resulting in electromigration dispersion or concentration. When analytes migrate from a high-conductivity sample zone into a low-conductivity buffer zone, they experience higher field (with a constant current); thus, the analytes entering the buffer zone accelerate away from those still in the sample zone. On the other hand, when analytes diffuse into the trailing zone, which also has lower conductivity and higher electric field, the analytes accelerate back into the sample zone. These effects result in a sample zone represented by a wide fronting peak (Figure 1.6).
Conversely, a sample zone with lower conductivity than the buffer yields a narrow tailing peak for opposite reasons. Gaussian peaks are observed only when the conductivities of the buffer and sample zones are equal. To minimize electromigration dispersion, samples are generally diluted with the BGE by at least 100 times to match the BGE [60,61].

![Diagram showing high, equivalent, and low conductivity sample zones with corresponding peaks.]

**Figure 1.6.** Effect of electromigration on the separation of cations.

### 1.3.9. Detection methods

Detection in CE is complicated by the dimensions and curvature of the capillary as well as the small volumes and associated low molar quantities of analytes [61,63,65]. In spite of these differences, various detection methods have been adapted from HPLC or developed for use in CE with the most common methods relying on optical or electrochemical detection of analytes [61,65]. Table 1.1 summarizes the LODs for common detection methods in CE [64].

Absorption detection monitors interaction between light and analytes which contain chromophores. Traditional absorbance measurement monitors absorption of a chromophore in relation to the concentration and molar absorptivity of an analyte as well as the path length occupied by the sample according to Beer’s law. In a capillary, the
small inner diameter (50-100 μm) reduces the path length and the circular structure causes the path length to vary throughout the region where absorption is being measured. Additionally, the fused silica capillary can lead to scattering and refraction of light that does not interact with analytes in the capillary; it is therefore necessary to tightly focus the incident light.

**Table 1.1.**

LODs for common CE detectors

<table>
<thead>
<tr>
<th>Method</th>
<th>Limit of Detection (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-Vis Absorption</td>
<td>$10^{-5}$ - $10^{-8}$</td>
</tr>
<tr>
<td>LIF</td>
<td>$10^{-9}$ - $10^{-13}$</td>
</tr>
<tr>
<td>Refractive</td>
<td>$10^{-5}$ - $10^{-7}$</td>
</tr>
<tr>
<td>Conductivity</td>
<td>$10^{-5}$ - $10^{-7}$</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>$10^{-8}$ - $10^{-9}$</td>
</tr>
<tr>
<td>Indirect</td>
<td>10 - 100 fold less than direct method</td>
</tr>
</tbody>
</table>

Fluorescence is the most common detection method for high-sensitivity CE systems. The high sensitivity associated with fluorescence detection results from the following factors: (1) the collection of fluorescence emission at a 90° angle from excitation, minimizing scattering from the capillary surface; (2) the Stoke’s shift between the excitation and emission wavelengths of the fluorophore allowing filters to be used to pass only emitted light, further reducing the background [61]; (3) fluorescence is a reoccurring phenomenon and a single fluorophore can fluoresce repeatedly resulting in increased signal. Fluorescence detectors require an excitation source, a detection cell, collection optics, and a photodetector. The most common excitation sources are lasers, which have high power densities, good stability, and coherent light that can be focused to a spot with
the size of the sample stream in the inner diameter of the capillary [61,63,65].

Mass spectrometry (MS) is increasingly becoming popular as a detection method for CE [77-81]. The combination of CE’s high efficiency and high speed with the high sensitivity and selectivity offered by MS is very attractive. CE is very tolerant of complex sample matrices, and therefore its combination with MS provides highly selective detection of compounds in complex mixtures. MS also helps to improve the general sensitivity of CE analyses with appropriate modifications. The benefit of combining MS detection with any separation technique is that it provides a secondary dimension of separations. Therefore, in CZE-MS, for example, CZE performs the first dimensional separation on the basis of analytes’ charge-to-size ratios and then MS carries out the second dimensional separation on the basis of their mass-to-charge ratios (MS). A variety of ionization and coupling methods have been attempted since the first report of CE–MS coupling by Olivares et al. [82]. The most popular method for routine coupling of CE with MS is electrospray ionization (ESI) [81]. Because ESI can generate multicharged ions, it is of great benefit in bringing large molecules, for example, proteins, into the molecular mass range of most mass spectrometers.


CHAPTER 2: PDMS-INTERCONNECTED MICROFLUIDIC SYSTEMS FOR RAPID SEPARATIONS OF NEUROTRANSMITTERS

Note: Most contents in this chapter have been published in Journal of Chromatography A 2014, 1324, 231-237.

2.1. Introduction

The complexity of microfluidic systems is increasing rapidly as various functional parts are coupled within a system. Interconnections play a critical role in the performance of a microfluidic system [2,3]. Commercially available interfaces such as 4-way mixers and flow gates are relatively expensive and difficult to further miniaturize. The conventional fabrication method for these interfaces requires precise micromachining and delicate alignment [4-6]. Compared to hard materials, soft materials such as silicone elastomers have been of great interest for integrated CE systems. Reported in this chapter is a rapid prototyping method to fabricate poly(dimethylsiloxane) (PDMS) interfaces for a flow-gated CE system.

In conventional CE, a sample is introduced into the separation capillary by immersing the capillary tip into the sample solution. To rapidly and repeatedly inject samples for successive separations or to couple CE with an LC column, however, a flow-gated injection strategy has been developed by using flow gates [7-9]. These flow gates are mechanically machined to form a cross configuration in which the opposite branches serve as sample supply and the separation capillaries while the other opposite two branches conduct flow gating and waste straining. By adjusting the relative rates of the gating and sample flows, sample is deflected from the inlet of the separation capillary. To inject a sample, the gating flow is briefly swerved and the sample fills the cross section
and meanwhile, an injection voltage is applied to electrokinetically introduce a sample plug into the capillary. By swerving the gating flow back, flow gating is re-established and separation buffer is brought to contact with the separation capillary. Therefore, the gating flow interface is a major part for the decent flow-gated injection procedure. Moreover, this flow gating configuration provides a convenient way to couple a LC microcolumn or another CE capillary for multi-dimensional separations [10,11].

![Image of a machined flow gate with polycarbonate.](image)

**Figure 2.1.** Image of a machined flow gate with polycarbonate.

Although machined interfaces (Figure 2.1) for flow-gated injection work well, they have numerous disadvantages. First, large inner diameters (typical 1/16 inches, i.e. 1.59 mm) over the outer diameter of silica capillaries (typically 360 μm) require a great gating flow rate to yield effective gating when the two opposite capillary tips are close (such as 40-100 μm) thus, in a given analysis time, it uses a large volume of buffer solution for gating, which will increase the cost of waste disposal; moreover, the buffer is usually supplied through a syringe, and refilling of the syringe will interrupt the experiment during
long-term monitoring of a biological or chemical process, while a large-volume syringe (e.g. 500 mL) and a related syringe pump are considerably expensive. Second, appropriate screw nuts and sleeves are required to stabilize the sample and separation capillaries, while the tightening process of the screws carries both capillaries forward, which poses difficulty in exactly setting the distance between the two capillary tips; so it takes time and is discouraging. Third, the 1/16” holes and the sleeves may fail to exactly match, which would not align the two capillary tips in a line thus taking longer time for the sample flow to fill the gap between the two capillaries before injection. To solve these problems, novel flow gates with improved performance are desired but currently unavailable.

PDMS is an elastic and transparent polymer which has been commonly used for prototyping of microfluidic chips [12-14]. Numerous articles have reported PDMS casting techniques for fabrication of interconnects as linear connectors for tube-to-tube or tube-to-chip connection [15,16]. Also, Bergstrom et al. developed PDMS interfaces for coupling HPLC to CE in 2D separations [17]; however, the two channels lie on different levels which cannot effect efficient flow gating. Therefore, we developed a simple procedure to prepare intersecting cross lines as molds for PDMS flow gate casting. Additionally, we prototyped reagent mixers and tube-to-tube connectors, both based on PDMS casting. Laser-induced fluorescence (LIF) detection is sensitive and thus commonly used in CE and microchip CE. However, most of analytes lack native fluorescence and require derivatization with fluorogenic reagents including naphthalene-2,3-dicarboxaldehyde (NDA) [18], o-phthalaldehyde (OPA) [19], fluorescamine (FC) [20], 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) [21], 3-(2-furoyl)quinoline-2-
carboxaldehyde (FQ) [22], 7-Nitrobenz-2-Oxa-1,3-Diazole (NBD) [23], and numerous other reagents [24,25]. Derivatization is performed either offline or online by mixing numerous reagents to start the reaction. Online derivatization is often preferred to dynamically monitor the concentration variation. Similarly, any analyses involving two or more reagents to form detectable species such as antibody-antigen binding studies and enzyme kinetics analyses also prefer online pre-column mixing and curing before being injected for electrophoretic separations [26]. Therefore, reagents mixers and tube-to-tube connectors are essential to interconnect multiple flow modules.

In this chapter, the method of rapidly prototyping PDMS interfaces will be described. Their performances were demonstrated by using these interfaces in an integrated CE system. These interfaces have smaller channel diameters, offer easy capillary alignment and stabilization, and are reusable, inexpensive, and transparent. To demonstrate the application of the PDMS-interconnected system, amino acid neurotransmitters were derivatized online with NDA at the presence of cyanide and then electrophoretically separated.

2.2. Experimental

2.2.1. Materials and reagents

Miniature stainless steel wires and nylon fishing lines were purchased from McMaster-Carr (Chicago, IL). Sodium tetraborate, ethylenediaminetetraacetic tetrasodium (EDTA), and all amino acids were obtained from Sigma (St. Louis, MO). Potassium cyanide, dimethylsulfoxide (DMSO), and salts (NaCl, KCl, MgSO$_4$, CaCl$_2$, Na$_2$HPO$_4$, and NaH$_2$PO$_4$) for the preparation of artificial cerebral spinal fluid (aCSF) were purchased from Fisher Scientific (Chicago, IL). NDA was ordered from Invitrogen (Eugene,
OR). Aqueous Solutions were prepared in DI water, and NDA stock solution was in DMSO. Fused silica capillaries with 360 μm OD and various IDs were purchased from Polymicro Technologies (Phoenix, AZ).

**Figure 2.2.** Schematic diagram showing the preparation procedure of molds for PDMS casting of the flow gate and the 4-way mixing interface. (a) Side view of the stainless steel wire with clamped slots; (b) top view of the clamped wire with expanded metal; (c) top view of the edge-grinded wire; (d) top view of the nylon line with a needle-punched hole; (e) top view of the assembled wire and nylon line after pressing both sides of the nylon line; (f) the wire/line assembly was suspended in a petri dish.

### 2.2.2. Preparation of molds

*Flow gate and 4-way mixer:* Both molds were prepared by using a similar procedure. A stainless steel wire with a diameter of 305 μm (0.012”) or 230 μm was tapered and pressed hard (but avoid breaking) in the middle by using a plier cutting part to produce slots on both sides (Figure 2.2a); then, both edges along the slots were sanded flat with a Dremel sander (Figure 2.2c). A nylon line with an appropriate diameter (230 μm, 430 μm, or 560 μm) was first gently pressed by using the plier cutting part to produce a flat section, and then a hole at the center of the pressed area was punched with a sewing needle on an inverted microscope (Figure 2.2d). Then, the tapered and slotted wire was inserted through the hole until reaching the slots, and the cross section was then
gently pressed with the flat part of a plier (Figure 2.2e). The assembly of the cross was placed in a 1-inch (diameter) plastic petri dish through 4 uniformly distributed holes on the side wall (Figure 2.2f). The nylon line was tightened and stabilized with a sticky tape.

**Figure 2.3.** Images of the flow gate and tubing assembly. (a) PDMS interface filled with blue dye. (b) Close look of the PDMS cross section; horizontal diameter 300 μm and vertical 430 μm. (c) Flow gate with assembled tubing. (d) Close look of the head-to-head capillaries in a flow gate.

*Linear connector:* A 200-μm (ID) silica capillary was smoothened on one end with sand paper. A 230-μm wire was tapered and inserted into the smoothened side of the capillary until it stopped. The linear assembly was placed in a 1-inch petri dish through two opposite holes on the side wall. For capillary-to-capillary connectors, 230-μm wires were directly used as molds.

### 2.2.3. Prototyping

PDMS prepolymer and curing reagent at the mass ratio of 10/1 were thoroughly mixed, degased under a vacuum, and poured into a petri dish with the mold suspended. The mixture was cured for >30 minutes at 80 °C. The wire was pulled out followed by the nylon line. The solidified PDMS block was taken out of the petri dish and then was placed back
to the oven and cured at 110 °C for > 1 hour to enhance PDMS rigidity. Finally, the PDMS blocks were cut into cubic shapes and were ready to use (see Figure 2.3b).

2.2.4. Instrumentation

As schematically shown in Figure 2.4, the lab-built CE detection system consisted of a Zeiss Axioskop 20 microscope, a 442-nm laser (Laserglow Technologies, Toronto, ON, Canada), a set of filters (Edmund Optics Inc., Barrington, NJ): a bandpass excitation filter (442±5 nm), a fluorescence dichroic filter (458 nm cut-on), and a bandpass emission filter (482±17 nm), a 40x oil-immersing objective (Carl Zeiss Microscopy, Thornwood, NY) and a PMT module (H10722-210, Hamamatsu Photonics, Japan). A high-voltage power supply (Model CZE1000R) was purchased from Spellman High Voltage Electronics Corporation (Hauppauge, NY). A 3-way solenoid valve (Cole-Parmer, Vernon Hills, IL) was used for gating flow switching. The system was controlled via custom-programmed LabVIEW software (National Instruments, Austin, TX). A single-syringe pump (Harvard Apparatus, Holliston, MA) was used to deliver separation buffer also serving as the gating flow. A 4-syringe pump (Chemyx Inc., Stafford, TX) was used to supply sample and derivatization reagents through individual gas-tight Hamilton syringes (Reno, Nevada). Capillaries were tapered by using circularly-cut sand paper mounted on the Dremel rotor. The capillary was held and rotated using two fingers with one end against the rotating sand paper at an angle of about 30-45°.

2.2.5. Amino acid separation

Amino acid solutions were diluted to expected concentrations with aCSF from stock solutions prepared in DI water. NDA in water/DMSO (50/50 in volume) was diluted to 5 mM with DI water and 10 mM tetraborate buffer at pH 9.2 from NDA stock solution in
DMSO. 10-mM KCN in 20-mM tetraborate buffer (pH 9.2) and 50-mM EDTA was also prepared from a 100-mM stock solution in DI water. Separation was performed by using a 10-μm (ID) capillary at an effective length of 10 cm and 16 cm in total. Amino acids were pumped through a syringe (sample in the syringe) or dialyzed through a side-by-side dialysis probe [27] (aCSF in the syringe) which was manually inserted to standard sample solutions. Flow-gated injection was performed at -5 kV (0.5 s) applied at the capillary outlet through a centrifuge tube filled with separation buffer, 20-mM tetraborate at pH 9.2, and the buffer waste was grounded through a stainless steel tube inserted in the flow gate (Figure 2.4). Separation was performed under -25-kV high-voltage (-HV, Figure 2.3).

2.3. Results and Discussion

2.3.1. Interface fabrication and capillary arrangement

Basically, the molds were assembled by using commercially available stainless steel wires and nylon lines. These wires can spring back when bended, and nylon lines with various diameters (230 μm, 430 μm, and 560 μm) have high strength. The pressed flat part of the nylon line simplified the punching procedure with a sewing needle. A tapered wire was inserted and had tight contact with nylon lines, which prevented flowing PDMS from filling gaps. The cross section should have small dimensions to reduce dead volumes, especially the 4-way mixing interface (3 to 1). The pressed slots on the wire accommodated the thickness of nylon lines on both sides (see Figure 2.2a) so the total thickness of the cross after final pressing was reduced to the same as the wire diameters (230 μm or 305 μm). The cross section of either the flow gate or the mixer might have tiny PDMS pieces (see Figure 2.3d) due to the roughness coming from the inherent nature of the assembled molds; however, this roughness rarely affected the function of interfaces.
based on visual observation of flows in interfaces and the reproducible peak height as shown in Figure 2.9c.

In the flow gate, tapered capillaries were inserted into the cross section with an appropriate distance (e.g. 40 μm) apart which was much smaller than the diameter (430 or 560 μm) of the gating flow channel as shown in Figure 2.3c, while the gating flow was guided by using two stainless steel tubes at diameters of 0.51/0.82 mm (ID/OD). Considering that the backpressure in the flow gate was released through the gating flow waste branch, the channel diameter for silica capillaries was 305 μm (instead of 230 μm) which reduced the elastic stress applied on the 360-μm (OD) capillaries thus permitting easier capillary insertion and distance adjustment. On the other hand, smaller PDMS channels than tubes secured tubes in position prevented liquid leaking.

![Image](A) Assembled mixing interface with sample in the middle.

![Image](B) Assembled mixing with sample on the side.

**Figure 2.4.** Images of 4-way mixing capillary assemblies. (a) 100 nL/min each; (b) 200 nL/min each; (c) 400 nL/min each; (d) 800 nL/min each. The green dye represented the sample. The clear branches had water. Reagent supply capillaries were 40/360 μm (ID/OD), and the confluence was in a 100/360-μm (ID/OD) capillary on the right.

In the mixing interface, the sample capillary was tapered and inserted to the center of the cross section and the other capillaries were also close to the cross section as shown
in Figure 2.5. The capillary configuration in Figure 2.5A had the sample branch in the middle and derivatization reagents on both sides. When the three flows were on, the sample was flushed away by the other two flows thus reducing the dead volume of the sample. Higher flow rates limited the diffusion time and a clear sample flow path was observed (Figure 2.5Ac). Figure 2.5B demonstrates an alternative of the flow arrangement, in which the sample was positioned on one side and was inserted to the center of the cross section. This arrangement may be used when the sample and one of the reagents are incompatible due to various reasons such as precipitation formation. The mold for the mixing interface used 230-µm wires and nylon lines; the fabricated PDMS channels had tight contact with the inserted 360-µm (OD) capillaries thus tolerating high back pressure and preventing liquid leaking. For 3-way mixing (2 to 1), one branch of the 4-way mixer could be blocked by using a sealed capillary or a solid bar with an appropriate diameter.

![Image of tube-to-tube connectors](image-url)

**Figure 2.5.** Images of tube-to-tube connectors. (a) Assembled mold; the capillary ID was 200 µm. (b) The interface with a needle and a capillary inserted. (c) Tight connection of two tubes to reduce the dead volume.

For tube-to-tube connection between identical diameters, single wires were used as molds, while connections of 22-gauge syringe needles (150/718 µm ID/OD) to 360-µm (OD) capillaries required differing diameters; thus, a mold was prepared by using a silica
capillary (200/360 μm ID/OD) and a wire (230 μm) as shown in Figure 2.5a. The completed PDMS connector had one side with a larger inner diameter to accommodate the syringe needle and the other side with a smaller diameter for the capillary (Figure 2.6b). The two tubes could be inserted close thus reducing the dead volume as shown in Figure 2.5c. The 718-μm needle in a 360-μm PDMS channel offered stronger sealing than the 360-μm silica capillary in a 230-μm channel. The combination tolerated up to 120 psi in back pressure before leaking was observed on the capillary side. This pressure was estimated by pumping a dye solution through a PDMS connector into a 100-μm capillary filled with air and sealed with super glue on the other end. A higher back pressure (over 200 psi) tolerance was observed when the ratio of the PDMS prepolymer/curing reagent was decreased to 5/1 in mass that offered harder PDMS.

2.3.2. Flow-gated injection

![Flow-gated injection process](image)

**Figure 2.6.** Flow-gated injection process in the assembled PDMS interface. (a) Normal gating; (b) waiting for gap refilling with gating flow off; (c) injection; (d) flushing with gating flow on; (e) separation voltage on.

Figure 2.7 shows the flow-gated injection process. Two 40-μm (ID) capillaries were inserted into the PDMS flow gate tip-to-tip at 40 μm apart. A 0.5-s interval between swerving off the gating flow and switching on the injection voltage allowed the sample
(green dye in buffer) to fill the gap thus the injected sample would closely represent the bulk sample solution (Figure 2.7b). Also, a 0.3-s interval before the application of the separation voltage was maintained to clear the sample around the separation capillary (Figure 2.7d). As can be seen in Figure 2.7e, a clean sample plug was injected and then electrophoretically pumped downstream for separation. The gating extent of the sample is determined by the flow rate ratio of the gating flow and the sample for a specific capillary gap. Figure 2.8A demonstrates the gating situation at various gating flow rates while the sample was maintained at 400 nL/min. As can be seen in Figure 2.8Aa, 40 μL/min provided ineffective gating, which would promote sample leaking into the separation capillary thus increasing the background signal, while 80 μL/min produced decent gating as shown in Figure 2.8Ab, and the flow rate ratio is 200, roughly 25-fold less than the minimum requirement (5000) in the machined flow gate as reported [8]. This gating flow reduction, on the one hand, was due to the smaller diameter (0.43 mm) of the PDMS flow gate channel than the machined one (1.59 mm); on the other hand, the tapered capillaries (360 μm OD) blocked a larger percentage of the sectional area of the PDMS channel. Apparently, a greater gap between the two capillary tips requires smaller gating flow as shown in Figure 2.8B, but it takes a longer stagnant time for the sample to refill the gap before injection thus lowering sample transfer efficiency and finally lengthening total analysis time.
2.3.3. Performance of interconnected system

To demonstrate the performance of the CE system interconnected through PDMS interfaces (Figure 2.3), NDA/cyanide/amino acids were used to perform online derivatization and separations. As reported [28-30] NDA and cyanide may react when stored together and therefore were introduced to the amino acids sample separately. As can be seen in Figure 2.4, three solutions were pumped through individual syringes connected to 40-μm (ID) capillaries via PDMS connectors; the other ends of the capillaries were connected to a 4-way PDMS mixer, with amino acids in the middle; the confluence flowed through a 100-μm (ID) capillary at the length of 32 cm allowing ~3 minutes for reaction. For flow-gated injection, a 40-μm (ID) capillary with 10 cm in length was used to supply sample to a 10-μm (ID) separation capillary. The 100-μm ID and 40-μm ID were connected with a fabricated 230-μm PDMS connector. The performance of the PDMS-
interconnected system was tested through separating amino acids. Figure 2.8a shows a typical electropherogram showing well-resolved 5 standard amino acids which were directly delivered through a sample syringe followed by online mixing, derivatization, injection, separation, and detection. Overlap injection was also performed as shown in Figure 2.9b which doubled the analysis throughput. As can be seen in both electropherograms (Figs. 2.9a and 2.9b), NDA-derivatization of primary amine-containing compounds produces by-products, while these by-product peaks are often covered by amino acid peaks when a complex mixture of sample is analyzed with capillary zone electrophoresis [27]. To test its long-term performance, more than 300 consecutive injections were performed and the results were plotted in Figure 2.9c, and an excellent reproducibility in peak height was demonstrated by the %RSD (relative standard deviation) of 1.6; high separation efficiency was also obtained with the theoretical plates of 250 k. Further, amino acids at various concentrations of 1.0, 2.0, 4.0, and 8.0 μm were sequentially dialyzed for 5 minutes through a microdialysis probe and the results of glutamate are summarized in Figure 2.9d, which suggests that the integrated system was able to dynamically monitor sample concentration variation with the response time of ~40 s at 90% of the maximum signal. Note that the 8.0-μm sample produced relatively greater variation, but its %RSD (4.5) is still in acceptable range in CE separations [31]; this greater variation possibly came from occasional instability of sample injection since the other analyte, aspartate, also presented a similar variation pattern (data not shown). These results demonstrate that the PDMS-interconnected system is potentially valuable for in vivo neurotransmitter monitoring [28,32].
MEKC was also performed using this CE system. CZE has difficulty in separating analytes with similar charge-to-size ratios. To demonstrate the separation power of MEKC, a sample containing twenty analytes was derivatized with NDA and KCN by offline mixing. The sample was delivered by using a syringe pumping system and injected with flow-gated injection. The BGE contained 50 mM tetraborate at pH 9.2, 50 mM sodium dodecyl sulfate (SDS) and 2 mM hydroxypropyl-beta-cyclodextrin (HP-β-CD). A typical electrophoreogram is shown in Figure 2.10. As can be seen, most analytes were baseline-
separated (valine has both D and L forms) within 90 seconds.

Figure 2.9. Separation of 20 analytes by MEKC. Sample contained 20 analytes which were used for derivatized with NDA and KCN at pH 9.2. Offline mixing was performed. BGE contained 50 mM borate, 50 mM SDS and 2 mM HP-β-CD.

2.4. Conclusions

The developed method for fabricating PDMS interfaces used for flow-gated injection, multiple-reagent online mixing, and tube-to-tube connection involved a rapid and inexpensive procedure to produce functional parts for CE and other analytical techniques. These parts offered convenience and ease for the coupling of complex integrated systems, minimized dead volumes, and reduced buffer consumption in the gating flow. Moreover, these transparent interfaces had apparent advantages for easy trouble shooting since many problems would occur at interfaces, especially the mixer and the flow gate; also, the gating flow and mixing processes could be directly observed so that the alignment and configuration of the capillaries in the cross section could be
optimized. Furthermore, the prototyped channels had smaller diameters than the inserted capillaries, which tightly stabilized these capillaries in position and tolerated a high flow pressure. It is anticipated that the developed procedure and interfaces could be adapted to CE-coupled analytical instrumentation and eventually decrease the cost and complexity of multiplexed analytical systems.
REFERENCES


CHAPTER 3: DETECTION OF CATECHOLAMINES IN CEREBOROSPINAL FLUID USING AN ON-LINE PRECONCENTRATION METHOD

Note: Most contents of this chapter have been published in Journal of Chromatography A 2016, 1450, 112-120.

3.1. Introduction

Catecholamines (CAs) including dopamine (DA), norepinephrine (NE), and epinephrine (E) are important neurotransmitters and neurohormones in nervous systems and adrenal glands [2,3]. The levels of CAs in healthy human beings maintain in specific statistical ranges. Levels below the lower limits or above the upper limits often reflect health problems [4]. In addition, the level variation with time or upon stimulation can be employed as a major indicator for pharmaceutical and physiological investigation. However, the concentration levels of CAs are in nano- or pico- molar ranges. To detect this low level accurately and rapidly, powerful tools are highly wanted. Currently, laser-induced fluorescence (LIF) and electrochemical (EC) detection represent the major two formats of the most sensitive detectors for CAs. For example, HPLC coupled with electrochemical or LIF detection is able to obtain pico-molar limits of detection (LODs) for CAs [5,6]. However, HPLC separation usually takes time and requires a large volume of samples, which is infeasible to the hard-to-obtain biological fluids such as cerebrospinal fluids (CSF). Compared with HPLC, CE consumes much smaller sample volumes and facilitates high-throughput analysis.

During last two decades, CE and microchip-based CE separation platforms have been applied to in vivo neurotransmitter monitoring [7-10]. The temporal resolution has been shortened to as low as 2 s when the rapid CE separation was coupled with
segmented flow transport of microdialysis dialysate [10,11]. However, the application of these platforms is often limited to analytes with high basal levels such as glutamate and taurine [12]. CAs such as DA and NE have sub-nanomolar concentrations in brain regions, and the low concentrations pose a challenge in detection after CE separation. Shou et al. have developed a sensitive CE method for DA monitoring in which a 1.0 nM LOD was achieved [13]. Nonetheless, the DA signal may often be immersed in interferences, which require optimal and stable conditions to secure DA detection after separation. Therefore, further improved LODs such as in pico-molar are strongly desired to expand the CE applicability in tackling complex biological samples.

To improve LODs, a sensitive detection method such as LIF should be used. Alternatively, sample preconcentration is a promising strategy and has been extensively used to enhance the detectability of analytes using various strategies [14]. Off-line sample preconcentration such as solid phase extraction (SPE) lacks automation and flexibility although it may effectively enrich specific analytes such as CAs [15,16]. The on-line preconcentration mode is more flexible and convenient to couple with rapid CE separations. Strategies for on-line sample preconcentration in CE including sample stacking, sweeping, isotachophoresis (ITP), and dynamic pH junction, are usually based on the variation of electrophoretic velocities of analytes when located in two distinct buffer zones, sample and BGE [17,18]. This variation in electrophoretic velocity can be manipulated by establishing an electric field gradient or by varying the charges on analyte molecules or analyte-involving entities. Analytes may electrophoretically migrate faster in the sample plug but slow down or stop at the boundary of the sample plug and the BGE, and finally all analytes are piled up and concentrated (e.g. sample stacking and ITP).
Alternatively, analytes may be electrophoretically stagnant (for neutral molecules) or migrate slowly; when a voltage is applied across the capillary, the components in the BGE invade into the sample plug and interact with analytes thus varying their electrophoretic velocities (e.g. sweeping and dynamic pH junction). These methods can be hyphenated or combined to have improved concentration enhancement. For instance, dynamic pH junction-sweeping hyphenation has been employed to enhance focusing and selectivity for a complex mixture [19,20]. Field-amplified stacking injection followed by sweeping has been widely used [21,22]. However, these on-line preconcentration techniques are developed mainly for conventional CE [14], but such techniques often pose challenges when conducted on microchip and flow-gated CE that equally or more urgently requires on-line sample preconcentration to enhance the detection sensitivity.

This chapter describes a novel and simple on-line technique for selective focusing of fluorescent CA derivatives on a flow-gated CE platform. Briefly, DA and NE in CSF or aCSF are first fluorogenically derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide in borate buffer, and then a sample plug is electrokinetically introduced into the separation capillary through the flow-gated injection. The fluorogenic derivatization mixture contains borate buffer while the BGE is void of borate. The basic principle for focusing CA derivatives is based on the complexation of CAs with borate, which generates anionic complexes. In an electric field, these complexes electrophoretically migrate to the rear boundary of the borate plug where complexes may dissociate to neutral CA derivatives due to lower borate concentrations. On the other hand, electrophoretic migration of borate plug increases the local borate concentration which allows reformation of complexes in the concentrated zone. This process continues and
all the CA derivatives will be focused to a narrow zone.

In practice, NDA-derivatization of primary amines in the presence of cyanide requires a basic buffer such as pH 9.2, while the media for LIF detection of DA prefers a lower pH such as 7.4 due to the pH-dependence of fluorescence as reported [13]. The multi-section electrolyte systems may have complicated effects including pH junction, transient ITP, conductivity variation, and surfactant sweeping. To verify the proposed focusing mechanism, individual effects were experimentally studied and theoretically discussed, and the capability of the method was demonstrated by applying it to the measurement of DA in CSF via microdialysis sampling. The technique is anticipated to be easily adapted to microchip platforms which provide additional advantages for sensitive detection and rapid separation of CAs.

3.2. Experimental

3.2.1. Chemicals and reagents

Dopamine hydrochloride, D/L-norepinephrine hydrochloride, sodium tetraborate, and all amino acids were purchased from Sigma (St. Louis, MO, USA). Potassium cyanide, boric acid (BA), dimethylformamide (DMF), sodium hydroxide, sodium chloride, tetrasodium salt of ethylenediaminetetraacetic acid (EDTA), and chemicals (NaCl, KCl, MgSO₄, CaCl₂, and Na₂HPO₄) for preparing artificial cerebrospinal fluid (aCSF) were purchased from Fisher Scientific (Chicago, IL, USA). NDA was ordered from Invitrogen (Eugene, OR, USA). Cerebrospinal fluid (CSF) collected from the striatum of Sprague Dawley rats was purchased from BioreclamationIVT (Long Island, NY, USA). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

The NDA stock solution at 20.0 mM was prepared in DMF, and the working NDA
was prepared by diluting the stock solution to 10.0 mM with deionized (DI) water on a daily basis. The stock solution of KCN (100.0 mM) was prepared in DI water, and the working KCN was diluted to 5.0 or 10.0 mM. The stock borate buffer (pH 9.2) was prepared by dissolving tetraborate (Na₂B₄O₇·10H₂O, 100.0 mM) in DI water, and the borate buffer concentrations in the following text indicate the total concentrations of tetrahydroxyborate (THB) anions and neutral boric acids (BAs) by supposing tetraborate dissociates to equal moles of THB and BA in aqueous solution. BA stock solution at 500.0 mM was prepared in DI water, and its pH was adjusted by using 100.0 mM NaOH. Stock solutions of NaCl at 2.0 M and EDTA at 100.0 mM were prepared in DI water without pH adjustment. DA and NE stock solutions at 10.0 mM were separately prepared in 20 mM HCl solutions with the pH 2.5 on a weekly basis, and they were stored in a refrigerator. Amine solutions (glycine, arginine, glutamine, glutamate, and aspartate) at 10 mM were prepared in DI water, and were diluted to appropriate concentrations with aCSF. The aCSF consisted of 145.0 mM NaCl, 2.7 mM KCl, 1.0 mM MgSO₄, 1.2 mM CaCl₂, and 2.0 mM Na₂HPO₄, and its pH was adjusted to 7.4 with NaOH [11]. The final cyanide solution contained 5.0 or 10.0 mM KCN, 20 mM EDTA, 200.0 mM NaCl, and 60.0 mM tetraborate unless otherwise stated.

3.2.2. Instrumentation

The custom-built detection system has been described in Chapter 2 [23]. Briefly, a 442-nm laser (Laserglow Technologies, Toronto, ON, Canada) beam was spectroscopically filtered through an interference bandpass filter at 442 ± 5 nM and then focused on the separation capillary through a 40x oil-immersion objective with the numerical aperture of 1.3 (Carl Zeiss Microscopy, Thornwood, NY, USA). The
fluorescence was collected by using the same objective and then transmitted to a photomultiplier tube (PMT) with the part # R11540 (Hamamatsu Photonics, Japan) after emission filtration with two bandpass filters (485 ± 12.5 nm) in the series arrangement. The current signal generated by the PMT was pre-amplified and then converted to voltages by an SR570 current preamplifier (Stanford Research Systems, Sunnyvale, CA, USA). Voltage signals were finally recorded by a LabVIEW program. Sample injection and separation were performed by applying a negative voltage on the capillary outlet reservoir with a high-voltage power supply (Model CZE1000R) purchased from Spellman High Voltage Electronics Corporation (Hauppauge, NY, USA). A sample plug was electrokinetically injected via the flow-gated injection, and then separated. This procedure was controlled by a LabVIEW program. For on-line mixing, a 4-syringe pump (Chemyx Inc., Stafford, TX, USA) was used to supply sample and derivatization reagents through individual gastight Hamilton syringes (Reno, Nevada, USA). The flow gate and other interfaces were fabricated with poly(dimethylsiloxane) (PDMS) as described earlier [23,24].

3.2.3. Experimental conditions

For condition optimization, off-line mixing was used unless otherwise stated. Off-line mixing was performed by pipetting samples and reagents to be mixed in a centrifuge tube. The separation capillary had an inner diameter (ID) at 10 μm and an outer diameter (OD) at 360 μm. It had 17.0 cm in total length and 10.0 cm in effective length. The inlet side of the separation capillary was grounded while the outlet side was applied with a negative high voltage. The separation buffer or BGE was supplied through a 50-mL plastic syringe powered by a Harvard Model 11 syringe pump. The gating flow of the separation...
buffer was switched on and off with a 3-way solenoid pinch valve purchased from Cole-Parmer (Vernon Hills, IL, USA). Three solutions were prepared separately for off-line or on-line mixing: (a) NDA, 10.0 mM in DMF/water at 50/50 by volume; (b) KCN, 5.0 or 10.0 mM in water, plus 200 mM NaCl, 20 mM EDTA, and 240 mM borate buffer at pH 9.2; (c) sample, aCSF plus CAs and amino acids, or aCSF for microdialysis sampling. The mixing ratio was 1:1:2 where ‘2’ was for sample. Silica capillaries with 100 μm in ID and 30.0 cm in length were used for on-line mixing and derivatization. The reaction capillary allowed >3.0 min for derivatization when the overall flow rates were set at 600 to 800 nL/min. Other capillaries had 40 μm in ID for fluid transport. Unless otherwise stated, the BGE consisted of 120.0 phosphate buffer (PB) at pH 7.4, 7.0 mM cholate, 0.5 mM deoxycholate, and 10% methanol by volume.

3.3. Results and Discussion

3.3.1. Mechanism of borate-assisted CA preconcentration

It is well known that boronic acids and borate react with cis-diols to form complexes [25-31]. This reaction is reversible, and both the forward and reverse reaction kinetics is rapid [25,27]. The complexation induces an additional negative charge to a complex entity which promotes its electrophoretic migration under an electric field. This property has been employed in CE separations of CAs and saccharides, in which the BGE contained boronic acids or borate [32,33]. Borate has also been used as a carrier for sweeping in which borate was added to BGE while the sample was void of borate; however, this sweeping strategy lacked efficiency [34]. In addition, Britz-Mckibbin et al. have addressed the importance of borate complexation with vicinal diols in analyte focusing via a dynamic pH junction [16,18]. However, they used equal concentrations of borate in both BGE and
Distinct from previously reported techniques, the present method requires borate in sample while the BGE free of borate, and consequently a novel focusing mechanism and a procedure are proposed and schematically demonstrated in Figure 3.1. First, a sample plug was electrokinetically injected into the capillary via the flow-gated injection. NE and DA formed fluorescent cyanobenz[f]isoindole (CBI) derivatives through the amine reaction with NDA in the presence of cyanide ions [35], and diols on DA and NE formed anionic complexes with borate [25]. Under an electric field, borate-CBI complexes electrophoretically migrated toward the anode. The original pH of the borate buffer in the derivatization mixture was 9.2 produced by 15 mM sodium tetraborate which generated equivalent concentrations of THB, $B(OH)\_4^-$, and BA, $B(OH)\_3$ [36,37]. THB

**Figure 3.1.** Schematic procedure showing on-line DA/NE-CBI preconcentration and separation. (a) Sample injection; (b) focusing; and (c) separation. Arrows indicate flow direction, and red arrows indicate sample while black ones indicate BGE gating flow. The sign “–” indicates the cathode with negative high voltage applied, and the waste side is grounded.
and anionic complexes electrophoretically migrated backward thus resulting in a borate
gradient at the borate plug front as shown in Figure 3.1a. Second, a separation voltage
was applied across the capillary for concentrating the sample plug. During this process,
the front borate gradient zone expanded. Simultaneously, the rear of the borate plug also
developed a borate gradient zone which was elongated with time. The anionic borate-CBI
was electrophoretically driven towards the rear boundary of the borate plug until the
complexes dissociated to produce neutral DA/NE-CBI derivatives when the borate
concentration was low in the rear borate gradient. Simultaneously, the borate plug also
electrophoretically migrated towards the anode. The borate plug migration increased the
borate concentration at the focused zone thus complex reformation occurred. The
association-and-dissociation process continued and all the NE and DA derivatives were
focused to a narrow zone as shown in Figure 3.1b. Finally, the NE/DA-CBI s were
separated by a surfactant mixture of cholate and deoxycholate as schematically
demonstrated in Figure 3.1c.

To better understand the dynamic complexation process of borate with diols under
an applied voltage, it is important to understand the borate buffer system. Borate buffer
can be prepared by dissolving tetraborate or boric acid with pH adjustment using HCl or
NaOH. At low concentrations, the major components in borate buffer are THB and BA [37]
which maintain at a dynamic equilibrium according to Equation 3.1 depending on the local
pH values.

\[
B(OH)_3 + 2H_2O \rightleftharpoons B(OH)_4^- + H_3O^+ \tag{3.1}
\]

It has been validated that cis-diols react with both THB and BA to produce the
same complex, but the reaction rate with neutral BA is usually greater than with anionic
THB [25]. The CA derivatization mixture contains borate at pH 9.2, and establishes two dynamic equilibriums as Equations 3.1 and 3.2.

\[
B(OH)_3 \text{ or } B(OH)_4^- + L(OH)_2 \rightleftharpoons B(OH)_2O_2L^- + H_3O^+ \text{ or } H_2O \quad (3.2)
\]

where \( L \) represents the ligand owning vicinal diols. During the concentrating process, borate plug electrophoretically migrates towards the anode with an electrophoretic velocity determined by the fraction of THB (negative) out of total borate including THB and BA (neutral). To focus the complex to the rear borate boundary, the electrophoretic velocity (or mobility) of the complex should be greater than the weighted velocity (or mobility) of borate. On the other hand, the pH of the sample plug affects the fraction of borate and thus the weighted borate mobility. Therefore, the pH of the sample plug and/or the pH of BGE affect the focusing results, which was experimentally studied and discussed in the following section.

### 3.3.2. pH effect on CA-CBI focusing

Conventionally, native NE and DA could be concentrated by using a dynamic pH junction [16,32]. Fluorogenic derivatization of NE and DA by NDA varies the property of the amine group thus complicating the pH junction effect on DA/NE-CBI focusing. The pH effect on CA-CBI focusing in the proposed method involves bilateral situations: high sample pH with low BGE pH and low sample pH with high BGE pH. Under an applied voltage, both situations generate pH junctions that might affect CA-CBI focusing, but the pH junction does not directly influence CA-CBI but alters the ratio of THB/BA which result in borate plug velocity variation as studied below.

Constant sample pH at 9.2 and various BGE pH values. The optimal pH range for the fluorogenic derivatization with NDA is 8.5-10.0 [38], and hence it is convenient to use
the selected pH for the sample plug injected for preconcentration, especially when on-
line mixing and derivatization are performed. Borate buffer at pH 9.2 prepared with
tetraborate was used for fluorogenic derivatization without pH adjustment [13]. To test the
BGE pH effect on NE-CBI focusing, various pH values of the BGE were utilized to conduct
sample focusing. The BGE contained PB (100-120 mM) plus 10% methanol by volume
(void of surfactants), and its conductivity was adjusted to be comparable to that of the
sample mixture. The results were summarized in Figure 2a. As can be seen, NE-CBI
signal decreased with the increase of the pH until finally the focusing became impossible
at pH 10.0. Two factors related to pH may affect the focusing of NE-CBI. The first factor
is the ionization of NE hydroxyl groups at pH 10.0 since the pKₐ of NE hydroxyl groups
was theoretically calculated as 9.63 [39]. The second factor is the increase of the THB/BA
concentration ratio when the BGE pH increases. The sample plug with pH 9.2 was
sandwiched between BGE plugs as demonstrated in Figure 3.1b. Under an applied
voltage, the pH of the borate plug decreases (or increases when BGE pH > sample pH)
from both sides of the plug, and finally the pH of the borate plug will be equal to the pH of
the BGE as compared with the reference [40] where a low pH 4.5 in the sample plug and
a high pH 10 in the BGE were used. Accordingly, the increase in pH of the BGE increases
the fraction of THB and the THB/BA ratio according to Equation 3.1. Higher concentrations
of THB increase the weighted electrophoretic velocity of the borate plug towards the
anode. The faster migration of the borage plug adversely affects the focusing of NE-CBI
whose focused zone is located in the rear boundary of the borate plug. As shown in Figure
3.2a, the migration time of NE-CBI increased relative to the Arginine peak with the pH
increase of the BGE, and the NE-CBI marked the rear boundary of the borate plug. On
the other hand, the THB/BA ratio may also affect the complexation as shown in Equation 3.2. Although both THB and BA result in the same anionic complexes, their reaction kinetics differs [25]. The decrease in BGE pH induces a greater fraction of neutral BA which may facilitate complexation of NE-CBI with BA [25], and the rapid complexation and/or dissociation may promote focusing. It was observed that lower BGE pH such as pH 7.0 generated better focusing of NE-CBI (data not shown). However, pH 7.4 was selected for the BGE considering the feasibility of EOF with a basic buffer.

Constant BGE pH at 7.4 and various sample pH values. The BGE consisted of PB at 120 mM and 10% methanol by volume. Each derivatization mixture was prepared to have boric acid at 60 mM with various pH values and phosphate for conductivity adjustment to match the BGE. The low sample pH affects the derivatization kinetics; therefore, a longer period (>30 minutes) was allowed for each derivatization mixture to proceed in dark. As shown in Figure 3.2b, the focused NE-CBI peaks were sharp at all the pH values tested, and had theoretical plates > 600 thousands. However, a brief peak height comparison shows that lower pH of the sample mixture slightly facilitated focusing, which agrees with the above discussion that the sample pH would finally be replaced by the BGE pH and low pH benefited the focusing before the BGE pH swept across the sample plug. In experiments, however, a pH 9.2 was used for the sample mixture due to the pH requirement for NDA derivatization of primary amines in the presence of cyanide [41]. It should be addressed that the transient ITP with the leading electrolyte of phosphate and the terminating electrolyte of borate existed and might play a role in NE-CBI focusing. Another series of experiments using NaCl (instead of phosphate) to adjust the conductivity of the sample mixture was performed and effective focusing was
observed with over one million theoretical plates (data not shown). These results indicate that high salt contents including NaCl in biological sample may aid CA-CSI focusing although it is not a major factor in the proposed method.

![Figure 3.2](image_url)

**Figure 3.2.** Focusing comparison. (a) Focusing comparison at various BGE pHs. NE 1.0 μm, Arg 0.5 μm, and Gln 2.0 μm in aCSF before mixing. BGE: 100-120 mM PB at various pHs and 10% methanol with conductivity comparable to the sample mixture. Sample recipe: NDA 10 mM, KCN 5 mM, and Sample at 1:1:2 by volume at pH 9.2. Injection at -10 kV x 10 s and separation at -25 kV. (b) Focusing comparison at various sample pHs. BGE: 120 mM PB at pH 7.4 plus 10% methanol. Sample mixture: NDA 2.5 mM, KCN 1.25 mM, 12.5% DMF, boric acid 60 mM, and PB 100-120 mM with conductivity comparable to BGE. Injection at -10 kV x 8 s and separation at -27 kV.
3.3.3. Borate concentration effect

**Figure 3.3.** Comparison of NE-CBI focusing results at various concentrations of borate in sample mixtures and at various injection times. (a) NE-CBI focusing at various concentrations of borate at a large scale. NE at 1.0 μM and Arg at 0.5 μM. Injection at -10 kV x 10 s and separation at -27 kV. (b) NE-CBI focusing at various concentrations of borate at a small scale. Injection at -5 kV for 5 s. (c) Injection time effect on NE-CBI focusing at borate 10 mM. Injection at -5 kV for various times. (d) Injection time effect on NE-CBI focusing at borate 5.0 mM. Injection at -5 kV for various. For (b, c and d), NE at 50.0 μM and Arg at 5.0 μM, and separation at -20 kV. For all electropherograms, separation buffer consisted of PB 120 mM at pH 7.4 and 10% methanol by volume. Derivatization mixtures were prepared by mixing three reagents: NDA, 10.0 mM in DMF/water at 50/50 by volume; KCN, 5.0 mM plus 200 mM NaCl, 20 mM EDTA, and various borate concentrations at pH 9.2; Sample in aCSF before mixing. Off-line mixing ratio was 1:1:2 by volume (2 for sample). Borate concentrations indicate after off-line mixing.

Borate in forms of THB and BA plays a major role in effective focusing of NE/DA-CBI. The concentration of borate affects the equilibrium and complexation kinetics according to Equation 3.2. Various concentrations of borate in the derivatization mixture are expected to result in variation of focusing results. Figs. 3.3a and 3.3b show experimental
results obtained by using various concentrations of borate. The NE-CBI peak decreased with the decrease of borate concentration as shown in Figure 3.3a where the NE concentration was 1.0 μm before mixing. On the other hand, borate at greater than 65 mM failed to further improve the NE-CBI signal. Figure 3.3a also shows that the overall migration velocity of the concentrated DA/NE-CBI zone fairly changed with the increase of the borate concentration in the sample mixture when the borate was above a critical concentration and the BGE pH was maintained at 7.4, which indirectly ascertains that the focused DA/NE-CBI zone migrates with the borate rear gradient.

What happens to CA focusing when borate concentration further decreases? A series of experiments was performed by using a higher concentration of NE at 50.0 μm (before mixing), and the results are shown in Figure 3.3b. As can be seen, borate at 5.0 mM had weak focusing capability, and thus some NE-CBIs remained between Arginine and NE plugs; 2.5 mM borate failed in focusing; and 1.0 mM borate was unable to resolve NE-CBI from the Arginine zone. According to Equation 3.2, small borate concentrations decrease the complexation kinetics and shift the equilibrium to the left side, thus resulting in a lower fraction of anionic complex. These results indicate that a critical borate concentration is required to produce stable complexes that enable focusing of NE-CBI.

Accordingly, when the borate concentration is low, the length of the injected borate plug also affects the focusing of NE-CBI because a short borate plug will be rapidly expanded and diluted under an electric field. The diluted borate concentration mainly depends on the original borate concentrations. Figure 3.3c was obtained by using 10 mM borate at various injection times. As can be seen, even a short injection at -5.0 kV x 0.2 s or 0.4 s was able to shift NC-CBI plug from the arginine zone, but effective focusing
was observed until the injection was greater than 1.2 s (see Figure 3c) which introduced a sample plug in the length of 0.14 cm. As comparison, initial borate concentration at 5 mM (see Figure 3.3d) failed in effective focusing even at -5 kV x 5 s injection. Therefore, it is always safe to use a high concentration of borate such as 60 mM for effective focusing.

![Figure 3.4](image)

**Figure 3.4.** BGE borate effect on NE-CBI focusing. Separation buffer consisted of PB 120 mM at pH 7.4 and 10% methanol plus various concentrations (as indicated in the figure) of boric acid with pH adjusted to 7.4 using NaOH. Sample contained NE at 5.0 μm, Arg 1.0 μm, and Gln at 1.0 μm before off-line mixing. Borate in the derivatization mixture was 60 mM after mixing. KCN at 5.0 mM before mixing. Injection at -10 kV x 10 s and separation at -25 kV.

According to the focusing mechanism, borate in the sample mixture is sufficient to effectively concentrate DA-CBI and NE-CBI. It is anticipated that the presence of borate in the BGE will fail in effective focusing of DA/NE-CBI. To test this prediction, a series of experiments was performed with various borate concentrations in BGE while maintaining sample borate at 60 mM pH 9.2. The borate added to BGE (120 mM phosphate plus 10% methanol by volume) was prepared with boric acid and its pH was adjusted to 7.4 using NaOH. As can be seen in Figure 3.4, a low concentration of borate such as 5.0 mM in BGE significantly decreased the focusing result, and higher concentrations such as 10 and 20 mM further weakened the focusing, which was attributed to the borate continuity.
in the sample plug and the BGE. It was also observed that the migration time of NE-CBI became longer with the increase of borate concentration in BGE, which was attributed to continual interaction of borate and NE-CBI prior to detection. In addition, the borate complexation quenches fluorescence of DA/NE-CBI derivatives [35,42]. To obtain optimal fluorescence intensity, borate-free BGE should be used. In present research, PB plus additives was employed as the BGE.

3.3.4. Sample and BGE conductivity effect

![Figure 3.5](image)

**Figure 3.5.** Sample and BGE conductivity effect. (I) Separation buffer PB 120 mM; No additional NaCl in sample. Separation buffer PB 120 mM; (II) Sample contains additional 50 mM NaCl. Separation buffer PB 100 mM; (III) No additional NaCl in sample separation buffer PB 100 mM; (IV) Sample contains additional 50 mM NaCl.

Conductivities of the sample plug and the BGE determine the local electric field when a voltage is applied across the capillary. A smaller conductivity of the sample solution than the BGE induces a greater electric field in the sample plug than the BGE, and thus significant injection bias will be observed (Figure 3.5). On the contrary, a greater conductivity of the sample mixture reduces injection bias but a slow and ineffective focusing process is anticipated due to the small electric field in the sample plug. As a compromise, the derivatization reagents were mixed on-line or off-line with the volume ratio of 1:1:2 (NDA, CN and aCSF). The mixture consisted of 60.0 mM borate, 50.0 mM...
NaCl, 5.0 mM EDTA, 2.5 mM NDA, 2.5 mM KCN, and 50% aCSF. This recipe produced a comparable conductivity to the BGE which consisted of 120 mM phosphate at pH 7.4, 7.0 mM cholate, 0.5 deoxycholate, and 10% methanol by volume. The maximum injection length of the effective sample plug was estimated to be 2.3 cm under the injection voltage of -10 kV for 20 s by using the EOF ($\mu_{eo} = 3.16 \times 10^{-4}$ cm$^2$/Vs determined by using Arg-CBI as the neutral marker) of the BGE and DA/NE-CBI electrophoretic mobilities ($\mu_{ep}^{NE} = -1.17 \times 10^{-4}$ cm$^2$/Vs and $\mu_{ep}^{DA} = -1.22 \times 10^{-4}$ cm$^2$/Vs) in the sample solution, and this sample plug length took 23% of the effective separation length (10 cm). The mobilities determined in the sample solution are listed in Table 3.1. Note that the total injected sample plug was estimated to be 3.7 cm which was related to the EOF. It is anticipated that a sample plug with smaller conductivity than the BGE via hydrodynamic injection would further improve the focusing result, which will be studied on a conventional capillary electrophoresis system.

<table>
<thead>
<tr>
<th></th>
<th>$\mu_{total}$ (cm$^2$/Vs)</th>
<th>$\mu_{ep}$ (cm$^2$/Vs)</th>
<th>Velocity (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>2.97 x 10$^{-4}$</td>
<td>0.00E+00</td>
<td>1.75 x 10$^{-1}$</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1.80 x 10$^{-4}$</td>
<td>-1.17 x 10$^{-4}$</td>
<td>1.06 x 10$^{-1}$</td>
</tr>
<tr>
<td>Dopamine</td>
<td>1.74 x 10$^{-4}$</td>
<td>-1.22 x 10$^{-4}$</td>
<td>1.03 x 10$^{-1}$</td>
</tr>
<tr>
<td>Glutamine</td>
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<td>-1.41 x 10$^{-4}$</td>
<td>9.16 x 10$^{-2}$</td>
</tr>
<tr>
<td>Glycine</td>
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<td>-1.72 x 10$^{-4}$</td>
<td>7.35 x 10$^{-2}$</td>
</tr>
<tr>
<td>Glutamate</td>
<td>3.58 x 10$^{-5}$</td>
<td>-2.61 x 10$^{-4}$</td>
<td>2.11 x 10$^{-2}$</td>
</tr>
</tbody>
</table>

### 3.3.5. Other factors on CA focusing

Other factors that may affect CA-CBI focusing include surfactant sweeping and
transient ITP. Cholate at 7.0 mM and deoxycholate at 0.5 were added to the BGE for resolving DA and NE, but even without both surfactants, NE-CBI was effectively focused as shown in Figure 3.2a and Figure 3.4. Since the sample mixture and the BGE involve different components, transient ITP may exist. This focusing method was developed to preconcentrated DA/NE in CSF that contains high salt contents including NaCl. As reported, high salt and organic solvent were able to facilitate sample stacking based on transient ITP [43]. However, experiments performed with NE dissolved in borate buffer (without additional salts) still obtained effective focusing (data not shown). Note that extra NaCl at 50 mM added to the derivatization mixture was used to match the conductivity of the BGE.

**Borate complexation effect on CA oxidation.**

It is worth mentioning that borate complexation with CAs is able to protect CAs from oxidation by oxygen. NDA is a useful fluorogenic derivatization reagent for primary amines [35,41,44-48]. The favorable pH for the derivatization has been determined to be in the range of 8.5-10.0 [38]. However, DA and NE tend to be quickly oxidized in this basic pH range thus decreasing the actual DA/NE amounts in the sample [32,49]. To reduce the oxidation loss of CAs, an acidic buffer and/or ascorbic acid are often used for the preparation of stock solutions. To compare the oxidation effect, DA and NE were separately dissolved in various solutions with different pH values. The color change with time was recorded with a camera. As shown in Figure 3.6a, the DA solution with PB at pH 9.1 quickly became yellowish (in 1 min) and darker with time, which indicates that DA was oxidized by dissolved oxygen gas to produce black precipitate of neuromelanin [50]. On the other hand, DA in borate buffer with pH 9.2 was still clear 40 min later, which indicates
that DA was protected by complexation with borate. Both aCSF at pH 7.2 and water at pH 6.0 retarded DA oxidation, but the acidic solution at pH 2.5 (HCl) was able to effectively prevent DA oxidation in days at room temperature. Stored overnight, all the solutions except HCl (pH 2.5) became yellowish or black with observable precipitate. The same experiments were performed for NE, and the results indicate that NE was more resistant to oxidation than DA at the same conditions, but pH 9.1 with PB greatly promoted NE oxidation as shown in Figure 3.6b. Therefore, borate buffer on the one hand secures suited pH at 9.2 for fluorogenic derivatization, and on the other hand prevents CA oxidation at a high pH.

![Figure 3.6](image1.png)

**Figure 3.6.** Oxidation of DA and NE in various solvents with different pH values. (a) DA solutions at 10.0 mM. (b) NE solutions at 10 mM. HCl at 20 mM pH 2.5; Borate in the form of tetraborate at 50 mM pH 9.2; aCSF pH 7.2; and PB = Na$_2$HPO$_4$ at 200 mM pH 9.1
3.3.6. Separation of DA/NE-CBI

After focusing, DA-CBI and NE-CBI appeared at the same location in the electropherogram when the BGE was free of surfactants. To resolve DA/NE, surfactants are required to interact with DA/NE derivatives thus varying their migration mobilities. Conventionally, SDS (sodium dodecyl sulfate) at a concentration above its critical micelle concentration (CMC) is often used for micellar electrokinetic chromatography (MEKC); and SDS below its CMC is also capable of resolving DA/NE [13]. However, when SDS was added to the BGE, the focusing capability was ruined presumably due to the interaction of DA/NE and SDS which migrated faster than borate-CA-CBI complexes. To secure DA/NE focusing and resolve them after, the combination of cholate and deoxycholate was employed considering their small electrophoretic mobilities relative to SDS. Cholate and deoxycholate are negatively charged with one carboxyl group at the BGE pH 7.4 and they have larger dimensions than SDS. During and after the sample injection, cholate and deoxycholate electrophoretically migrated towards the anode and

![Typical electropherogram showing the separation of DA-CBI and NE-CBI. DA, NE, Gln, and Arg 2.0 μm each in aCSF before off-line mixing. Separation buffer consisted of 120 mM PB at pH 7.4, 7.0 mM cholate, 0.5 mM deoxycholate, and 10% methanol. Injection at -10 kV x 5 s, and separation at – 25 kV.](image-url)
across the sample plug, and finally caught up with and resolved the concentrated DA/NE-CBI zones as shown in Figure 3.7. The resolution of DA/NE-CBI became better with the increase of the concentrations of cholate and/or deoxycholate.

![Figure 3.8. DA-CBI peak sizes increase with DA concentrations. Various concentrations of DA and 20 nM NE were prepared in aCSF. Sample, aCSF as perfusate for microdialysis in the standard sample. KCN at 10 mM before on-line mixing. Injection at -20 kV x 8 s, and separation at -28 kV.](image)

**3.3.7. Concentration enhancement and LODs**

The concentration enhancement factor was estimated to be about 100 folds with standard analytes dissolved in aCSF. It was estimated by using the peak height at the focusing mode versus the normal injection mode. The best signal was obtained at the injection of -10 kV x 20 s with the injection plug length of 2.3 cm. Further increase of the injection length failed to increase the peak height presumably because the time might not be enough to complete the focusing prior to detection. The normal injection was performed at -5.0 kV x 0.2 s. It should be addressed that there was focusing even at the normal injection since the sample mixture contained borate at 60 mM. The LODs for DA/NE with standard samples dissolved in aCSF were estimated to be 20 pM for NE and
50 pM for DA at 3-fold S/N ratio.

### 3.3.8. Calibration curve

![Electropherogram](image)

**Figure 3.9.** CSF sample with spiking. (a) Comparison of typical electropherograms obtained with CSF sample and after spiking. The inset shows the magnified DA-CBI peaks. (b) DA peak height variation before and after spiking. Initial microdialysis was from CSF 50 µL, then 10 minutes microdialysis in CSF + 30 nM DA spiking (1.0 µL), and finally back to the CSF. KCN at 10 mM before on-line mixing. Injection at -20 kV x 8 s, and separation at -26 kV.

The main application of the focusing method is to be applied to in vivo DA/NE monitoring when coupled with microdialysis sampling. The peak sizes have to be proportional to the concentration of the analytes in real samples. A series of experiments with various DA concentrations from 2.0 nM to 80 nM was performed by using the preconcentration strategy coupled with microdialysis and on-line mixing/derivatization. The dialysis probe was prepared according to the reference [11], and had an active length at 4.0 mm and a diameter at 200 µm. As shown in Figure 3.8, DA peak increased with the
increase of the concentrations from 2 nM to 80 nM while the NE (20 nM) peak was maintained constant. The calibration equation was determined to be $H=0.028C + 0.221$ in terms of peak height $H$ versus DA concentration of $C$, and with the square correlation coefficient $R^2=0.9866$, which shows good response linearity.

### 3.3.9. CSF sample with spiking

CSF is more complex than standard samples dissolved in aCSF. There are tens of amine-containing compounds and salts sampled through microdialysis [13]. The sampled amines are simultaneously derivatized thus demonstrating fluorescence signals in the electropherogram. These interferences might complicate the detection of DA and NE that are in the sub-nanomolar ranges or lower. To monitor the DA concentration variation, CSF from the rat striatum was utilized as the mimic of the real rat brain. A dialysis probe was immersed into the CSF, the perfusate of aCSF was used for microdialysis sampling, and dialysates were analyzed with the preconcentration method. As shown in Figure 3.8, the DA peak maintained at a low level but shot up when the sample was spiked. It decreased to the original level when the probe was moved back to the CSF. The signal ratio was calculated to be 5.5 thus the DA concentration in the CSF sample was estimated to be 6.7 nM. It has been determined that the DA level in rat striatum was approximately 20 nM [13]. The lower DA level in the CSF sample was probably due to the oxidation loss during its collection and storage. As observed in Figure 3.9a, the background signal was high at the DA-CBI location compared with the BGE at the location of 10 s. This elevated background was from NDA-Cyanide by-products, and unknown interferences in CSF. Anyhow, this simple demonstration confirms the applicability of the developed method for neurotransmitter monitoring of DA in real samples. Future work will apply this
preconcentration method to in vivo NE and DA monitoring in rat brain.

3.4. Conclusions

The integrated microfluidic system is coupled with serial procedures including microdialysis, on-line mixing and fluorogenic derivatization, on-line sample preconcentration, rapid separation, and sensitive LIF detection. It is crucial to improve the detection sensitivity in CE separations for a variety of applications, especially when the separation is performed on a microchip platform [14]. Here, we have developed a novel, simple, but effective on-line preconcentration strategy for focusing DA and NE fluorescent derivatives in a complex matrix on the integrated microfluidic system. This technique takes advantage of borate complexation with cis-diols of CAs, which is able to tune the charge of the analytes and consequently the electrophoretic velocities. The focusing mechanism has been experimentally clarified in terms of pH, borate concentration, and other factors. Distinct from readily available on-line preconcentration techniques, the present mode is exclusively valuable for on-line preconcentration of CAs, and broadly useful for sensitive detection of analytes with vicinal diols such as sugar and other carbohydrates [51,52]. Moreover, this simple preconcentration method can be easily adapted to a microchip CE format where the flow-gated injection is widely used.

It is also important to note that the combination of the sensitive LIF detection and on-line sample preconcentration of fluorescent CA derivatives is an effective but elusive strategy to enhance the detection sensitivity in electrophoretic separations. On the integrated microfluidic platform, we are able to decrease the LODs to the pico-molar range for DA and NE in biological matrices. The separation of DA and NE is observed in 30 s after 8 s for injection. This time scale agrees well with the typical temporal resolution
obtained with CE and microchip CE platforms [11,41]. The greatly improved LODs and the rapid separation of DA and NE are expected to promote the application of CE and microchip CE for in vivo DA/NE measurements in various brain regions with improved temporal resolution.
REFERENCES


CHAPTER 4: RAPID DETERMINATION OF CYANIDE IN HUMAN URINE BY CE-LIF

Note: Most contents in this chapter have been published in Journal of Chromatography A 2015, 1414, 158-162.

4.1. Introduction

Cyanides in the form of hydrogen cyanide (HCN) and its alkali metal salts (such as NaCN and KCN etc.) are extremely toxic chemicals which widely exist in nature and industrial processes [2,3]. Deliberate or accidental cyanide poisoning is rare but can be fatal to involved humans or animals [4]. The most common accidental cyanide exposure is the inhalation of smoke from residential and industrial fires that produce cyanides through nitrogen-containing combustibles [5]. The natural sources of cyanides mainly attribute to the nitrogen metabolic products of bacteria, fungi and algae and to the degradation of cyanogenic glycosides in some plants including bitter almonds, cassava roots, and whole sorghum [6]. Tobacco smoking also produces cyanide thus increasing cyanide levels in blood and urine of smokers but rarely causing intoxication. On the other hand, cyanides have quite important industrial applications including pesticides, electroplating, mineral refinery, and polymer synthesis [2,7], in which cyanides are possibly released into environment. Therefore, cyanide exposure is difficult to avoid, and thus the quick identification of potential cyanide intoxication and its appropriate treatment is crucial to save lives.

Various methods have been developed for cyanide detection, and are reviewed [6,8-10]. These methods may be based on spectrophotometry, potentiometry, or
fluorometry [8]. Involved instrumentation can be HPLC (high-performance liquid chromatography), CE (capillary electrophoresis), GC (gas chromatography), UV/Vis absorbance, or sensors. Although most of these techniques meet application requirements, there are operational limitations such as complexity and multiple steps of sample pretreatment. Therefore, simple and rapid methods are highly desired to facilitate the quick diagnosis of cyanide poisoning so that involved patients could be timely and appropriately treated. The indicators of cyanide intoxication can be biological markers (thiocyanate and ATCA) or direct measurement of cyanide ions ($\text{CN}^-$) [9]. The requirement of the detection sensitivity depends on the sources of biological fluids. For instance, cyanide ion concentrations are in micromolar ranges in blood while in nanomolar in urine due to the fast digestion of cyanide to thiocyanate (the typical half-life of $\text{CN}^-$ is approximately 20-60 min) [9].

Tobacco smokers expose themselves to cyanide and would have elevated levels of cyanide in blood and urine. Rapid and accurate determination of the cyanide levels would help to investigate the effect of smoking between smokers and nonsmokers. Here we report a direct electrophoretic method for rapid determination of cyanide in urine. In this method, urine samples are mixed online with fluorogenic reagents, NDA and glycine, to produce fluorescent derivatives. The mixture is then injected for electrophoretic separations. The separation is completed in 25 s and successive injections could be performed. Cyanide concentrations were rapidly measured by using the method of standard addition.
4.2. Experimental

4.2.1. Materials and reagents

Sodium tetraborate and all amino acids were purchased from Sigma (St. Louis, MO, USA). Potassium cyanide, acetonitrile (ACN), dimethylsulfoxide (DMSO), dimethylformamide (DMF), ethylenediaminetetraacetic acid (EDTA), creatine, urea, and sodium hydroxide were purchased from Fisher Scientific (Chicago, IL, USA). NDA was ordered from Invitrogen (Eugene, OR, USA). Glycine amide [5-(aminoacetamido)fluorescein] (FL-Gly) was purchased from Setareh Biotech (Eugene, OR, USA). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Aqueous solutions were prepared in deionized (DI) water, and NDA stock solution was in DMF. The stock solution KCN (100 mM) was prepared in DI water. Cyanide standard solutions were prepared by diluting the stock KCN solution. Buffer solutions with pH 9.2 were prepared in DI water using sodium tetraborate (Na₂B₄O₇·10H₂O). Amine solutions (glycine, alanine, glutamine, and glutamate) were prepared in DI water, and were diluted to appropriate concentrations with DI water or borate buffer with EDTA (5 mM).

4.2.2. Derivatization of cyanide

The derivatization was performed through offline or online mixing of three solutions: NDA in DMF/water at 50/50 by volume, glycine in buffer with EDTA (5.0 mM), and KCN in buffer or in urine at room temperature. The mixing ratio of NDA/glycine/samples was 1:1:1 or 1:1:2 by volume. The concentrations of NDA, Gly and CN⁻ indicate original concentrations in three separate solutions before mixing. Urine
samples were collected fresh on the experimental day and diluted by adding NaOH solution (final 15 mM in urine) to conserve cyanides. The samples with standard additions were prepared via KCN standard solutions; and the final volume of each sample consisted 50% urine plus NaOH (15 mM) and standard KCN (various concentrations).

4.2.3. Instrumentation

The microfluidic detection system has been described in Chapter 2 [11]. Briefly, a 442-nm laser (Laserglow Technologies, Toronto, ON, Canada) beam was focused on the separation capillary through a 40x oil-immersion objective (Carl Zeiss Microscopy, Thornwood, NY, USA), and the fluorescence was collected by using the same objective and then transported to a photomultiplier tube (Hamamatsu Photonics, Japan) after emission filtration (482±17 nm). Sample injection and separation was performed by using a high-voltage power supply (Model CZE1000R) purchased from Spellman High Voltage Electronics Corporation (Hauppauge, NY, USA). A sample plug was electrokinetically injected through flow gated injection, and then separated, which was controlled by a LabVIEW program. A 4-syringe pump (Chemyx Inc., Stafford, TX, USA) was used to supply sample and derivatization reagents through individual gas-tight Hamilton syringes (Reno, Nevada, USA). The flow gate and other interfaces were fabricated with poly(dimethylsiloxane) (PDMS) as described in Chapter 2 [11].

A 17-cm long capillary with the effective length of 10 cm, ID 10 μm, and OD 360 μm was used to perform separation and detection. The flow-gated injection was run at -5 kV x 0.5 s with the high voltage applied to the outlet side of the capillary, and the inlet
side was grounded. Separations were performed under -23 kV, and the separation buffer was 20-mM tetraborate with pH 9.2.

4.3. Results and Discussion

4.3.1. Fluorogenic derivatization of cyanide ions

NDA was first developed to efficiently derivatize primary amines at the existence of cyanide ions in 1986 [12]. Recent years, wide applications have been seen of NDA-derivatization of amino acid neurotransmitters for sensitive laser-induced fluorescence (LIF) detection [13-15]. To measure amines in a sample, excessive NDA and CN\(^-\) (typically in the mM range) were often used to derivatize amines to the greatest extent. On the other hand, this derivatization strategy has also been employed to measure cyanide ions by using high concentrations of NDA and an amine compound such as glycine and taurine [16-20]. Similarly, excessive NDA and the amine were used to facilitate the derivatization of cyanide ions thus quantitatively determining the levels of cyanide in waste water, blood, and urine.

However, NDA and the primary amine react to produce imines without the present of cyanide ions. The resulted water-insoluble species made the solution faintly yellow, brown, and finally black as shown in Figure 4.1a. NDA at 5.0 mM and glycine or taurine at 20 mM were mixed at 1:1 by volume at pH 9.2; the mixture changed color rapidly (~ 2 min). Experiments also showed that higher concentrations of amines and/or NDA accelerated the color change, which indicates that the reaction rate is amine- and/or NDA concentration dependent. Jackson et al. observed similar phenomenon when they mixed NDA and taurine [2], and the formation of NDA-ditaurine species was proposed considering the NDA molecule had two aldehyde groups which react with primary amines.
When the reaction mixture was analyzed with CE, capillary clogging could occur due to the precipitation and should be avoided. To confirm the reaction between NDA and primary amines, FL-Gly and NDA were mixed and then separated with CE, and the signal was detected with LIF at 491-nm excitation and 520-nm emission. As shown in Figure 4.1b, the derivative imines of FL-Gly and NDA were observed as a wide peak with fronting, which might include derivatives of NDA with bi-glycine and/or mono-glycine. Higher concentrations of NDA (such as 80 μM versus 20 μM of FL-Gly) produced the maximum NDA-FL-Gly peak while the FL-Gly peak disappeared. Further increased concentrations (0.5 or 1.0 mM) of NDA did not change the derivative signal magnitude and peaks shape, which indicates that the derivative of NDA and FL-Gly was independent of NDA concentration when the concentration of NDA was enough to derivatize all FL-Gly in the mixture. Similarly, it is anticipated that glycine reacted with NDA to form dark precipitate (Figure 4.1a). Therefore, it was proposed to mix Gly and NDA online.

**Figure 4.1.** NDA and the primary amine react to produce imines without the present of cyanide ions. (a) Images of dark color for taurine (20.0 mM) and glycine (20.0 mM) mixing with NDA (5.0 mM) after 2.0 min. (b) Electropherograms of FL-Gly (20.0 μM) at various concentrations of NDA added. Buffer 20.0 mM borate at pH 9.2.

To enhance the analytical throughput, online mixing of the derivatization reagents and samples was performed by using three syringes for liquid delivery, respectively, to a
4-way mixer prepared with PDMS [11]. This format was able to avoid sequential pipetting involved in offline mixing, could maintain derivatization conditions, especially the reaction time (time from the mixer to the flow gate), and could reduce the risk of capillary clogging by participation. A reaction capillary of 100 μm (ID) x 30 cm was used to have 2-4 min for the derivatization reaction with the total flow rate in the range of 600-1200 nL/min. A new sample could be switched rapidly while maintaining the derivatization reagents.

![Figure 4.2. Different organic solvents for dissolving NDA. Peak heights of the derivative by NDA, glycine and CN⁻. NDA (5.0 mM) was dissolved in organic solvent/water at 50/50 by volume; glycine was dissolved in 60.0 mM borate buffer pH 9.2; and DI water. The three were mixed offline at 1:1:1 ratio by volume. CN⁻ was contributed by the organic solvent and/or water.](image)

The NDA stock solution could be prepared in methanol or acetonitrile [2,14,17]. However, acetonitrile is unsuitable because it contains micro-amount of hydrogen cyanide as shown in Figure 4.2, which was also observed by Sano et al. [21]. The solubility of NDA in methanol is insufficient, and the presence of methanol often promotes bubble formation because of its volatility. These bubbles could interrupt sample mixing, injection and separation. To efficiently dissolve NDA but to tolerate salt contents when mixing, polar solvents are potential options such as DMSO and DMF. However, DMSO, like acetonitrile, contains hydrogen cyanide as shown in Figure 4.2. Therefore, DMF was finally selected.
as the solvent to prepare NDA stock solutions and was used for the following experiments.

4.3.2. Optimization of conditions

NDA is slightly soluble in water and its work solution was prepared in DMF/water at 50/50 by volume. A concentration of NDA at 2.0-10.0 mM was usually used for the detection of primary amines [15]. To perform online mixing and derivatization, the reaction time was manipulated by selecting flow rates and the length of the reaction capillary (100 μm ID). Additionally, the appropriate combination of NDA and amine concentrations was optimized at the selected flow rates (800 nL/min in total) and reaction capillary (30 cm long). The mixing ratio of NDA/Gly/sample was 1:1:1 or 1:1:2 by volume which produced equivalent concentrations of [NDA]/3, [Gly]/3, and [CN]/3, or [NDA]/4, [Gly]/4, and [CN]/2 in the reaction mixture, respectively (Note: without special notation, concentrations indicate before mixing). As shown in Figure 4.3a, the mixture obtained by mixing 2.0 mM NDA, 80.0 mM Gly and 5.0 μm CN‾ reached the fluorescence plateau in 3.0 min, while it took 11 min for the mixture prepared with CN‾ in urinary matrix. Apparently, the urinary matrix slowed down the reaction kinetics. To accelerate the reaction, the NDA concentration was increased to 8.0 mM, and the reaction time was shortened to 3.0 min (Figure 4.3a). Therefore, 8.0 mM NDA was selected for online mixing when using 1:1:2 mixing ratio which produced final reaction NDA concentration at 2.0 mM. Similarly, the concentration of Gly was optimized while maintaining the concentration of NDA at 8.0 mM and cyanide at 5.0 μm in urine. As can be seen in Figure 4.3b, 80 mM of Gly produced the optimal signal. Therefore, 80 mM Gly was used for 1:1:2 mixing ratio.
The target sample was the biological fluid of urine which contains inorganic ions (Na\(^+\), K\(^+\), Cl\(^-\), Mg\(^{2+}\), Ca\(^{2+}\), NH\(_4\)\(^+\), SO\(_4\)\(^{2-}\), and PO\(_4\)\(^{3-}\), etc.), amino acids, urea, creatine, and creatinine etc. The compositional complexity of urine could have matrix effect on CN\(^-\) measurement. For example, amino acids in urine may have competition with standard Gly for the limited amount of cyanide ions, which might reduce the analyte peak size related to Gly. However, normal levels of most amino acids in urine are less than 0.2 mM, and the total concentration of primary amines is less than 1.0 mM. In addition, relative large concentrations of urea (~15 mM), creatine (~0.5 mM), and creatinine (~0.5 mM) exist in urine. Fortunately, these compounds have conjugated amines that rarely react with NDA at the existence of cyanide ions thus lacking effect on cyanide detection. Glycine was selected because former research has proved that Gly has fast reaction kinetics over others [12,20,22]; and taurine may also be a promising option that has been reported [18]. The concentration of Gly on the one hand affects the derivatization kinetics, and on the other hand it reduces the effect from competitive primary amines inherent in urine. As
shown in Figure 4.3b, an increase in Gly concentration could increase the signal of CN⁻-related fluorescent derivatives until it reached the maximum at 80 mM. However, high concentrations of Gly also promoted the reaction between NDA and Gly which form imines or precipitate thus leading to experimental concerns (Figure 4.1). Therefore, 80 mM Gly (20 mM after mixing) was selected together with 8.0 mM NDA which achieved CN⁻ recovery of ~88% in urine.

### 4.3.3. Potential interferences

![Graph showing potential interferences on CN⁻ derivative peak height.](image)

**Figure 4.4.** Interference effect on CN⁻ derivative peak height. NDA (5.0 mM) in DMF/water (50/50 by volume), glycine (80.0 mM) in borate buffer (80.0 mM, pH 9.2), and KCN (1.0 μM in DI water) only, KCN (1.0 μM) plus Na₂S, Na₂SO₃, mercaptoethanol, and KSCN at 3 μM, respectively. Offline mixing ratio was 1:1:2 (2 for KCN) by volume.

Besides urine matrix effect, other potential interferences include thiocyanate (SCN⁻), thiols (R-SH), sulphide (S²⁻), and sulphite (SO₃²⁻) [10,23]. As shown in Figure 4.4, these potential interferences at a 3-fold concentration of CN⁻ slightly decreased the CN⁻ derivative peak height. S²⁻, thiol, and SCN⁻ decreased CN-derivative signal by 10% while SO₃²⁻ effect was trivial. On the other hand, no additional peak was observed, which indicates that the potential interfering species did not react with NDA/Gly or their products were non-fluorescent.
Considering the matrix effect and potential interferences existing in urinary samples, the method of standard addition was employed to quantitatively determine the CN⁻ levels in urine. The compositional levels from different individuals at various times may also vary, so the matrix effect on CN⁻ varies, which would be observed on the various slopes of calibration curves as seen in Table 4.1.

Table 4.1.

Summarized Results of Smokers and Non-Smokers

<table>
<thead>
<tr>
<th></th>
<th>Calibration Curve</th>
<th>R²</th>
<th>CN⁻ (nM) in Urine*</th>
<th>Ave ± s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smoker 1</td>
<td>( y = 0.606x + 129 )</td>
<td>0.9943</td>
<td>224</td>
<td></td>
</tr>
<tr>
<td>Non-smoker 2</td>
<td>( y = 0.556x + 112 )</td>
<td>0.9981</td>
<td>212</td>
<td>217 ± 6</td>
</tr>
<tr>
<td>Non-smoker 3</td>
<td>( y = 0.624x + 127 )</td>
<td>0.9998</td>
<td>214</td>
<td></td>
</tr>
<tr>
<td>Smoker 1</td>
<td>( y = 0.709x + 300 )</td>
<td>0.9970</td>
<td>445</td>
<td></td>
</tr>
<tr>
<td>Smoker 2</td>
<td>( y = 0.687x + 294 )</td>
<td>0.9972</td>
<td>450</td>
<td>518 ± 123</td>
</tr>
<tr>
<td>Smoker 3</td>
<td>( y = 1.058x + 663 )</td>
<td>0.9973</td>
<td>660</td>
<td></td>
</tr>
</tbody>
</table>

* CN⁻ concentrations were calculated for urine of 95% sample volumes.

4.3.4. Cyanide measurements of smokers and non-smokers

The optimized conditions and the method of standard addition were applied to CN⁻ detection in urinary samples from smokers and nonsmokers. The samples were collected freshly with 15 mM NaOH added. A series of urine sample solutions was prepared by adding standard KCN solution in water, and the original urine took 95% in volume. Three solutions: NDA, Gly, and urine sample, were delivered through three individual gas-tight syringes at the volume ratio of 1:1:2 which was performed by using a Hamilton syringe pump. The three flows mixed at a PDMS mixer with a reaction capillary of 100 μm ID x 30 cm in length. The total flow rate in the reaction capillary was 800 nL/min which secured the reaction time ~3 min. As shown in Figure 4.5, CN⁻ derivative peak height increased
with the concentration of standard additions. Each series of samples produced a
calibration curve, and the results are summarized in Table 4.1. The urinary samples were
donated by three male regular smokers and three male non-smokers in their ages of 20-
30 years old. The smokers got urinary CN\(^{-}\) levels of 518±123 nM, while non-smokers had
217±6 nM. These results are in good agreement with published results [9,24]. Student’s
t test suggested that the level difference of smokers and non-smokers were significant.

Note that peak areas were preferred for the quantification of CN\(^{-}\) in urine. Urinary
matrix contained large quantities of electrolytes which rendered conductivity mismatch
between the derivatization mixture and the separation buffer. This mismatch resulted in
peak broadening and peak height variation non-proportional to the concentration of
standard addition. Peak area correction was not performed since the migration time of
each series of standard additions had %RSD at <1.3%.

![Figure 4.5. Electropherograms of urine with standard additions. The KCN concentrations added are indicated. The major peak indicates the derivative of NDA, KCN and glycine. NDA (8.0 mM) in DMF/water (50/50 by volume), glycine in borate buffer (80.0 mM, pH 9.2) and EDTA (5.0 mM) and KCN in urine/water (95/5 by volume). Online mixing ratio was 1:1:2 (2 for KCN) by flow rate.](image-url)
4.4. Concluding Remarks

The reported method takes advantage of the property of CN- fluorogenic derivatization with NDA at the existence of a primary amine. The strategy of online mixing and derivatization with three separate syringes for reagent and sample delivery simplifies and accelerates the analysis procedure. The reaction took about 2 - 4 min for derivatization, and the rapid separation facilitates analytical throughput which is important when early diagnosis is desired. This strategy also avoids the oxidative loss of cyanide ions in the sample with the online mixing. The method of standard addition with multiple points might weaken the benefits of fast separations in 25 s, but the two-point standard addition may be employed to estimate the cyanide levels in urine in minutes. It is also anticipated that the developed method could be adapted to the detection of cyanide ions in whole blood samples after appropriate modifications and condition optimizations.
REFERENCES


CHAPTER 5: OVERALL CONCLUSION

5.1. Summary

Flow-gated CE-LIF is a powerful tool for trace detection of amino acids with high throughput and high efficiency. This dissertation presents a flow-gated CE system and its applications in rapid separation and sensitive detections of neurotransmitters and cyanides in biological fluids.

First, to reduce the difficulty of practical applications of the flow-gated CE system, a strategy to rapidly fabricate PDMS interfaces was developed. Operational conditions for this PDMS-interconnected CE system were studied and optimized, including separation buffer, derivatization reagents, sample buffer, separation voltage, sample and BGE conductivities. The experimental results showed that these interfaces provided easy visual trouble shooting, and produced long-term injection/separation stability. In addition, these interfaces are reusable, inexpensive, convenient for connection, and reliable.

To enhance detection sensitivity for catecholamines, a novel on-line sample preconcentration method was developed exclusively for fluorescent derivatives of dopamine and norepinephrine. This method took advantage of the complexation between diols and borate, thus one negative charge could be added to the complex entity. The sample derivatization mixture was electrokinetically injected into a capillary via the flow-gated injection, and then DA and NE derivatives were selectively focused to a narrow zone by the reversible complexation. Separation of NE and DA derivatives was executed by incoming surfactants of cholate and deoxycholate mixed in the front BGE plug. This online preconcentration method was able to lower the LOD to a pico-molar range. It is anticipated that the method would be valuable for in vivo monitoring of DA and NE in
various brain regions of live animals on flow-gated CE or microchip platforms.

As a demonstration of real world application, a rapid method was developed on the integrated CE system for the determination of cyanide ions in urine samples. Cyanide ions were derivatized with NDA and a primary amine for LIF detection. Three separate reagents, NDA, glycine, and cyanide sample, were mixed online, which secured uniform conditions between samples for the cyanide derivatization and reduced the risk of precipitation of mixtures. Conditions were optimized, and the derivatization was completed in 2-4 minutes and the separation was observed in 25 seconds. The limit of detection (LOD) was 5.0 nM for standard cyanide in buffer. The cyanide levels in urine samples from smokers and nonsmokers were determined by using the method of standard addition. The results were in good agreement with published data. The developed method was rapid, accurate, and is anticipated to be applicable to cyanide detection in waste water as well as in blood with appropriate modifications.

5.2. Future Work

The integrated flow-gated CE system is a powerful tool for dynamically monitoring species of interest, especially in complex biological samples. The PDMS-interconnected CE system can be used for in vivo neurotransmitter monitoring. Furthermore, a two-branch switch can be coupled with the CE system for monitoring two brain locations via alternate injections. This could allow scientists to study multiple regions in a brain, which could provide a strategy for the investigation of brain functional connectivity in terms of specific neurotransmitters.

The methods for measurement of cyanide ions will be applied to blood plasma or whole blood sample after appropriate modifications and condition optimizations.
Microdialysis probes will be integrated to the sample supplies, since blood samples are complex biological fluids with relative high concentration of proteins.

The on-line sample preconcentration method developed for DA and NE may be transferred to a microchip CE system with appropriate modifications.